Synthetic Genetic Interactions With Temperature-Sensitive Clathrin in *Saccharomyces cerevisiae*: Roles for Synaptojanin-Like Inp53p and Dynamin-Related Vps1p in Clathrin-Dependent Protein Sorting at the *trans*-Golgi Network

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ABSTRACT

Clathrin is involved in selective protein transport at the Golgi apparatus and the plasma membrane. To further understand the molecular mechanisms underlying clathrin-mediated protein transport pathways, we initiated a genetic screen for mutations that display synthetic growth defects when combined with a temperature-sensitive allele of the clathrin heavy chain gene (*chc1-521*) in *Saccharomyces cerevisiae*. Mutations, when present in cells with wild-type clathrin, were analyzed for effects on mating pheromone α-factor precursor maturation and sorting of the vacuolar protein carboxypeptidase Y as measures of protein sorting at the yeast *trans*-Golgi network (TGN) compartment. By these criteria, two classes of mutants were obtained, those with and those without defects in protein sorting at the TGN. One mutant with unaltered protein sorting at the TGN contains a mutation in *PTC1*, a type 2c serine/threonine phosphatase with widespread influences. The collection of mutants displaying TGN sorting defects includes members with mutations in previously identified vacuolar protein sorting genes (*VPS*), including the dynamin family member *VPS1*. Striking genetic interactions were observed by combining temperature-sensitive alleles of *CHC1* and *VPS1*, supporting the model that Vps1p is involved in clathrin-mediated vesicle formation at the TGN. Also in the spectrum of mutants with TGN sorting defects are isolates with mutations in the following: *RIC1*, encoding a product originally proposed to participate in ribosome biogenesis; *LUV1*, encoding a product potentially involved in vacuole and microtubule organization; and *INP53*, encoding a synaptojanin-like inositol polyphosphate 5-phosphatase. Disruption of *INP53*, but not the related *INP51* and *INP52* genes, resulted in α-factor maturation defects and exacerbated α-factor maturation defects when combined with *chc1-521*. Our findings implicate a wide variety of proteins in clathrin-dependent processes and provide evidence for the selective involvement of Inp53p in clathrin-mediated protein sorting at the TGN.

EUKARYOTIC cells contain multiple membrane-bounded compartments, each with a distinct composition and cellular function. To maintain the integrity of each organelle, the cell selectively transports proteins to the appropriate resident compartment. Examples of such selective transport occur in the secretory pathway. Proteins that reside in secretory pathway organelles can be actively retained and/or retrieved from subsequent compartments, while secreted and plasma membrane proteins traverse the pathway (Rothman and Wieland 1996; Kaiser *et al.* 1997). Further sorting occurs at organelles such as the *trans*-Golgi network (TGN) where pathways branch to multiple destinations (Traub and Kornfeld 1997). The complex molecular mechanisms necessary for different steps in selective protein transport remain incompletely defined.

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Coat proteins constitute a class of molecules associated with the cytoplasmic face of organelles and transport vesicles. Coat protein complexes are believed to drive membrane deformation during vesicle formation and impart specificity to protein transport reactions by selecting appropriate proteins for incorporation as cargo into nascent transport vesicles (Schekman and Orci 1996; Robinson 1997). Distinct coat complexes have been defined, including COPI, COPII, and clathrin coats. COPI and COPII coats have well-characterized roles in selective transport at the early stages of the secretory pathway and probably participate in other secretory and endocytic steps (Barlowe 1998; Lowe and Kreis 1998). In contrast, clathrin coats are involved in receptor-mediated endocytosis, localization of resident membrane proteins to the TGN, and transport of proteins to the lysosome/vacuole (Wilsbach and Payne 1993a; Schmid 1997; Molloy *et al.* 1999).

Clathrin and clathrin adaptors (APs) are the major structural components of clathrin coats (Schmid 1997). Clathrin is a three-legged molecule, termed a triskelion,

made up of heavy (Chc) and light chains (Clc). Triskelions assemble together to form the outer polyhedral shell of the coat. Clathin APs are heterotetramic complexes that bridge the clathrin shell to the membrane. APs act in recruitment of clathrin to appropriate membranes and in cargo selection during vesicle formation. At least two distinct AP complexes interact with clathrin, AP-1 at the TGN and AP-2 at the plasma membrane (Hirst and Robinson 1998). Whether the newly described AP-3 complex participates in clathrin-coated vesicle formation is currently unclear (Simpson *et al.* 1996; dell'Angelica *et al.* 1998). In addition to APs, a growing list of accessory proteins has been identified that associate with clathrin coats and / or function in clathrin-coated vesicle formation (Pishvaee and Payne 1998). Recent studies in yeast demonstrate clathrin function in the absence of AP complexes (Huang *et al.* 1999; Yeung *et al.* 1999). These findings further implicate the participation of additional factors in clathrin-coated vesicle formation.

The yeast *Saccharomyces cerevisiae* contains a single clathrin heavy chain gene (*CHC1*) and a single clathrin light chain gene (*CLC1*; Payne and Schekman 1985; Silveira *et al.* 1990; Lemmon *et al.* 1991). Strains lacking clathrin heavy chain (*chc1*Δ) grow slowly or, in certain genetic backgrounds, are inviable (Payne and Schekman 1985; Lemmon and Jones 1987). In addition to slow growth, phenotypes of viable *chc1*Δ strains include mislocalization of TGN membrane proteins and retarded receptor-mediated endocytosis (Payne *et al.* 1988; Payne and Schekman 1989). To characterize the immediate effects of inactivating clathrin heavy chain, a recessive, temperature-sensitive allele of *CHC1*, *chc1-521*, was generated previously. At the nonpermissive temperature, *chc1-521* cells rapidly exhibit protein trafficking defects commensurate with those in *chc1*Δ cells, and over time also grow slowly (Seeger and Payne 1992a; Tan *et al.* 1993). These results support roles for clathrin in Golgi membrane protein localization and receptor-mediated endocytosis. In contrast to *chc1*Δ cells, which sort newly synthesized soluble vacuolar proteins from the TGN at near wild-type levels, *chc1-521* cells display an immediate sorting defect leading to secretion of vacuolar protein precursors (Seeger and Payne 1992b). However, by an undefined mechanism, vacuolar protein sorting gradually recovers in *chc1-521* cells incubated at the nonpermissive temperature, reaching efficiencies observed in *chc1*Δ and wild-type cells. Taken together, the combined phenotypes of *chc1* mutants argue that clathrin is required for protein sorting at the TGN and receptor-mediated endocytosis at the plasma membrane.

Screens for mutations that enhance specific phenotypes in a sensitized mutant background can be an effective strategy to identify genes whose products act in the same pathway (Guarente 1993). Previously, this type of approach was carried out in a screen for mutations that confer lethality in a *chc1*Δ strain, yielding lesions in numerous loci (Munn *et al.* 1991). However, the specificity of the mutations was uncertain because of the possibility that subtle defects in pathways unrelated to clathrin could cause inviability when present in already debilitated *chc1*Δ cells. To circumvent this issue, we have taken advantage of the properties of cells expressing the temperature-sensitive *chc1-521* allele to characterize clathrin-dependent pathways. In these cells, the severity of growth and protein transport defects depends on the incubation temperature (Seeger and Payne 1992a). At the permissive temperature of 24°, *chc1-521* cells grow at wild-type rates and display little or no abnormality in clathrin-mediated protein traffic. At 30°, mutant cells continue to grow at near wild-type rates, but compromised clathrin function is evidenced by the partial mislocalization of TGN membrane proteins. Incubation at 37° is required to produce growth and trafficking defects approximating those of *chc1*Δ cells. This graded response to temperature suggested that the *chc1-521* allele would sensitize cells to mutations in other components of clathrin-dependent pathways. In support of this idea, disruptions of genes encoding subunits of the AP-1 complex, which have no observed detrimental effects alone, accentuate growth and TGN membrane protein localization defects in *chc1-521* cells (Phan *et al.* 1994; Rad *et al.* 1995; Stepp *et al.* 1995; Yeung *et al.* 1999). Consistent with a specific, if subtle, role for AP-1 at the Golgi apparatus, AP-1 subunit gene disruptions do not influence endocytosis in *chc1-521* cells.

Here we describe isolation and characterization of mutations that cause severe growth defects when combined with *chc1-521* at the semipermissive temperature of 30°. This type of genetic interaction in yeast, where two mutations in combination result in significantly greater defects than either alone, is referred to as synthetic (Guarente 1993). Accordingly we have termed the mutations *tcs* (*t*emperature-sensitive *c*lathrin *s*ynthetic mutation). The collection of *tcs* mutations defines a set of gene products that may participate in clathrin-dependent transport pathways. Identification of *tcs* mutations in the genes encoding the dynamin-related GTPase Vps1p and the synaptojanin-like inositol-5-phosphatase family member Inp53p prompted a more extensive examination of their genetic interactions with clathrin. The results offer functional evidence supporting roles for Vps1p and Inp53p in clathrin-mediated protein trafficking at the TGN.

MATERIALS AND METHODS

Plasmids and nucleic acid techniques: Plasmid constructions were performed using standard molecular biology techniques (Sambrook *et al.* 1989). pULE-CHC1, carrying the *LYS2*, *URA3*, and *CHC1* genes, was constructed as follows. An *Xba*I-*Hin*dIII fragment carrying the *LYS2* gene was inserted into

pRS316 (Sikorski and Hieter 1989) to create pRS316-LYS2A. A *Cla*I-*Sal*I fragment with the 5′ end of *CHC1* and a *Cla*I fragment with the 3′ end of *CHC1* from pCHCc102 (Payne *et al.* 1987) were sequentially inserted into pRS316-LYS2A to produce pULE-CHC1. A 6.9-kb *Sma*I-*Sal*I fragment containing *CHC1* was removed from pCHC-XS (Munn *et al.* 1991) and ligated into pRS423 (Christianson *et al.* 1992) to create p423-CHC1. YIpCHC521ΔCla contains the *Cla*I-*Sal*I fragment of the *chc1-521* allele (Seeger and Payne 1992b) in the integrating plasmid YIp5 (*URA3*). To generate p6-2(15a)BP, a 4.5-kb *Pvu*II-*Bam*HI fragment containing the *RIC1* open reading frame was isolated from a genomic library clone (the *Bam*HI fragment was generated by ligation of the *Sau*3A-cleaved genomic fragment to the *Bam*HI-cleaved vector) and subcloned into the *Bam*HI and *Sac*I sites in pRS315 (Sikorski and Hieter 1989) after converting the *Sac*I end to a blunt form with T4 DNA polymerase (blunt-end). p426-RIC1 resulted by ligation of a *Bam*HI-*Xba*I fragment and an *Xba*I-*Hin*dIII fragment from p6-2(15a)BP into pRS426 (Christianson *et al.* 1992) cleaved with *Bam*HI and *Hin*dIII. A *Sal*I-*Not*I fragment containing *RIC1* was subcloned from p426-RIC1 into pRS315 to form p315-RIC1. pric1Δ1 is pBluescript II KS$^+$ (Stratagene, La Jolla, CA) containing a 4.0-kb *Hin*dIII-*Bam*HI *RIC1* fragment from p6-2(15a)BP where a 2.2-kb *Eco*RI-*Spe*I fragment containing most of the *RIC1* coding sequence is replaced by the *TRP1* gene. A 3.6-kb *Bam*HI-*Sal*I genomic fragment containing YDR027c/*LUV1* was subcloned into pRS315 to produce p45-5(SB). ptcs3-Δ1 is pBluescript II KS$^+$ containing the 3.6-kb YDR027c/*LUV1* fragment where a 2.5-kb *Bgl*II-*Cla*I fragment containing most of the *LUV1* coding sequence is replaced by the *TRP1* gene. pPTC1-2 was constructed by subcloning a 1.26-kb *Eco*RI-*Bam*HI genomic fragment containing *PTC1* into pRS314 (Sikorski and Hieter 1989). pptc1-Δ1 is pBluescript II KS$^+$ containing the 1.26-kb *PTC1* fragment where a 0.81-kb *Nde*I-*Bam*HI fragment containing most of the *PTC1* gene was replaced with *TRP1*. p313-INP53 contains a 4.6-kb *Hin*cII-*Xba*I genomic fragment which includes the *INP53* open reading frame subcloned into pRS313 (Sikorski and Hieter 1989) cleaved with *Eco*RV and *Xba*I. The 4.6-kb fragment from p313-INP53 was liberated by cleavage with *Xho*I and *Sac*I and ligated into pRS315 to generate p315-INP53.

Strains, media, and genetic techniques: Strains used in this study are shown in Table 1. GPY982 was constructed by transforming SEY6210 with YIpCHC521ΔCla linearized with *Xba*I and selecting for integrants on SD −ura medium. Loss of URA3 along with the wild-type 3′ end of *CHC1* was selected on 5-fluoroorotic acid (5-FOA) medium. GPY1010-5B is a meiotic progeny from a cross of SEY6211 and GPY982.

To generate a disruption in the *INP53* gene, the primer pairs 5′-TGGGGCGAAGAATATCTAGTTATCCACTCCTTCA TAGAATGATTGTACTGAGAGTGCACC-3′, 5′-GGCGCAAAT CCTGATCCAAAC-3′ and 5′-CATTTTGGGGTCAATGGCTG CCATGAGTCTAAAGTCATATCATCTGTGCGGTATTTCAC ACCG-3′, 5′-CGGCTGGTCGCTAATCGTTG-3′ were used to generate two overlapping PCR products using pRS303 (Sikorski and Hieter 1989) as the template. These PCR products were cotransformed into SEY6210, GPY982, and/or GPY1056. Disruption of the *INP53* locus by homologous recombination was confirmed by Southern blot analysis and/or PCR. Strains disrupted at the *INP51* locus were generated in a similar fashion using the primer pairs 5′-GTTGTGTTAATCGTATGAA TTCGAAGCACATTTCACTACAATGATTGTACTGAGAGTG CACC-3′, 5′-GGCGCAAATCCTGATCCAAAC-3′ and 5′-GTG GCTCATCTTCGTTCTCAACGAATGGATTGGGATCTCCA TCTGTGCGGTATTTCACACCG-3′, 5′-CGGCTGGTCGCTA ATCGTTG-3′ in PCR reactions using pRS303 as the template. *INP52* was disrupted with the *HIS3* gene similarly using primer pairs 5′-ACGCAAAGGCAGCAGAATCAAAAACAAATACTC AGTAGCTATGATTGTACTGAGAGTGCACC-3′, 5′-GGCGC AAATCCTGATCCAAAC-3′ and 5′-GTAACACAATTTAATT GGGGTCGCAAGGCTTCAATGGATGAACATCTGTGCGGT ATTTCACACCG-3′, 5′-CGGCTGGTCGCTAATCGTTG-3′ using pRS303 as template. *INP52* was disrupted with the *TRP1* gene using the primer pairs 5′-ACGCAAAGGCAGCAGAAT CAAAAACAAATACTCAGTAGCTATGATTGTACTGAGAGT GCACC-3′, 5′-GGTATTCTTGCCACGACTCATC-3′ and 5′-GTAACACAATTTAATTGGGGTCGCAAGGCTTCAATGGAT GAACATCTGTGTGGTATTTCACACCG-3′, 5′-CAGAATGT GCTCTAGATTCGg-3′ using pRS304 (Sikorski and Hieter 1989) as template.

RIC1 was disrupted to generate GPY1480 by transforming SEY6210 with pric1-Δ1 digested with *Xho*I. Similarly a *Sal*I-*Bam*HI fragment from ptcs3-Δ1 was used to disrupt *LUV1*, and an *Eco*RI fragment from pptc1-Δ1 was used to disrupt *PTC1*. To create a *ptc1 hog1* double mutant (GPY1371), a *Cla*I-*Bam*HI fragment from pDHG16 (provided by H. Saito, Harvard Medical School, Boston, MA) was transformed into a strain carrying the *ptc1* allele found in our screen which had been backcrossed to a wild-type strain. All disruptions were verified by Southern blot analysis or by PCR. Strains bearing a chromosomal copy of the *vps1-ts* allele were constructed with pCAV40, an integrating vector containing the *vps1-ts* allele (provided by T. Stevens, University of Oregon, Eugene, OR). pCAV40 was digested with *Eco*RI and transformed into *CHC1* cells (GPY1100) or *chc1-521* cells (GPY418). Ura$^+$ transformants were plated onto 5-FOA-containing media, and resulting colonies were assayed for secretion of carboxypeptidase Y (CPY) at 37°. A collection of *VPS* mutants was kindly provided by B. Horazdovsky (The University of Texas Southwestern Medical Center, Dallas, TX).

YPD medium is 1% Bacto-yeast extract, 2% Bactopeptone, and 2% dextrose. SD is 0.67% yeast nitrogen base without amino acids and 2% dextrose. Supplemented SD is SD with 40 μg/ml adenine, 30 μg/ml leucine, 30 μg/ml lysine, 20 μg/ml histidine, 20 μg/ml uracil, and 20 μg/ml tryptophan. SD −ura, SD −his, and SD −trp are supplemented SD without uracil, histidine, or tryptophan, respectively. SD CAA medium is supplemented SD with 5 mg/ml vitamin assay casamino acid mix. SD CAA −ura is SD CAA without uracil. SDYE is supplemented SD with 0.2% yeast extract. 5-FOA and α-aminoadipate (α-AA) media were prepared as described previously (Rose *et al.* 1990). Cell densities in liquid culture were measured in a 1-cm plastic cuvette using a Beckman Instruments DU-62 spectrophotometer (Beckman Instruments, Fullerton, CA). One A_{500} unit is equivalent to 2.3×10^7 cells/ml.

Standard techniques for yeast mating, sporulation, and tetrad analysis were used (Guthrie and Fink 1991). DNA transformations were performed as previously described (Gietz and Schiestl 1995).

Mutagenesis and genetic screen for *tcs* mutants: The plasmid loss strategy relied on the toxic effects of 5-FOA and α-AA in cells that express the *URA3* and *LYS2* products, respectively (Basson *et al.* 1987). GPY1056 was grown at 30° in SD CAA −ura medium to stationary phase and plated at a density of 1000 cells/plate. Plates were exposed to UV irradiation, and irradiated cells were allowed to recover at 30°. This treatment resulted in 16% cell viability. Approximately 16,000 UV-irradiated colonies were replica plated onto α-AA medium and incubated at 30°. Colonies that did not grow on α-AA-containing medium were patched onto YPD agar, allowed to grow at 30°, and replica plated onto 5-FOA-containing medium. Out of 77 potential *tcs* mutants, 25 remained unable to grow on 5-FOA when transformed with a *HIS3 CHC1* plasmid, indicating that these mutants are sensitive to 5-FOA even when expressing wild-type *CHC1*. The remaining 52 mutants were successively backcrossed to GPY1056 or GPY1057 three times. Segregants from the third backcross were used for all assays

TABLE 1

Strains used in this study

Strains	Genotype	Reference
SEY6210	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.* (1988)
SEY6211	*MAT***a** *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9*	Robinson *et al.* (1988)
GPY418	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521*	Phan *et al.* (1994)
GPY775	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521 vps1-ts*	This study
GPY832	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 vps1-ts*	This study
GPY982	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521*	This study
GPY1019-5B	*MAT***a** *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 chc1-521*	This study
GPY1056	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 + pULE-CHC1*	This study
GPY1057	*MAT***a** *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 chc1-521 + pRS315 + pULE-CHC1*	This study
GPY1100α	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1*	Payne and Schekman (1989)
GPY1344	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-9 ptc1::TRP1*	This study
GPY1345	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-9 chc1-521 ptc1::TRP1 + pULE-CHC1*	This study
GPY1371	*MAT***a** *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs11-1(ptc1) hog1::TRP1 + pULE-CHC1*	This study
GPY1480	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ric1::TRP1*	This study
GPY1550	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 tcs3::TRP1*	This study
GPY1876	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 inp53Δ::HIS3*	This study
GPY1877	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 inp53Δ::HIS3 + pULE-CHC1*	This study
GPY2062	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 inp52Δ::TRP1*	This study
GPY2064	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 inp51Δ::HIS3 + pULE-CHC1*	This study
GPY2078	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 inp51Δ::HIS3*	This study
GPY2141	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 inp51Δ::HIS3*	This study
GPY2142	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 inp52Δ::HIS3*	This study
GPY2143	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 inp53Δ::HIS3*	This study
GPY2162	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 inp52Δ::HIS3 + pULE-CHC1*	This study
GPY2172	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs1-1(ric1) + pULE-CHC1*	This study
GPY2173	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 tcs2-1(vps1) + pULE-CHC1*	This study
GPY2174	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs3-1(luv1) + pULE-CHC1*	This study
GPY2175	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs4-1 + pULE-CHC1*	This study
GPY2176	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs5-1(vps5) + pULE-CHC1*	This study
GPY2177	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs6-1(vps21) + pULE-CHC1*	This study
GPY2178	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 chc1-521 tcs7-1(pep12) suc2-Δ9 + pULE-CHC1*	This study
GPY2179	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs8-1(vps17) + pULE-CHC1*	This study
GPY2180	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 TCS9-1 + pULE-CHC1*	This study
GPY2181	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 chc1-521 tcs10-1 + pULE-CHC1*	This study
GPY2182	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs11-1(ptc1) + pULE-CHC1 +pRS315*	This study
GPY2183	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs12-1(inp53) + pULE-CHC1*	This study
GPY2184	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs13-1 + pULE-CHC1*	This study
GPY2185	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs14-1 + pULE-CHC1*	This study
GPY2186	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 LYS2 or lys2-801 suc2-Δ9 chc1-521 tcs15-1 + pULE-CHC1*	This study

except for *tcs2*, *TCS9-1*, and *tcs10* for which the original mutagenized strains were used.

Filter overlay blot: Secretion of p2CPY was assessed using a filter overlay blot assay performed essentially as described previously (Wilsbach and Payne 1993b) except antibody against p2CPY was used (provided by T. Stevens).

Metabolic labeling and immunoprecipitation: For metabolic labeling of α-factor, cells were grown to midlogarithmic phase in SD CAA −ura at 24° or 30°. Labeling and immunoprecipitation was performed as described by Seeger and Payne (1992b) except that labeling time was as indicated in figure legends. α_2-Macroglobulin (10 μg/ml) was added to experiments shown in Figure 7 to stabilize secreted pheromone.

FM4-64 labeling: Yeast cells were labeled with FM4-64 essentially as described by Vida and Emr (1995). Cells growing in midlogarithmic phase were incubated in the presence of 40 μm FM4-64 at a cell concentration of 2×10^8 to 4×10^8 cells/ml for 15 min at 30°. Cells were harvested by centrifugation at 700 × *g* for 3 min, resuspended at 1×10^8 to 2×10^8 cells/ml in fresh medium, and allowed to internalize dye for 45 min at 30°. Cells were then collected by centrifugation, resuspended at 5×10^7 to 1×10^8 cells/ml, and viewed on concanavalin-A-coated microscope slides using a Nikon FX-A Microphot microscope.

Cloning *tcs* mutants: *tcs* mutants were transformed with a single copy genomic library (no. 77162; American Type Culture Collection, Manassas, VA), and transformants were screened for growth on 5-FOA-containing medium. DNA from 5-FOA-resistant transformants was prepared as described previously (Rose *et al.* 1990), and plasmids were introduced into *Escherichia coli* by electroporation. Genomic inserts were identified by DNA sequencing. The minimal complementing region of each genomic clone was determined by subcloning individual open reading frames followed by complementation analysis on 5-FOA-containing medium.

RESULTS

Isolation of mutations that display synthetic lethality with *chc1-521*: A plasmid loss strategy was adopted to identify *tcs* mutations in *chc1-521* cells at 30°. For this approach, a centromere-containing plasmid carrying wild-type *CHC1*, *URA3*, and *LYS2* genes was introduced into cells with *chc1-521*, *ura3*, and *lys2* mutant alleles at the chromosomal loci. Previously, we observed that viable *chc1*Δ cells transformed with a *URA3 CHC1* plasmid were inviable on 5-FOA, presumably because the 5-FOA selection procedure imposes sufficient stress to inhibit the growth of debilitated clathrin-deficient cells that lose the plasmid (G. Payne, unpublished results). On the basis of this finding we anticipated that if a *tcs* mutation together with *chc1-521* at 30° causes a severe loss of clathrin function, then the mutant cells should be inviable on 5-FOA or α-AA.

To isolate *tcs* mutants, plasmid-containing cells were mutagenized with ultraviolet irradiation and allowed to grow into colonies, and the colonies were screened for growth on α-AA- and 5-FOA-containing medium. Of 16,000 colonies formed from mutagenized cells, 52 *tcs* candidate strains were backcrossed three times to the parental strain, and those that yielded 2:2 segregation

TABLE 2

***tcs* mutants**

	Mutant allele[a]	% Unmature α-factor secreted[b]	Secretion of p2CPY[c]	Vacuolar morphology[d]
Class 1	*tcs1/ric1* (1)	50	++	Fragmented
	tcs2/vps1 (5)	44	+++	Class F-like
	tcs3/luv1 (1)	30	++	Fragmented
	tcs4 (1)	13	+/−	Wild type
	tcs5/vps5 (2)	19	+++	Fragmented
	tcs6/vps21 (1)	8.5	+++	Large vacuole
	tcs7/pep12 (2)	9	+++	Fragmented/large vacuole
	tcs8/vps17 (1)	10	+++	Fragmented/large vacuole
	TCS9-1	3.5	+++	Large vacuole
	tcs12/inp53 (1)	0	+/−	Wild type
Class 2	*tcs10* (1)	0	−	Wild type
	tcs11/ptc1 (1)	0	−	Wild type
	tcs13 (1)	0	−	Wild type
	tcs14 (1)	0	−	Wild type
	tcs15 (1)	0	−	Fragmented

[a] Number of alleles isolated is indicated in parentheses.

[b] The experiment shown in Figure 1 was subjected to phosphorimage analysis and quantified using a Molecular Dynamics phosphorImager and ImageQuaNT software. Percentage of unmature α-factor was calculated by dividing the amount of highly glycosylated precursor plus intermediate cleavage products by the total amount of α-factor. Values were normalized to wild type.

[c] Qualitative assessment of experiment shown in Figure 2.

[d] Qualitative description of vacuolar morphology assessed by staining with FM4-64 from Figure 3 and data not shown.

of the 5-FOA-sensitive phenotype were analyzed further. Complementation tests defined one dominant mutation (*TCS9-1*) and 14 recessive *tcs* complementation groups (Table 2). The majority of complementation groups contained a single isolate, indicating that the screen was subsaturating (Table 2).

A subset of *tcs* mutants affects clathrin-mediated protein trafficking events: The effect of *tcs* mutations in a *CHC1* background on clathrin-dependent protein transport processes was assessed. First, maturation of the mating pheromone α-factor precursor was examined as a measure of proper localization of the TGN membrane protein Kex2p. Kex2p is responsible for the cleavages that initiate proteolytic maturation of the α-factor precursor in the TGN (Fuller *et al.* 1988). In cells with defective clathrin heavy chain, Kex2p is mislocalized to the cell surface (Payne and Schekman 1989). The resulting depletion of TGN-localized Kex2p leads to inefficient α-factor precursor maturation and secretion of the highly glycosylated form of pheromone. Other mutations, such as vacuolar protein sorting (*vps*) mutations, that affect trafficking between the TGN and endosomes can also lead to Kex2p mislocalization and attendant defects in α-factor precursor maturation (Wilsbach and Payne 1993b). Therefore, secretion of highly glycosylated α-factor precursor serves as a convenient and reliable indicator of Kex2p mislocalization. Secreted forms of α-factor were detected by radiolabeling *tcs* cells with [^{35}S]methionine, immunoprecipitating α-factor from the culture supernatant, and subjecting the immunoprecipitates to SDS-PAGE. The mature 13-amino-acid α-factor peptide migrates to the bottom of the gel, while the highly glycosylated form remains near the top (Figure 1). Incompletely processed forms of α-factor can also be observed migrating immediately above the mature form. Of the 15 mutant strains, 9 secreted detectable levels of highly glycosylated α-factor precursor, ranging from a severe defect in *tcs1-3* mutants to a minor defect in the *TCS9-1* mutant (Figure 1; Table 2). These data suggest that *tcs1* through *TCS9-1* mutations affect localization of the TGN membrane protein Kex2p.

The integrity of vacuolar protein transport was determined by monitoring biosynthesis of the vacuolar hydrolase, CPY. Newly synthesized CPY is translocated into the endoplasmic reticulum and core glycosylated to form a 67-kD species (p1CPY). Upon transit through the Golgi apparatus, p1CPY is further glycosylated to form a 69-kD species (p2CPY; Stevens *et al.* 1982). In the TGN, p2CPY is recognized by a receptor, Pep1p/Vps10p, and diverted from the secretory pathway into vesicles targeted to endosomes. In prevacuolar endosomes, Pep1p/Vps10p is thought to dissociate from p2CPY allowing vesicle-mediated retrieval of Pep1p/Vps10p to the TGN (Marcusson *et al.* 1994; Cooper and Stevens 1996). Dissociated p2CPY continues to the vacuole where proteolytical maturation generates a 61-kD species (Stevens *et al.* 1982). Inefficient localization of Pep1p/Vps10p to the TGN and/or defects in the TGN to endosome to vacuole pathway result in secretion of p2CPY (Bryant and Stevens 1998). The *tcs* mutants

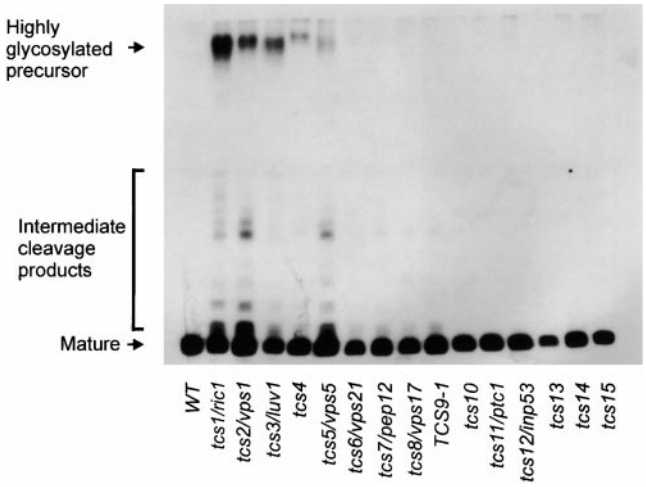

Figure 1.—Maturation of α-factor is incomplete in a subset of *tcs* mutants. Wild-type (*WT*, GPY1056) and *tcs1-15* mutant strains (GPY2172-GPY2186) carrying the *chc1-521* allele and a plasmid-borne copy of *CHC1* and *URA3* were grown in SD CAA −ura overnight at 30°. *tcs13* was grown in SDYE media overnight at 24° and shifted to 30° before metabolic labeling due to poor growth in SD CAA −ura at 30°. Cells were metabolically labeled with [^{35}S]methionine/cysteine for 45 min at 30°. α-Factor was immunoprecipitated from the culture supernatant and subjected to SDS-PAGE and autoradiography.

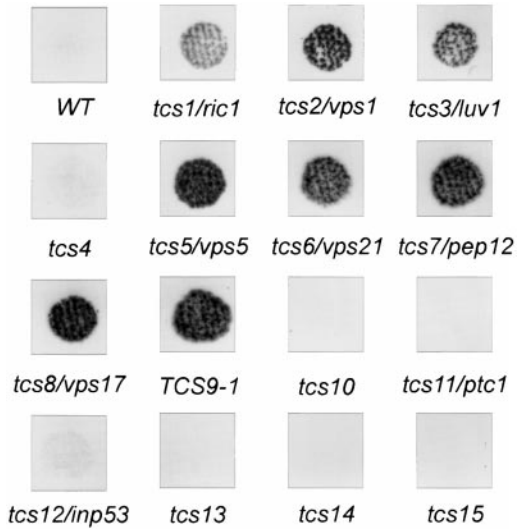

Figure 2.—p2CPY is secreted by a subset of *tcs* mutants. Wild-type (*WT*, GPY1056) and *tcs* mutant strains (GPY2172-GPY2186) carrying the *chc1-521* allele and a plasmid with *CHC1* and *URA3* were grown in SD CAA −ura media to saturation at 24°. Cells were diluted to 1×10^6 cells/ml, and 3 μl was spotted onto YPD agar. After 2 days of growth at 24°, cells were replica plated onto YPD agar, overlaid with a nitrocellulose filter, and incubated overnight at 30°. Filters were probed with a monoclonal antibody to p2CPY.

were tested for secretion of p2CPY using a filter overlay and immunoblotting with a p2CPY-specific monoclonal antibody. Missorting of p2CPY was detected in *tcs1* through *TCS9-1* and in *tcs12* strains (Figure 2). Qualitatively strong defects were apparent in *tcs2*, *tcs5*, *tcs6*, *tcs7*, *tcs8*, and *TCS9-1* strains (Figure 2; Table 2). *tcs1* and *tcs3* strains exhibited milder phenotypes, while *tcs4* and *tcs12* strains secreted slight but reproducible levels of p2CPY (Figure 2; Table 2). Except for *tcs12* (but see below), the same set of mutants were defective in α-factor precursor maturation and p2CPY sorting, though the relative extent of the defects in each process differed between strains (Table 2). We define this set as class 1 mutants. The synthetic growth defects of *tcs* mutations with the *chc1-521* allele and effects of the class 1 mutations by themselves on α-factor maturation and p2CPY sorting suggest that products of the class 1 *TCS* genes influence protein transport pathways between the TGN and endosomes.

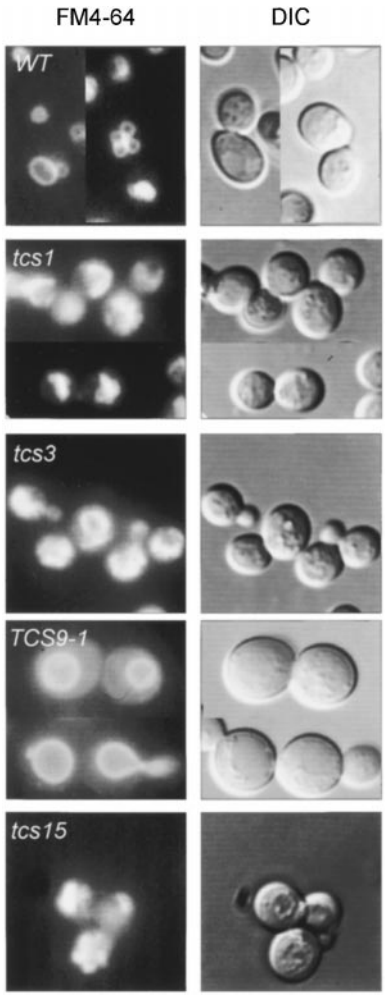

Figure 3.—A subset of *tcs* mutants displays abnormal vacuolar morphology. Wild-type (*WT*, GPY1056), *tcs1* (GPY2172), *tcs3* (GPY2174), *TCS9-1* (GPY2180), and *tcs15* (GPY2186) strains were grown overnight at 30° in SD CAA −ura to midlogarithmic phase. Cells were incubated with FM4-64 for 15 min and resuspended in fresh medium devoid of dye and allowed to internalize the dye for 45 min at 30° before viewing. FM4-64 fluorescence (left) and differential interference contrast (DIC) optics (right) are shown.

***tcs* mutations in *VPS* genes:** Studies of *vps* mutants that missort p2CPY have resulted in identification of >50 genes involved in vacuolar protein transport from the TGN to vacuoles (Bryant and Stevens 1998). To determine whether *tcs* mutations occurred in previously known *VPS* genes, p2CPY secretion was used as an assay in complementation tests between *tcs* mutants and a gallery of *vps* mutants. Diploids from noncomplementing crosses were induced to undergo meiosis and subjected to linkage analysis. By these criteria *vps1*(*tcs2*), *vps5*(*tcs5*), *pep12*/*vps6*(*tcs7*), *vps17*(*tcs8*), and *vps21*(*tcs6*) were present in the class 1 *tcs* collection (Table 2).

Trafficking defects in *vps* strains can result in morphological changes to the vacuole. On the basis of vacuole morphology, the *vps* mutants have been classified into six groups, A–F (Raymond *et al.* 1992). The *vps* mutants identified in the *tcs* screen represent three classes, B, D, and F. Class B (*vps5*, *vps17*) is characterized by fragmented vacuoles, class D (*pep12*, *vps21*) by large single vacuoles, and class F (*vps1*) by large vacuoles encircled by smaller vacuoles. Vacuolar morphology in the *tcs* collection was visualized with the lipophilic vital dye FM4-64, which stains the vacuole membrane (Vida and Emr 1995). In general, the vacuole morphology of strains with *tcs* mutations in known *VPS* genes corresponded to earlier classifications of *vps* mutants. Highly fragmented vacuoles, similar to class B *vps* mutants, were also observed in *tcs1*, *tcs3*, and *tcs15* cells (Figure 3). In addition, *TCS9-1* cells displayed an abnormally large percentage of cells containing single, large vacuoles (Figure 3). An increase in cell size was also observed in the *TCS9-1* strain (Figure 3, compare DIC panel with other panels). The vacuole morphology defects observed in *tcs* strains are consistent with a role of these gene products in membrane traffic to the vacuole.

Of the three *tcs* mutant strains exhibiting both strong

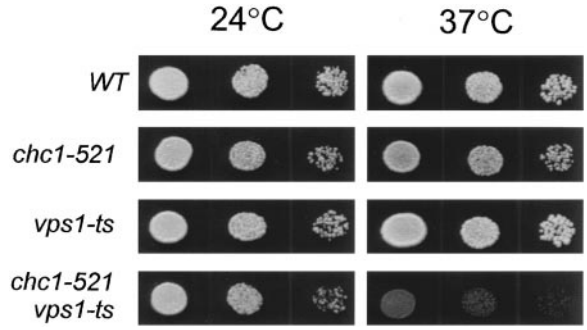

Figure 4.—A temperature-sensitive allele of *VPS1* accentuates the growth defect of *chc1-521* cells. Wild-type (*WT*, GPY1100), *chc1-521* (GPY418), *vps1-ts* (GPY832), and *chc1-521 vps1-ts* (GPY775) strains were grown overnight to saturation at 24° in YPD. Serial dilutions of each culture were spotted on YPD agar and incubated at 24° or 37°.

α-factor maturation and CPY sorting defects (*tcs1*, *tcs2*, *tcs3*), only *tcs2* represented a known *vps* locus, *vps1*. *VPS1* encodes a member of the dynamin family of GTPases (Vater *et al.* 1992). In mammalian cells, dynamin is involved in scission of invaginated clathrin-coated pits at the plasma membrane to form free clathrin-coated vesicles (Schmid 1997; Schmid *et al.* 1998). By analogy, Vps1p and clathrin could function together at the TGN in yeast. Consistent with this idea, similar defects in TGN membrane protein localization have been described in *vps1* and *chc1-521* mutants (Wilsbach and Payne 1993b; Nothwehr *et al.* 1995). To probe the relationship of Vps1p and clathrin in more detail, we constructed a strain containing *chc1-521* and a temperature-sensitive allele of *VPS1* (*vps1-ts*; Vater *et al.* 1992). Growth and α-factor maturation were evaluated in the double mutant, congenic single mutants, and the wild-type strain. At 24°, growth of the single mutants was commensurate with the wild-type strain, but the double mutant grew at a slightly slower rate. At 37°, the *chc1-ts* cells grew somewhat more slowly than the *vps1-ts* and wild-type strains. Compared to the single mutants, growth of the double mutant was severely affected at 37° (Figure 4). A striking synthetic effect of the double mutant combination on α-factor precursor maturation was also apparent (Figure 5). At 30°, only mature α-factor was secreted by the wild-type and *vps1-ts* strains. The congenic *chc1-521* strain used in this comparison secreted a minor amount of highly glycosylated precursor and intermediate cleavage products (16%). In contrast, the double mutant secreted a substantial level of precursor forms (47%). The synergistic effects of *CHC1* and *VPS1* conditional alleles indicate a sensitive functional interdependence of Vps1p and clathrin in cell growth and Kex2p localization in the TGN. These results, together with the identification of *tcs* mutations in other *VPS* genes whose products are known to function in transport between the TGN and endosomes, demonstrate that the *tcs* screen can be an effective approach to define proteins which act in clathrin-dependent transport pathways.

***RIC1* and *LUV1*/*RKI1* are class 1 *TCS* genes:** Four class 1 *tcs* mutants (*tcs1*, *tcs3*, *tcs4*, and *TCS9-1*) did not correspond to a previously identified *vps* complementation group. Two of these mutants, *tcs1* and *tcs3*, displayed growth defects at elevated temperatures (37°) as well as α-factor maturation defects and CPY sorting defects at lower temperatures (24° and 30°). On the basis of the relatively severe α-factor maturation defects in these strains, we focused on identifying the mutant loci. To isolate wild-type versions of *tcs1* and *tcs3*, mutant strains were transformed with a genomic library carried by a centromere-containing plasmid. The resulting transformants were screened for the ability to grow on medium containing 5-FOA. Genomic DNA fragments complementing the 5-FOA growth defect were subsequently dissected to identify the complementing gene (Figure 6).

A single open reading frame containing *RIC1* complemented all mutant phenotypes of *tcs1* mutants (Figure 6; data not shown). A mutant allele of *RIC1* was identified by Mizuta *et al.* (1997) in a screen for genes involved in ribosome synthesis. This *ric1* mutant was reported to be temperature sensitive for growth and to exhibit reduced levels of transcripts encoding both ribosomal protein genes and ribosomal RNA after shift to the nonpermissive temperature (37°; Mizuta *et al.* 1997). However, many secretory pathway mutants display similar ribosome synthesis defects, raising the possibility that reduced synthesis of ribosome components in *ric1* cells is a secondary consequence of a defect in protein trafficking (see discussion; Mizuta and Warner 1994). The primary sequence of the 1056-amino-acid Ric1 protein reveals no significant homology to known proteins or motifs. Disruption of *RIC1* (*ric1*Δ) produced phenotypes identical to those of the *ric1* mutant isolated in the *tcs* screen (data not shown). A cross between the *ric1*Δ strain and a strain carrying the *ric1*/*tcs1* allele resulted in a diploid which displayed *ric1* mutant phenotypes, providing further evidence that the mutant locus in the *tcs1* strain is *RIC1*.

Complementation of the *tcs3* mutant resulted in isolation of *LUV1*/*RKI1*. Introduction of *LUV1* on a centro-

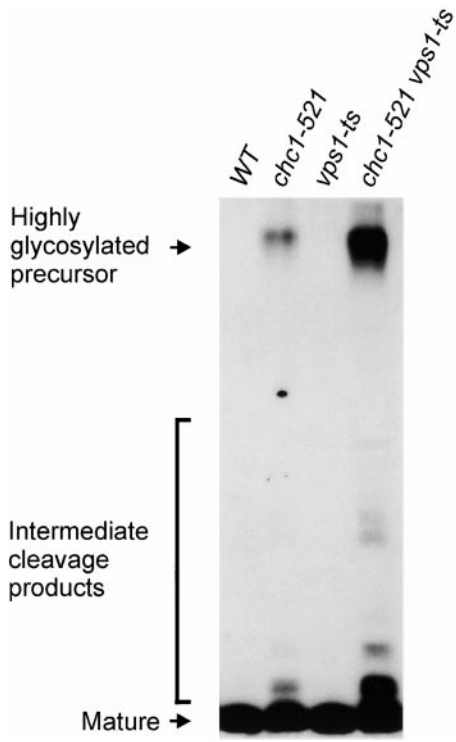

Figure 5.—The α-factor maturation defect of *chc1-521* cells is enhanced by the *vps1-ts* allele. Wild-type (*WT*, GPY1100), *chc1-521* (GPY418), *vps1-ts* (GPY832), and *chc1-521 vps1-ts* (GPY775) strains were incubated at 24° to midlogarithmic growth. After a 2-hr shift to 30°, cells were metabolically labeled at 30° for 45 min, and α-factor was immunoprecipitated as described in the legend to Figure 1.

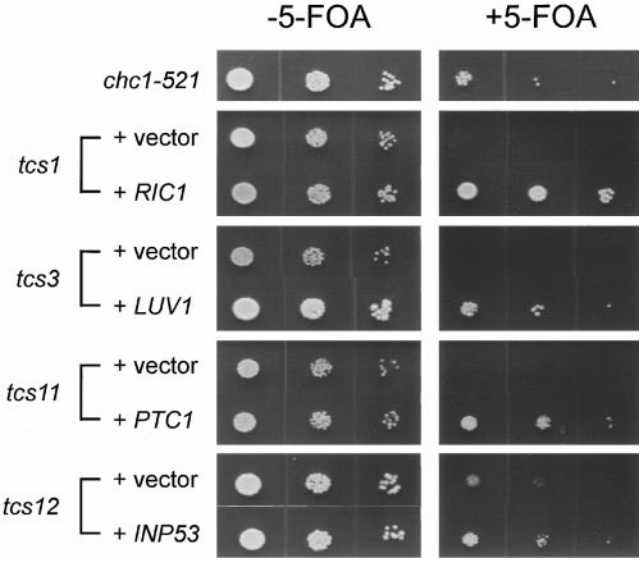

Figure 6.—Complementation of *tcs* mutants with genomic DNA fragments. Strains carrying *tcs* mutant alleles were transformed with plasmids containing the indicated gene isolated from a genomic library or the parental vector (+ vector). Transformants were grown to saturation at 24° in medium selecting for the appropriate plasmid, and serial dilutions were spotted onto supplemented SD (−5-FOA) and 5-FOA-containing media (+5-FOA) and allowed to grow at 30° for 3 days.

meric plasmid was sufficient for complete complementation of the *tcs3* mutant phenotypes (Figure 6; data not shown). *LUV1* also has been isolated through a screen for mutations that cause synthetic growth defects with a calcineurin mutant (M. Conboy and M. Cyert, personal communication) and (as *RKI1*) in a screen for mutations affecting microtubules (Smith *et al.* 1998). *LUV1* encodes an open reading frame that has the potential to encode a 101.5-kD protein with a predicted coiled-coil domain. A BLAST database search with the Luv1p amino acid sequence identified two related open reading frames of unknown function in other organisms: *Schizosaccharomyces pombe* (E value = 4×10^{-12}, accession no. CAB16266) and *Arabidopsis thaliana* (E value = 3×10^{-9}, accession no. CAA16926; Altschul *et al.* 1997). We constructed a strain carrying a disruption of *TCS3*. The phenotypes of this strain mirrored those of the original *tcs3* mutant, including a growth defect at elevated temperatures, secretion of highly glycosylated α-factor, and secretion of p2CPY (data not shown). Mutant phenotypes were not complemented in a diploid from a cross between a *tcs3*Δ strain and a strain carrying the original *tcs3* allele. We conclude that mutation of the evolutionarily conserved product of *LUV1*/*RKI1* is responsible for the phenotypes caused by *tcs3*.

***PTC1* is a class 2 *TCS* gene:** A genomic fragment was also isolated that rescued the *chc1-521*-dependent 5-FOA growth defect of the class 2 *tcs* mutant *tcs11*. *PTC1* was sufficient for rescue of the 5-FOA growth defect (Figure 6). A cross between a *ptc1*Δ *chc1-521* strain carrying a *CHC1 URA3* plasmid and a *tcs11 chc1-521* strain also carrying this plasmid resulted in a diploid unable to grow on 5-FOA-containing medium. The lack of complementation argues that *tcs11* is a mutant allele of *PTC1*. *PTC1* encodes a type 2C serine/threonine phosphatase implicated in multiple cellular functions, including osmotic stress response, tRNA biosynthesis, and mitochondrial inheritance (van Zyl *et al.* 1989; Maeda *et al.* 1993, 1994; Roeder *et al.* 1998). A strain with a disrupted copy of *PTC1* displayed normal α-factor maturation, CPY sorting, endocytosis as assayed by α-factor internalization, and vacuolar morphology (data not shown). Therefore, at present the only connection between *PTC1* and protein trafficking is the synthetic growth defect with *chc1-521*.

We considered the possibility that synthetic growth defects of *ptc1* and *chc1-521* derive from the role of Ptc1p in osmotic stress response. Cells challenged by high external osmolarity increase internal osmolarity by activating a MAP kinase signal transduction pathway (the HOG pathway) that includes the MAP kinase Hog1p and the MAP kinase kinase Pbs2p (Brewster *et al.* 1993). Genetic experiments suggest that Ptc1p can downregulate the HOG pathway through activities on Hog1p and/or Pbs2p (Maeda *et al.* 1994). Thus, the absence of Ptc1p could allow increased basal activity of the HOG pathway MAP kinase, leading to an increase in internal osmolarity. If plasma membrane composition was altered by the defect in clathrin, an increase in internal osmolarity might lead to cell lysis. As a test of this possibility, Hog1p was eliminated by gene disruption in the *ptc1*Δ *chc1-ts* strain carrying the *CHC1 URA3* plasmid. As a control, *HOG1* was also disrupted in a *chc1-521* carrying the *CHC1 URA3* plasmid. Disruption of *HOG1* did not suppress the 5-FOA growth defect in the *ptc1*Δ *chc1-ts* strain and did not itself cause synthetic growth defects with *chc1-521*. These findings argue against the idea that synthetic lethality caused by *ptc1* is due to upregulation of the HOG pathway.

***INP53* is a *TCS* gene:** The library fragment that complemented *tcs12* carried *INP53*/*SJL3*/*SOP2*, one of three genes (*INP51*, *INP52*, or *INP53*) encoding synaptojanin-like inositol polyphosphate 5-phosphatases (Srinivasan *et al.* 1997; Singer-Kruger *et al.* 1998; Stolz *et al.* 1998; Guo *et al.* 1999). *INP53* was identified previously in a screen for mutations that suppress vacuolar targeting of a mutant form of the plasma membrane ATPase (Luo and Chang 1997). The mutant allele of *INP53* identified in the screen, designated *sop2*, caused a subtle α-factor maturation defect at 24°. The *tcs12* mutant allele isolated in our screen was originally assigned to class 2 based on the absence of an α-factor maturation defect at 30°. Recognition of *tcs12* as *inp53* prompted us to reevaluate the α-factor maturation phenotype of *inp53* cells and compare the effects of *inp53* to *inp51* and *inp52*. For

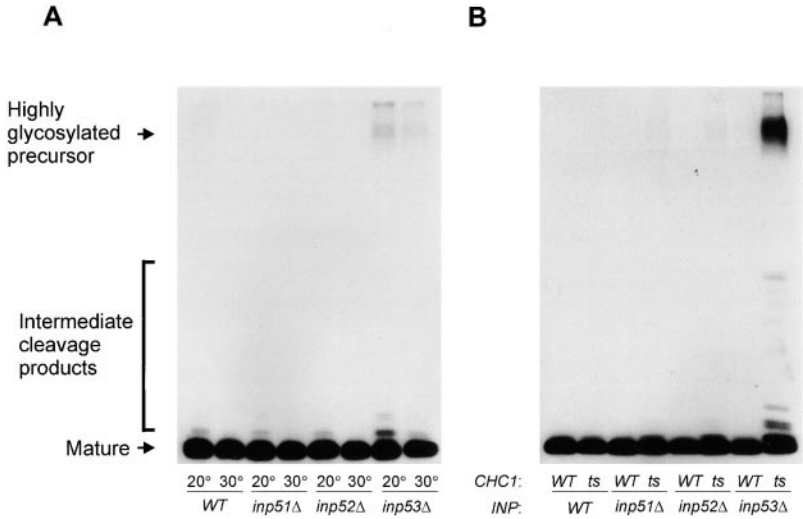

Figure 7.—Low temperature and the *chc1-521* allele accentuate the α-factor maturation defect of *inp53*Δ cells. (A) Wild-type (*WT*, SEY6210), *inp51*Δ (GPY2078), *inp52*Δ (GPY2062), and *inp53*Δ (GPY1876) strains were grown at 24° to midlogarithmic phase, shifted to 20° or 30° for 15 min, and labeled with [^{35}S]methionine/cysteine for 45 min at 20° or 30 min at 30°. α-Factor was immunoprecipitated from the culture supernatant and subjected to SDS-PAGE and autoradiography. (B) Wild-type (*WT*, SEY6210), *chc1-521* (GPY982), *inp51*Δ (GPY2078), *chc1-521 inp51*Δ (GPY2141), *inp52*Δ (GPY2062), *chc1-521 inp52*Δ (GPY2142), *inp53*Δ (GPY1876), and *chc1-521 inp53*Δ (GPY2143) strains were grown to midlogarithmic phase in SDYE media and metabolically labeled with [^{35}S]methionine/cysteine for 45 min at 24°. α-Factor was immunoprecipitated from the culture supernatant and subjected to SDS-PAGE and autoradiography.

this purpose, isogenic strains were generated carrying disruptions of either *INP51*, *INP52*, or *INP53*. We examined α-factor maturation after shifting cells from 24° to either 30° or 20°, choosing 20° by reasoning that a defect in a process involving the lipid bilayer might be exaggerated at lower temperatures. Wild-type, *inp51*Δ, and *inp52*Δ strains secreted exclusively mature α-factor at 30° (Figure 7A). At this temperature, a minor maturation defect was apparent in the *inp53*Δ strain (Figure 7A). At 20°, the defect in *inp53*Δ cells was more pronounced, whereas the other two *inp* mutants were essentially unaffected by this temperature shift (Figure 7A). These results prompted reassignment of *tcs12* as a class 1 mutation and suggest a specific role for Inp53p in Kex2p localization.

To explore the specificity of genetic interactions between *inp53* and *chc1-521*, the *INP* genes were individually disrupted in a *chc1-521* strain carrying the *CHC1 URA3* plasmid. As shown in Figure 8A, only the combination of *inp53*Δ with *chc1-521* resulted in inviability on 5-FOA medium at 30°. Combining *inp51*Δ with *chc1-ts* did not prevent growth on 5-FOA, but the colonies were smaller than those from the *chc1-521* control strain. The *inp52*Δ allele had no effect when combined with *chc1-521*. As an alternative approach to examining synthetic growth defects, a diploid strain heterozygous for *inp53*Δ and homozygous for *chc1-521* was induced to sporulate, and the meiotic progeny were subjected to tetrad analysis. Each tetrad yielded four viable segregants, indicating that the *inp53 chc1-521* combination is not lethal when double mutants are obtained by this method. This finding was not totally unexpected given earlier findings with *chc1*Δ strains, indicating that loss of a complementing plasmid on 5-FOA is a more stringent growth condi-

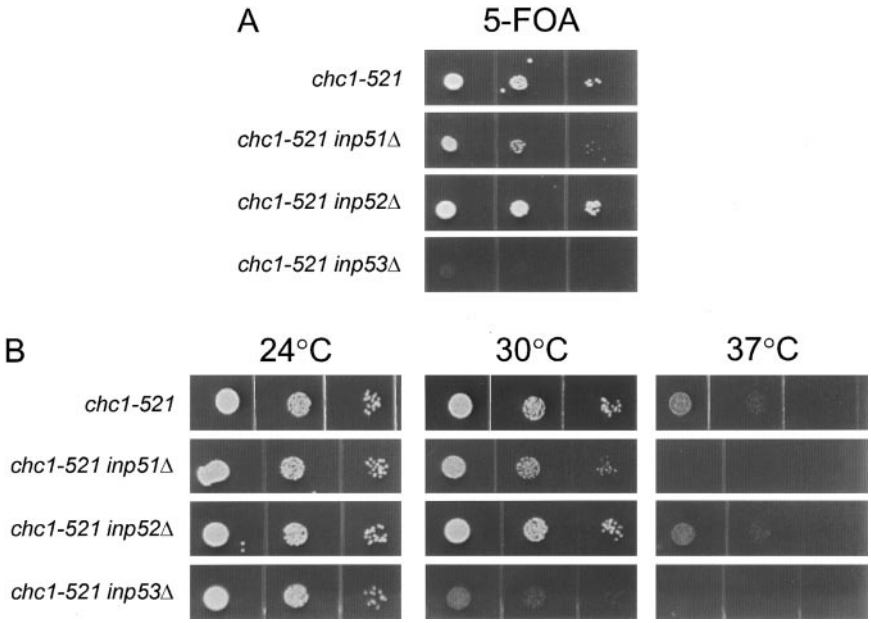

Figure 8.—*inp53*Δ accentuates the growth defect of *chc1-521* cells. (A) *chc1-521* (GPY1056), *chc1-521 inp51*Δ (GPY2064), *chc1-521 inp52*Δ (GPY2162), and *chc1-521 inp53*Δ (GPY1877) strains, all of which carry the pULE-CHC1 plasmid, were grown to saturation in YPD media at 24°. Serial dilutions of each culture were spotted onto 5-FOA media and allowed to grow at 30°. (B) *chc1-521* (GPY982), *chc1-521 inp51*Δ (GPY2141), *chc1-521 inp52*Δ (GPY2142), and *chc1-521 inp53*Δ (GPY2143) strains were grown to saturation at 24° in YPD media. Serial dilutions of each culture were spotted onto YPD agar and allowed to grow at 24°, 30°, or 37°.

tion than direct incubation of a mutant on standard medium (see above). The viability of *inp53*Δ *chc1-521* meiotic progeny encouraged us to disrupt the individual *INP* genes directly in *chc1-521* haploids, generating a set of congenic double mutants. Growth of each double mutant at 24° was equivalent to the wild-type growth of the parental *chc1-ts* strain (Figure 8B). A striking growth defect was observed when the *inp53*Δ *chc1-521* strain was incubated at 30° and 37° (Figure 8B). Growth of the *inp51*Δ *chc1-521* strain was also compromised at the elevated temperatures but less than growth of the *inp53*Δ *chc1-521* strain. The *inp52*Δ *chc1-521* strain mimicked the *chc1-521* parental strain at 30° and 37°. The specificity of the *inp chc1-521* interactions was probed further by analyzing α-factor maturation in each double mutant at the permissive growth temperature, 24° (Figure 7B). Little or no defect was apparent in any of the single mutants (Figure 7B). In the double mutants, pairing *chc1-521* with either *inp51*Δ or *inp52*Δ had only a marginal effect on α-factor maturation, but combining *chc1-521* and *inp53*Δ produced a strong pheromone maturation defect (42% precursor forms). These results are consistent with effects of the individual *inp* mutations on α-factor maturation and point to a specific role for Inp53p in clathrin-mediated TGN localization of Kex2p. It remains to be determined whether, like *inp53*, viable combinations of other class 2 *tcs* mutations with *chc1-521* can be isolated and whether such combinations display TGN sorting defects.

DISCUSSION

As a genetic strategy to identify proteins involved in clathrin-dependent protein transport pathways, we carried out a screen for mutations that cause synthetic growth defects in a strain expressing a partially functional clathrin heavy chain. The *tcs* mutations recovered in this screen divide into two classes based on their effects on protein trafficking in the TGN/endosome system. Class 1 mutations cause defects in α-factor maturation, a reliable signature of Kex2p mislocalization, and defects in biosynthetic sorting of CPY to the vacuole. Measurements of Kex2p stability in selected class 1 mutants indicate higher than normal rates of Kex2p vacuolar degradation (data not shown), supporting conclusions based on the α-factor maturation assay. Class 2 mutations do not affect Kex2p localization or CPY sorting.

One of the genes represented in the *tcs* collection was *INP53*, encoding a type II inositol polyphosphate 5-phosphatase. Classification of Inps is based primarily on substrate specificity of animal cell enzymes, and type II Inps characteristically are able to hydrolyze the C5 phosphate from the inositol moiety of both phosphatidylinositol 4,5-bisphosphate (PI[4,5]P_2) and phosphatidylinositol 3,4,5-trisphosphate (PI[3,4,5]P_2) (Erneux *et al.* 1998). A subset of type II INPs is further distinguished by the presence of a domain related to the yeast protein Sac1p, a protein implicated in phospholipid metabolism and vesicular trafficking (Cleves *et al.* 1989; Whitters *et al.* 1993). Recently, the Sac1 domain was found to encode a novel polyphosphoinositide phosphatase activity, indicating that this subgroup of type II Inps has the potential to express two distinct inositol phosphatase activities from one polypeptide (Guo *et al.* 1999). Synaptojanin, the founding member of the Sac1 domain-containing INP subfamily, has been proposed to act in clathrin-dependent endocytosis in nerve terminals (McPherson *et al.* 1996; Haffner *et al.* 1997). Of the four yeast proteins with homology to INPs, three, Inp51–53, also carry an N-terminal Sac1p domain and are alternatively referred to as synaptojanin-like (Sjl; Srinivasan *et al.* 1997; Singer-Kruger *et al.* 1998; Stolz *et al.* 1998). However, the Sac1-like domain in Inp51p is catalytically inactive (Guo *et al.* 1999). Systematic analyses of endocytic and vacuolar sorting pathways in strains with individual disruptions of the *INP* genes have been carried out. These studies revealed only subtle vacuolar morphology anomalies in *inp52* or *inp53* cells, but no defects in CPY sorting or in endocytic uptake of the lipophilic endocytic tracer FM4-64 (Srinivasan *et al.* 1997; Singer-Kruger *et al.* 1998; Stolz *et al.* 1998). Double mutant combinations have no effect on CPY sorting but result in varying levels of endocytic defects; the *inp51 inp52* combination is more detrimental than *inp52 inp53*, while the *inp51 inp53* pair is innocuous (Srinivasan *et al.* 1997; Singer-Kruger *et al.* 1998; Stolz *et al.* 1998). Although results from double mutants offer some distinction between the Inps, particularly with respect to endocytosis, the absence of informative phenotypes in single mutants has hindered functional definition of specific roles for any of the individual Inps in vesicular transport events. Our results indicate that, of the three synaptojanin-like *INP* genes, only disruption of *INP53* results in an α-factor maturation defect at 20° and 30°, accentuation of α-factor maturation defects when combined with *chc1-521* at 24°, and an inability to grow on 5-FOA when introduced into *chc1-521* cells carrying a *CHC1 URA3* plasmid. Additionally, on standard media, the combination of *inp53*Δ with *chc1-521* showed stronger synthetic growth defects than either *inp51*Δ or *inp52*Δ. These findings argue that Inp53p specifically affects the clathrin-dependent TGN to endosome traffic pathway. Our studies confirm and extend the work of Luo and Chang (1997), who detected minor levels of secreted α-factor precursor and marginally decreased steady-state levels of Kex2p in *inp53* cells at 24°, but did not analyze mutations in other *INP* genes. By analogy to the proposed function of synaptojanin in endocytic clathrin-coated vesicle traffic, we suggest that Inp53p participates in clathrin-coated vesicle traffic from the TGN.

The synthetic growth defect of *chc1-521 inp51*Δ cells, although less severe than that of *chc1-521 inp53*Δ cells,

suggests that Inp51p could also participate in a clathrin-dependent transport process. Since *inp51*Δ alone, or in combination with *chc1-521*, did not alter α-factor maturation, Inp51p is more likely to be involved in endocytosis. In support of this idea, *inp51* causes synthetic lethality when combined with a temperature-sensitive allele of *PAN1* (Wendland and Emr 1998). Mutations in *PAN1* affect endocytosis, and the Pan1 protein is homologous to the mammalian clathrin accessory protein, Eps15 (Wendland *et al.* 1996; Tang *et al.* 1997). Furthermore, *inp51* combined with *inp52* causes severe endocytosis defects (Singer-Kruger *et al.* 1998). Unlike *inp51* and *inp53*, *inp52* did not display synthetic effects with *chc1-521*. Thus, if Inp52p is involved in clathrin-dependent transport, its contribution is not substantial enough to be detected by our assays.

A number of class 1 *tcs* mutations occurred in known *VPS* genes, including *VPS1*. The genetic interaction between *VPS1* and *CHC1* extends to temperature-sensitive alleles, which produce synthetic defects in growth and α-factor maturation, suggesting a strong connection between the functions of Vps1p and clathrin. Vps1p is a member of the dynamin family of GTPases (Vater *et al.* 1992). In mammalian cells, dynamin assembles into a ring around the necks of invaginated clathrin-coated pits at the plasma membrane (Takei *et al.* 1995). It is generally agreed that GTP hydrolysis is necessary to sever the connection between the nascent vesicle and plasma membrane, though the model that dynamin itself is the severing agent has been recently challenged (Sweitzer and Hinshaw 1998; Sever *et al.* 1999). Given the interaction of dynamin and clathrin in mammalian cell endocytosis, it is reasonable to posit a similar relationship between Vps1p and clathrin. Consistent with this view, *vps1* and *chc1* cells share a similar, and distinctive, defect in TGN membrane protein localization. In both types of mutant cells, TGN membrane proteins are mislocalized to the cell surface. In *chc1* cells, the additional endocytic defect causes accumulation at the cell surface, whereas in *vps1* cells the TGN proteins are internalized and delivered to the vacuole (Seeger and Payne 1992b; Nothwehr *et al.* 1995). Since TGN membrane protein localization involves cycling between the TGN and endosomes, plasma membrane mislocalization in *vps1* and *chc1* cells suggests that these mutations block the endosome-targeted pathway at the TGN. The observation of synthetic effects on α-factor maturation by temperature-sensitive alleles of *VPS1* and *CHC1* now establishes a functional connection between the gene products in TGN membrane protein localization. Additional experiments, particularly subcellular localization of Vps1p and clathrin, will be needed to test for physical links.

Mutant alleles of four other well-characterized *VPS* genes were identified in the *tcs* screen (*vps21*, *pep12*/*vps6*, *vps5*, and *vps17*). Vps21p, a small GTPase of the Ypt/rab family, and Pep12p, an endosomal t-SNARE, are proposed to act in targeting and fusion of TGN-derived vesicles with endosomes (Horazdovsky *et al.* 1994; Becherer *et al.* 1996). Interestingly, Vps5p and Vps17p form a subcomplex within a multimeric assembly of Vps proteins thought to constitute a vesicle coat essential for endosome to TGN traffic (Horazdovsky *et al.* 1997; Seaman *et al.* 1998). Considering the current model that clathrin functions in vesicular transport between the TGN and endosomes, it is not surprising to find genetic interactions between *chc1-521* and mutations that affect other stages of TGN-endosome membrane trafficking. Since the *tcs* screen was not carried out to saturation, a more systematic examination will be needed to determine whether the *vps*/*tcs* mutations identify a subset of *VPS* genes particularly sensitive to clathrin deficiencies or whether genetic interactions with *chc1-521* will prove to be a ubiquitous *VPS* feature.

Two class 1 genes, *RIC1* and *LUV1*/*RKI1*, have not been implicated previously in vesicular transport. RIC1 was originally identified in a screen for temperature-sensitive mutants with reduced synthesis of ribosome components. At the elevated temperature, transcript levels of both ribosomal proteins and RNA were lowered (Mizuta *et al.* 1997). However, a similar reduction in ribosomal protein and rRNA synthesis has been documented for *ts* mutations affecting different stages of the secretory pathway, revealing a regulatory pathway connecting secretory pathway defects to ribosome biosynthesis (Mizuta and Warner 1994). Several observations suggest that the defects in synthesis of ribosomal components in *ric1* cells are similarly indirect, stemming from a primary defect in vesicle trafficking. First, although the *ric1*/*tcs1* strain is temperature sensitive for growth, we observed α-factor maturation defects, CPY missorting, and vacuole fragmentation at permissive growth temperatures where no obvious defects in protein synthesis were apparent. Second, in the genetic background used in the *tcs* screen, disruption of *RIC1*, or introduction of the *ric1* allele identified in the ribosome synthesis screen, yielded the same spectrum of trafficking defects at permissive growth temperatures that were detected in the *ric1*/*tcs1* cells (E. Bensen and G. Payne, unpublished results). The primary sequence of Ric1p does not offer clues about molecular function. Further experiments analyzing *ric1* mutants and the Ric1 protein are in progress.

TCS3/*LUV1*/*RKI1* (which we will refer to as *LUV1*) has been identified in multiple screens. In our studies, the original *tcs3* allele and a *LUV1* disruption cause a substantial α-factor maturation defect, CPY missorting, and vacuole fragmentation at permissive growth temperatures, suggesting a role for Luv1p in vesicle traffic between the TGN and endosomes. A *luv1* allele was also isolated in a screen for mutations that cause synthetic growth effects with a disruption of the regulatory subunit of calcineurin encoded by *CNB1* (M. Conboy and M. Cyert, personal communication). This analysis dem-

onstrated vacuole fragmentation in *luv1* cells, partial secretion of the Golgi form of CPY, and sensitivity of cell growth to a variety of ions (M. Conboy and M. Cyert, personal communication). In addition, Luv1p has been implicated recently in microtubule function. Cells harboring *luv1*Δ/*rki1*Δ are hypersensitive to microtubule-depolymerizing drugs, and at the nonpermissive growth temperature they display a loss of microtubule structures. Furthermore, Luv1p can bind to Rbl2p, a protein associated with free β-tubulin subunits in cells (Archer *et al.* 1995; Smith *et al.* 1998). The pleiotropic defects in cells lacking Luv1p make it difficult to ascribe a direct role for the protein in any one of the affected processes. It may be that Luv1p provides a single cellular function that impacts multiple aspects of cell physiology. Alternatively, the protein may directly function in distinct pathways. For example, the view that Rbl2p acts in managing assembly of tubulin heterodimers could indicate a general function for Luv1p in regulating assembly of multimeric protein assemblies. Isolation of a conditional allele of *LUV1* and further examination of Luv1p-interacting partners may allow these possibilities to be distinguished.

The class 2 *tcs* mutation in the gene encoding the type 2C serine/threonine protein phosphatase Ptc1p caused synthetic growth defects with *chc1-521* but did not affect TGN-endosome protein transport. Synthetic effects can result from mutations in genes whose products act in the same process or pathway, but they can also occur when mutant proteins function in distinct processes (Guarente 1993). Particularly when considering effects on cell growth, the possibility of indirect interactions cannot be minimized. For class 1 mutations, autonomous effects on clathrin-mediated trafficking steps link the products of these *TCS* genes to protein sorting at the TGN. For class 2 mutations, connections to clathrin are more tenuous. One possibility is that these mutations affect endocytosis, but preliminary experiments do not indicate strong effects on the trafficking of the mating pheromone **a**-factor receptor (E. Bensen, unpublished results). Published studies of Ptc1p function do not offer significant clues to the basis of its synthetic effects with *chc1-521*. Ptc1p has been proposed to participate in a multitude of cellular functions, including osmoregulation, tRNA synthesis, DNA recombination, cell wall β-glucan assembly, and mitochondrial inheritance (van Zyl *et al.* 1989; Maeda *et al.* 1994; Huang and Symington 1995; Jiang *et al.* 1995; Roeder *et al.* 1998). It was possible that the synthetic growth defects in *ptc1 chc1-521* cells are due to upregulation of basal osmotic stress response (HOG) pathway activity, thereby eliciting an increase in internal osmolarity that could indirectly intensify defects caused by the *chc1-521* allele. However, disruption of *HOG1* did not restore growth to *chc1-521 ptc1* cells, making this scenario unlikely. This experiment also suggests that the effects of *ptc1* are not mediated through the recently discovered role of Hog1p in Golgi membrane protein localization (Reynolds *et al.* 1998). Thus, given the pleiotropic activities of Ptc1p, a direct role for this phosphatase in clathrin-dependent trafficking is possible but remains to be established.

In summary, characterization of *tcs* mutants demonstrates that the screen for mutations that cause synthetic growth interactions with *chc1-521* constitutes a robust method for identifying proteins that influence clathrin-dependent transport pathways. Our findings strengthen connections between Vps and clathrin function, offer new insights into the specificity of inositol polyphosphate 5-phosphatases, and raise the possibility of membrane-trafficking roles for proteins thought to act in other processes. These results provide a genetic foundation to guide molecular analysis of the proteins identified through the *tcs* screen, and suggest that further application of the screen will allow novel insights into clathrin-mediated traffic routes.

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