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Identification of yeast proteins that can compensate for loss of the Gcs1 ARF GAP

by

Xiangmin Wang

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

at

Dalhousie University

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by Xiangmin Wang

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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For my family

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ABSTRACT

The yeast Gcs1 protein is a GTPase-activating protein for yeast Arf1 and Arf2, and it is involved in intracellular vesicular transport. Mutations in the *GCS1* gene produce a novel growth defect. At a restrictive temperature, *gcs1* mutant cells are specifically impaired for resumption of cell proliferation from stationary phase. In contrast, actively dividing *gcs1* cells can maintain ongoing cell proliferation at the temperature that is restrictive for stationary-phase mutant cells. To further study the function of Gcs1 and the cellular processes taking place during the resumption of cell proliferation from stationary phase, I identified seven yeast genes which, when present in multiple copies, suppress the growth defect of *gcs1* mutant cells. Of these seven genes, *YCK1*, *YCK2*, and *YCK3* encode three different membrane-associated casein kinase I isoforms, *CDC55* encodes a regulatory subunit of protein phosphatase 2A, *YPT31* and *YPT32* encode small GTP-binding proteins that belong to the YPT/Rab family of proteins, and *IMH1* encodes a protein with extensive coiled-coil motifs.

Membrane association of the casein kinase I enzymes is important for their ability to suppress the growth defect of *gcs1* mutant cells, and a decrease in the Yck1 + Yck2 activity exacerbates that growth defect. These observations indicate that membrane-associated casein kinase I and Gcs1 are involved in a common process. Similarly, a decrease in the Ypt31 + Ypt32 activity also exacerbates the growth defect of *gcs1* mutant cells, suggesting that Ypt31 + Ypt32 and Gcs1 are also functionally related. Furthermore, increased casein kinase I activity suppresses the growth defect caused by insufficient Ypt31 + Ypt32 activity. Therefore, it is likely that increased activities of Ypt31 + Ypt32 and casein kinase I activate a parallel pathway that bypasses the need for Gcs1 during the resumption of cell proliferation from stationary phase. The non-essential Imh1 protein is structurally similar to Rabaptin-5, a direct effector of Rab5, and Imh1 has been reported as a low-copy suppressor of *ypt6*, suggesting that Imh1 might also be involved in vesicular transport.

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ABBREVIATIONS

bp	base pairs
ER	endoplasmic reticulum
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine Nucleotide Exchange Factor
GTP	Guanosine triphosphate
h	hour
kbp	kilobase pairs
MBN	Mung Bean Nuclease
min	minute
ORF	open reading frame
PCR	polymerase chain reaction
rpm	revolutions per minute
sec	second
TGN	*trans*-Golgi network

I. INTRODUCTION

1. Preface

Microorganisms, such as the budding yeast *Saccharomyces cerevisiae*, proliferate in response to nutrients. When nutrients are depleted, yeast cells cease cell proliferation and enter the nonproliferating state termed stationary phase. Stationary-phase cells acquire physiological properties that are different to those of actively proliferating cells, so that they can maintain viability for extended periods of time under starvation conditions. When stationary-phase cells encounter fresh nutrients, they can resume cell proliferation again (Hartwell, 1974; Pringle and Hartwell, 1981; Werner-Washburne *et al.*, 1993). Since starvation is one of the most common stresses encountered by living organisms and much of the microorganism biomass in the world is estimated to exist under nutrient-depleted conditions (Lewis and Gattie, 1991), the ability of yeast cells to interconvert between the proliferating state and the nonproliferating quiescent state is therefore important for their survival and propagation. A failure either in entering stationary phase or in resumption of cell proliferation causes yeast cells to lose viability under starved conditions and to be unable to proliferate when there are ample nutrients available.

The previous isolation of *gcs1* mutant cells, conditionally defective only for the resumption of cell proliferation from stationary phase (Drebot *et al.*, 1987), provided genetic evidence that stationary phase is a unique developmental state, and that the requirements for stationary-phase cells to resume cell proliferation are different from those for maintaining ongoing cell proliferation. Recent work from our laboratory has revealed that the Gcs1 protein, which is needed for stationary-phase cells to resume cell proliferation, is a component of the intracellular vesicular transport machinery (Ireland *et al.*,1994; Poon *et al.*, 1996). In this thesis, I have extended the previous genetic and molecular studies of the *gcs1* mutant phenotype by further exploring the cellular processes that regulate the transition between stationary phase and active cell

proliferation. To provide the context in which this work has been undertaken, this introduction first describes the experimental system and then briefly reviews our current understanding of stationary phase as a morphologically and genetically unique state. Genetic and molecular characterization of the *gcs1* mutant phenotype and the function of Gcs1 is then introduced, followed by a consideration of why intracellular vesicular transport is important for yeast cell growth. The final section of this introduction details the objectives of this study.

2. *Saccharomyces cerevisiae* as a model system for studying the regulation of cell proliferation

The budding yeast *Saccharomyces cerevisiae* (or yeast) is a unicellular fungus, and several features of its life cycle make it an ideal model system for genetic studies of eukaryotic cellular processes, including cell proliferation.

First, yeast cells can reproduce almost as rapidly as bacteria, so that genetic studies on the segregation of mutations affecting the cell-division cycle can be carried out in a much shorter period of time compared with mammalian systems.

Second, yeast cells can proliferate either in the haploid or in the diploid form, and these two forms are interconvertable. The diploid cell when starved for nitrogen can undergo meiosis to form haploid cells. On the other hand, haploid cells of two different mating types can mate (conjugate) to form diploid cells. The haploid form makes it easy to study the phenotype of a mutation without the complication of a second copy of the gene in the cell. Therefore mutations that cause recessive phenotypes can also be studied in haploid cells. On the other hand, diploid cells can be induced to undergo meiosis in a process termed sporulation, allowing analysis of the resultant meiotic haploid progeny to determine genetic linkage relationships and to analyze genetic interactions (Mortimer and Hawthorne, 1969). These analyses are important for studying functional relationships

among different gene products and for dissecting genetic pathways.

Third, the morphological changes that occur as cells progress through the cell-division cycle make yeast a particularly useful organism for studying cell proliferation. As the name "budding" implies, *S. cerevisiae* divides by the formation of a protrusion, or bud, on the cell surface, which gradually enlarges to form a new cell. The presence of a bud is a morphological signal that the cell has initiated a division process, and the emergence of the bud also coincides with the beginning of DNA synthesis (Williamson, 1965; Hartwell, 1974; Johnston *et al.*, 1980). Stationary-phase cells exhibit a uniform cell morphology of single cells without a bud (Hartwell, 1974), indicating that these cells have ceased proliferation and that this cessation of cell proliferation occurs in the cell-cycle phase prior to DNA synthesis.

Fourth, molecular genetic techniques are very well developed for studies with yeast. Yeast cells can efficiently take up and maintain plasmid DNA through the process of transformation. High-efficiency transformation techniques, as well as techniques for the cloning by functional complementation, make yeast amenable for the isolation of genes of interest. Gene-disruption and gene-replacement techniques allows us to generate desired mutant genes directly in chromosomes so as to study their effects on cellular processes *in vivo* (reviewed in Struhl, 1983).

The genetic and molecular characterization of the yeast cell-division process has made great contributions to the understanding of eukaryotic cell-cycle control machinery in general, suggesting that understanding the stationary phase in yeast will also provide insight into the quiescent state of other eukaryotic cells.

3. Stationary phase as a unique state

A) Characteristics of stationary-phase cells

As discussed above, stationary-phase yeast cells can retain the ability to resume cell proliferation after being quiescent for long periods under conditions of nutritional stress.

While little is known about the mechanisms by which stationary-phase cells survive such long periods of nutritional limitation, it has been shown that stationary-phase cells acquire characteristics that differ from those of actively proliferating cells (reviewed in Werner-Washburne *et al.*, 1993). Therefore, stationary phase is a distinct physiological state.

First, stationary-phase cells have distinct physiological characteristics. In response to nutrient starvation, yeast cells accumulate storage carbohydrates such as glycogen and trehalose (Lillie and Pringle, 1980). While glycogen is considered to be a reserve carbohydrate that is mobilized to supply glucose during the onset of stationary phase, trehalose has been suggested to protect cells from desiccation and other stresses rather than simply to serve as a reserve carbohydrate (Panek, 1991; Wiemken, 1990). The accumulation of storage carbohydrates is also observed when cells face other stresses such as heat shock, and its role in maintaining cell viability in stationary phase needs to be further studied. The cell wall of stationary-phase cells is also resistant to enzymatic digestion due to a thickened cell-wall structure (DeNobel *et al.*, 1990). It has been suggested that changes in the structure of cell-wall mannoprotein contributes to this thickening of the cell wall (Valentin *et al.*, 1987). This altered cell-wall structure and the accumulation of storage carbohydrates is correlated with increased resistance of stationary-phase cells to heat stress (thermotolerance). The heat shock protein Hsp104 has also been suggested to contribute to the ability of stationary-phase cells to survive brief exposures to high temperatures (~ 55°C) (Sanchez *et al.*, 1992).

Second, biosynthetic activities are dramatically decreased in stationary-phase cells, and overall transcription and translation are decreased approximately 20-fold (Choder, 1991; Boucherie, 1985). Most mRNA species that are abundant in actively proliferating cells are barely detectable in stationary-phase cells. It has been shown that this general transcriptional repression requires a functional topoisomerase I enzyme, which is encoded by the *TOP1* gene (Choder, 1991). Mutations in the *TOP1* gene cause a failure

of stationary-phase cells to repress transcription. Since topoisomerase I affects DNA supercoiling, it might have an important role in repositioning nucleosomes which may contribute to the decrease in transcriptional activity. The rate of protein synthesis in stationary-phase falls to 10% or less of that observed in actively proliferating cells (Bissinger *et al.*, 1989). This general decrease in biosynthetic activity might reflect the nutrient limitation faced by stationary-phase cells, as proliferating cells require fresh nutrient for an energy supply to maintain a high level of biosynthetic activity.

Third, stationary-phase cells exhibit patterns of gene expression different from those of actively proliferating cells. While the overall transcriptional activity has been shown to decrease approximately 20-fold as cells enter stationary phase (Choder, 1991; Boucherie, 1985), not all mRNA levels decrease in cells as they enter stationary phase. For instance, *ENO1* (McAlister and Holland, 1985) and *BCY1* (Doherty and Werner-Washburne, personal communication) mRNA levels are quite consistent in both actively proliferating and stationary-phase cells, whereas the abundance of *UBI4* (Finley *et al.*, 1987) mRNA actually increases several fold when cells enter stationary phase. Other mRNAs that are not readily detectable in actively proliferating cells accumulate to relatively high levels in stationary-phase cells, including the mRNAs from *ACH1*, encoding acetyl coenzyme A hydrolase (Lee *et al.*, 1990); *CTT1*, encoding catalase (Bissinger *et al.*, 1989); *PRB1*, encoding the vacuolar protease B (Moehle *et al.*, 1990); *UBC5*, encoding a ubiquitin-conjugating protein (Seufert and Jentsch, 1990); the heat shock genes *HSP26* (Petko and Lindquist, 1986), *HSP12* (Praekelt and Meacock, 1990), *HSP82* (Borkovich *et al.*, 1989), and *HSP104* (Sanchez and Lindquist, 1990); the *HSP70*-related gene *SSA3* (Werner-Washburne *et al.*, 1989); and *CYC7*, encoding the minor isoform of the mitochondrial cytochrome *c* (Pillar and Bradshaw, 1991). Of all these genes, *UBI4* and *HSP104* have been shown to be important for the long-term survival of stationary-phase cells under nutrient-depleted conditions (Finley *et al.*,1987; Sanchez *et al.*, 1992). The functions of other genes in maintaining the viability of stationary-phase

cells need to be further studied.

B) *gcs1* provides genetic evidence that stationary phase is a unique developmental state

While stationary-phase cells do have many distinct characteristics as discussed above, many of those characteristics are not unique to stationary-phase cells. Accumulation of storage carbohydrates, resistance to enzymatic digestion of cell wall and increased thermotolerance can also be manifested by cells that are proliferating slowly in glucose-limited chemostat cultures (Elliot and Futcher, 1993; Plesset *et al.*, 1987; Bujega *et al.*, 1982). Accumulation of trehalose and increased thermotolerance are also observed in proliferating cells after heat shock. Therefore, until recently it was not clear whether stationary phase is merely a cessation of ongoing cell division or a unique state that represents an offshoot of the cell-division cycle. The characterization of a novel phenotype caused by a mutation in the *GCS1* gene has provided genetic evidence that stationary phase is a unique developmental state.

It has been found previously in our lab that while *gcs1* mutant cells behave just like wild-type cells at the permissive temperature of 29°C, they have an interesting growth defect when grown at the restrictive temperature of 15°C. That is, when stationary-phase *gcs1* mutant cells are transferred to fresh medium, they fail to resume cell proliferation at the restrictive temperature of 15°C. In contrast, actively proliferating *gcs1* mutant cells, when transferred to the low temperature of 15°C, can continue to proliferate indefinitely as long as fresh nutrients are provided (Drebot *et al.*, 1987; Ireland *et al.*, 1994). These observations show that *gcs1* mutant cells have a conditional defect only for the resumption of cell proliferation from stationary phase, and that the requirements to resume cell proliferation are different from those required for maintaining cell proliferation. Therefore, stationary-phase cells are in a unique state, and represent a departure from the cell-division cycle. To understand the special requirements that must

be met for stationary-phase cells to resume cell proliferation, it would be useful to study the functions of Gcs1, as *GCS1* remains the only known gene in which mutations produce the novel phenotype described above.

4. Gcs1 is a GAP for yeast Arf1 and Arf2

The novel *gcs1* mutant phenotype can be produced by mutations in a single gene encoding the Gcs1 protein (Ireland *et al.*, 1994). The Gcs1 protein contains a putative "Zinc-finger" domain as defined by the "C-x-x-C-x_{16}-C-x-x-C" amino acid sequence. This C_2-C_2 motif is conserved within a family of yeast proteins and was an important key to the determination of Gcs1 function. While others in our lab were engaged in studying the function of Gcs1, I undertook a genetic approach to identify gene products that might have functions related to that of Gcs1. During that time, Dan Cassel's group (Technion, Haifa, Isreal) isolated a rat protein similar in structure to Gcs1, especially around the putative finger region. The rat protein was shown to be a GTPase-Activating Protein (GAP) for a mammalian GTP-binding protein named Arf1, and the finger motif of this rat protein was shown to be important for its GAP activity: a mutation changing one of the cysteines to alanine abolished the Arf1 GAP activity of that rat protein (Cukierman *et al.*, 1995). Mammalian Arf1, a substrate for the rat GAP, has been shown to be involved in regulating intracellular vesicular transport (reviewed in Donaldson and Klausner, 1994). Its activity has been implicated in ER-to-Golgi and intra-Golgi vesicular transport (Helms *et al.*, 1993; Tanigawa *et al.*, 1993) and is also important for secretory vesicle biogenesis from the *trans*-Golgi network (Barr and Huttner, 1996). Arf1 has also been found to be associated with endosomes (Whitney *et al.*, 1995), and this raises the possibility of its involvment in endocytosis. Collaborative work between our laboratory and Cassel's laboratory showed that the Gcs1 protein also has GAP activity *in vitro* towards mammalian Arf1 (Poon *et al.*, 1996). This finding has raised the interesting question of whether Gcs1 is a GAP for yeast Arf1 homologues.

In yeast there are seven Arf-like proteins, including a protein termed Sar1 (Nakano and Muramatsu 1989), which is more similar to Arf than to other small Ras-like GTP-binding proteins. Yeast Arf1 and Arf2 form an essential family, and might regulate yeast intra-Golgi vesicular transport. A mammalian *ARF1* gene expressed in yeast can compensate for the loss of yeast Arf1 and Arf2 activity (Stearns *et al.*, 1990a, b). Therefore the mammalian Arf1 can provide yeast Arf1 +Arf2 activity in yeast, suggesting that yeast Arf1 and Arf2 are homologues of the mammalian Arf1, and that the function of Arf1 is conserved between yeast and mammalian cells. In collaboration with Cassel's group, we decided to determine whether Gcs1 is an authentic GAP for yeast Arf1 and Arf2. My contribution to this work was to express yeast Arf1, Arf2 and Arf3 proteins in *E. coli* for use in the *in vitro* GAP assay. Our collaborative study found that not only did Gcs1 provide effective GAP activity for yeast Arf1 and Arf2 *in vitro*, but that there were strong genetic interactions among mutations in the *GCS1*, *ARF1* and *ARF2* genes. These results strongly support the view that Gcs1 is an authentic GAP, *in vivo,* for yeast Arf1 and Arf2 (Poon *et al.*, 1996). Mutations that abolish the Arf1- and Arf2-GAP activity, such as the *gcs1-1* and the *gcs1Δ* null mutations, produce the novel growth defect of *gcs1* mutant cells (described previously). Therefore it is almost certain that the Arf1-GAP activity of Gcs1 is important for the resumption of cell proliferation from the stationary phase at the low temperature of 15°C.

5. The function of Arf1 in vesicular transport

As mentioned above both yeast and mammalian Arf1 have been suggested to play a role in intracellular vesicular transport. So what is vesicular transport and what are the possible functions for Arf1 in this process? As we know, unlike procaryotic cells, eukaryotic cells developed highly complex intracellular membrane compartments, such as the endoplasmic reticulum (ER), the Golgi apparatus, lysosome, and endosome. Transportation of macromolecules among these intracellular membrane compartments as

well as into and out of the cell is mediated by a series of membrane-capsulated vesicular intermediates. This form of vesicular transport allows eukaryotic cells to transport large molecules across membranes. On the contrary, prokaryotic cells can only transport small molecules, such as digestive enzymes and small metabolites, directly across the plasma membrane without jeopardizing the integrity of the cell. In eukaryotic cells, the intracellular vesicular transport system consists, in part, of two opposing pathways, the secretory and endocytic pathways, which are essential for a cell to communicate with its environment. In the secretory pathway, newly synthesized proteins pass through multiple intracellular membrane compartments, such as the ER, the Golgi apparatus and the secretory vesicles, before they arrive at the cell surface. This passage through multiple membrane components allows cells to regulate the delivery and the modification of different proteins. In the endocytic pathway, macromolecules are taken up from the cell surface by a process called endocytosis, and delivered to lysosomes (or to the yeast vacuole) where they can be stored or digested. Transportation along the endocytic and secretory pathways is mediated by a series of vesicular intermediates. In general, vesicles formed from the donor membrane of one compartment subsequently dock onto and fuse with a receptor membrane of another compartment, so that proteins and membrane components can be transported from one compartment to another. A great number of studies have revealed that various intracellular membrane compartments participate along the secretory and endocytic pathways. However, detailed mechanisms of the regulation of vesicular transport at different stages along the secretory and endocytic pathways, as well as the integration of these two pathways, remains largely undefined. Recently, biochemical studies of secretion in neuron cells and genetic studies on yeast secretion have revealed that the regulatory mechanisms of vesicular transport along the secretory pathway are conserved between yeast and neuron cells (reviewed in Ferro-Novick and Jahn, 1994; Bennett and Scheller, 1993). Therefore, understanding the regulation of vesicular transport in yeast will also help us to understand the regulation of

vesicular transport in other eukaryotic organisms.

Genetic and biochemical studies have yielded evidence that Arf1 is important for vesicular transport and we now know that a major function of Arf is to drive, in a GTP-dependent cycle, the assembly of sets of cytosolic coat proteins onto the ER or Golgi membranes as well as to determine the timing of the release of the coat proteins back to the cytosol (reviewed in Donaldson and Klausner, 1994). The activity of Arf1 is closely related to the set of coat proteins called COPI, which is a non-clathrin "coat" complex that is thought to function in both ER-to-Golgi and intra-Golgi vesicular transport (reviewed in Salama and Scheckman, 1995). Arf1 in its GTP-bound form becomes associated with membranes and stoichiometrically binds COPI, and this process drives the budding and formation of coated vesicles. The hydrolysis of Arf1-bound GTP, catalyzed by an Arf1 GTPase activating protein (perhaps Gcs1), triggers the release of Arf1 and COPI coat complex from the vesicles. This decoating process precedes the docking of the vesicles onto a target membrane. Presumably this decoating process allows vesicle-targeting proteins, the v-SNAREs, to be exposed so they can interact with target-membrane-associated proteins, the t-SNAREs, allowing vesicles to dock onto target membranes (reviewed in Ferro-Novick and Jahn, 1994).

Yeast Arf1 and Arf2 proteins have been implicated in one or more steps of vesicle transport (Stearns *et al.*, 1990a, b). Moreover, the mammalian Arf1 has been localized to the Golgi compartments (Helms *et al.*, 1993; Tanigawa *et al.*, 1993), to secretory vesicles originating from the *trans*-Golgi network (Barr and Huttner, 1996), and to endosomes (Whitney *et al.*, 1995). While COPI and Arf1 association is well documented for ER-to-Golgi and intra-Golgi vesicular transport, recent findings that Arf1 protein (but not COPI) is involved in secretory vesicle biogenesis from the *trans*-Golgi network suggest that Arf1 might be able to associate with different coat proteins (Barr and Huttner, 1996). It is apparent that more studies are needed to further define the functions of Arf1 in vesicular transport.

6. Vesicular transport and the yeast cell growth

The finding that Gcs1 is a GAP for yeast Arf1 and Arf2 suggests that Gcs1 is involved in regulating intracellular vesicular transport. Since the proper coordination of this intracellular vesicular transport process is very important for yeast cell growth, it is not such a surprise that mutations in the *GCS1* gene produce the novel growth defect described above.

First, the secretory pathway is responsible for the modification and delivery of secretory and plasma-membrane proteins. Mannoprotein, a cell-wall structural protein, undergoes several steps of glycosylation as it passes through the ER and the Golgi apparatus before reaching the cell surface (Ballou *et al.*, 1982). As discussed above, when cells enter stationary phase the cell wall becomes thicker due to changes in mannoprotein structure (Valentin *et al.*, 1987). Therefore the secretory pathway is directly involved in building new cell wall and in changing cell-wall structure. Other proteins are secreted that allow cells to grow on different media. For example, invertase is needed for cells to grow on sucrose (Meyer and Matile, 1975), and α-galactosidase is needed for cells to grow on melibiose (Post-Beittenmiller *et al.*, 1984). Haploid cells of α mating type also secrete α-pheromone so that they can conjugate with **a**-mating-type haploid cells to form diploid cells (Wickner, 1981). As cell growth progresses, newly synthesized plasma-membrane proteins (ion channels, transporters) rely on the secretory pathway for transport to the plasma membrane.

Second, the fusion of secretory vesicles with the plasma membrane drives bud growth. Continuous growth of the bud as a cell progresses through the cell cycle also requires supply of membrane components. Newly synthesized membrane components in the form of secretory vesicles are transported into the bud, and the fusion of these vesicles with the inner surface of the bud drives the growth of the bud surface (Schekman and Novick, 1982).

Third, mutations that affect vesicular transport also affect yeast growth. Conditional mutations that cause defects in different stages of vesicular transport along the secretory pathway, defined as *sec* mutations, have been isolated (Novick and Schekman, 1979). Cells bearing *sec* mutations become blocked in the secretion process and arrest cell growth quickly when shifted to a restrictive temperature. This arrest provides genetic evidence that vesicular transport and yeast cell growth are directly related (Novick and Schekman, 1979). Mutations affecting the secretory pathway have also been shown to affect the maintenance of stationary phase. For example, the Ypt1 protein is necessary for transport from the ER to the Golgi apparatus. Cells that have a mutated *YPT1* gene lose viability when they enter stationary phase, suggesting that vesicular transport is important for the entry into or the maintenance of stationary phase (Segev *et al.*, 1988). Since stationary-phase cells have cell-wall and membrane activities that are different from those of actively proliferating cells, it is likely that vesicular transport activity is also important for stationary-phase cells to change the membrane and cell-wall activities during the resumption of cell proliferation from stationary phase.

7. Objectives

The objective of this study was to use a genetic approach to identify gene products that have related function with Gcs1. I have concentrated on isolating wild-type genes that, when present in multiple copies, bypass the need for Gcs1 during the resumption of cell proliferation from stationary phase. These genes are defined as multicopy suppressors (Robinson *et al.*, 1992). Through this approach, I further elucidated the cellular function of Gcs1. When I started this study the Gcs1 protein had been characterized at the nucleotide sequence level, but we did not yet know the biological function of Gcs1. The identification of copy suppressors helped us to identify cellular processes required for stationary-phase cells to resume cell proliferation. With our recent finding showing that Gcs1 is a GAP for Arf1 and Arf2, the identification of these multicopy suppressors will

not only help us identify proteins that might also be involved in the same vesicular transport stage(s) as Arf1 and Gcs1, but also help us to identify the stage(s) at which Gcs1 functions. Some of my work described in this thesis has been published (Wang *et al.*, 1996; Poon *et al.*, 1996)

II. MATERIALS AND METHODS

1. Strains and plasmids

The genotypes and sources of yeast strains used in this study are listed in Table 1.

Mating-type switching was carried out as follows. A starting strain was first transformed with plasmid pHO, which contains the *HO* gene driven by the *GAL1-10* promoter and the *URA3* gene for plasmid selection. The resultant transformant was first grow in YM1 medium containing glucose to a concentration of 10^6 cells/ml and then was washed and incubated in YM1 medium containing galactose for another 4 h so that the *HO* gene is induced. The *HO* gene encodes a site-specific endonuclease which recognizes a site at the mating-type locus and produces a double-stranded break at that position. Subsequent DNA-repair processes at the locus resulted in mating-type switching. Therefore the induction of *HO* promotes mating-type switching (Herskowitz and Jensen, 1991). The mixture of cells of different mating types, resulted from mating-type switching, was spread on medium containing glucose, and the mating types of cells comprising individual colonies were tested by a halo assay (Fink and Styles, 1972) using tester strains MIS1 and RRC631. Selected cells of the desired mating type were grown in YM1 liquid medium to allow them to lose the pHO plasmid, the cell mixture was then spread on YEPD solid medium and individual colonies that have lost the plasmid, as indicated by the loss of uracil prototrophy, were finally chosen.

YX5 is a haploid *gcs1Δ* mutant in the YI228 genetic background. It was constructed by deleting the *GCS1* gene in YI228 cells that were also carrying a low-copy wild-type *YCK2* plasmid, pXW125, and subsequently losing the pXW125 plasmid. The *GCS1* deletion plasmid, pBN-Δ4, has been described (Ireland *et al.*, 1994).

The *YCK3* disruption plasmid pNUF1Δ was digested with *Not*I and transformed into 21R-B cells to generate strains NRK1B and NRK2B. Due to the special structure of pNUF1Δ (Figure 6), strains NRK1B and NRK2B contain differently modified *YCK3* loci.

In NRK1B a *LEU2* gene is inserted into the *YCK3* ORF, while in NRK2B cells part of the *YCK3* gene and part of *GLO3* (an adjacent gene), were deleted and replaced with *LEU2*.

Yeast strains containing disrupted genes were subjected to Southern analysis to confirm the structures of those loci.

Plasmids used or constructed in this study, together with descriptions of the ways they were made, are listed in Table 2. The YEp213-based yeast genomic library used for the isolation of dosage suppressors was provided as a gift by Dr. D. Thomas.

E. coli strain DH5αF' [*F'/endA1 hsdR17 supE44 thi-1 recA1 gyrA(Nal^r) relA1Δ(lacZYA-argF)$_{u169}$ (φ80/lacZΔM15)*; Bethesda Research Laboratories] was used for cloning purposes. *E. coli* strain DE3 (F$^-$ *ompT* r_b^- m_b^-; Novagen) expressing yeast N-terminal myristyl transferase from plasmid pBB131 (Randazzo *et al.*, 1992) was used for the production of myristylated recombinant yeast Arf proteins. *E. coli* strain JF1754 (*hsdr leuB hisB-436 metB gal lac*; McNeil *et al.*, 1980) was used to express recombinant Ypt31 protein.

2. Media and culture conditions

Yeast cells were grown in YM1 complex liquid medium (Hartwell, 1967) or the defined medium YNB (Johnston *et al.*, 1977) supplemented with 2% glucose or galactose. Amino acids (40 μg/ml) and nucleotide bases (20 μg/ml) were added to the YNB medium as required to satisfy auxotrophies. Cultures were incubated in gyratory shaking water baths. For the maintenance of 15°C cultures, the water bath was equipped with a refrigeration unit. YEPD solid medium was used as a rich medium and YNB solid medium supplemented with amino acids (40 μg/ml), nucleotide bases (20 μm/ml) and glucose (2%) was used to maintain cells with nutritional auxotrophies. Liquid sporulation medium (1% potassium acetate plus 0.1% glucose) was used for the sporulation of diploid cells. Yeast strains were maintained for long-term storage as frozen stocks in 15% glycerol at -70°C.

Table 1. Yeast strains used in this study

STRAIN	GENOTYPE	SOURCE
W303-1A	*MAT***a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	M. Hoekstra
W303-1B	*MAT*α *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	M. Hoekstra
21R	*MAT***a** *leu2-3,112 ura3-52 ade1*	Ireland *et al.*, 1994
21R-B	*MAT*α *leu2-3,112 ura3-52 ade1*	*MAT*α version of 21R
GWK8A	*MAT***a** *gcs1Δ::URA3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Ireland *et al.*, 1994
GRK4-7	*MAT***a** *gcs1Δ::URA3 leu2-3,112 ura3-52 ade1*	Ireland *et al.*, 1994
MDgcs1-3X	*MAT***a** *gcs1-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52*	Drebot *et al.*, 1987
GWK9A	*MAT***a** *gcs1Δ::URA3 leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	*GCS1* deleted in W303-1A
GWK9B	*MAT*α *gcs1Δ::URA3 leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	*GCS1* deleted in W303-1B
GCS1-2H1R	*MAT*α *gcs1-2::HIS3 leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	*GCS1* disrupted in W303-1B
GCS1-2L9	*MAT*α *gcs1-2::LEU2 leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	*GCS1* disrupted in W303-1B
YI228	*MAT***a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 yck2Δ::ura3 yck1Δ::HIS3 (yck2-2ts TRP1)*	Vancura *et al.*, 1994
YI228B	*MAT*α *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 yck2Δ::ura3 yck1Δ::HIS3 (yck2-2ts TRP1)*	MATα version of YI228

Table 1, continued

YX3	*MATa/MATα leu2-3,112/leu2-3,112 ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 yck2Δ::ura3/yck2Δ::ura3 yck1Δ::HIS3/yck1Δ::HIS3 (yck2-2ts TRP1)*	YI228 x YI228B
YX4	*GCS1/gcs1Δ::URA3* version of YX3	*GCS1* deleted in YX3
YX5	*MATa leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 gcs1Δ::URA3 yck2Δ::ura3 yck1Δ::HIS3 (yck2-2ts TRP1)*	*GCS1* deleted in YI228
NRK1B	*MATα leu2-3,112 ura3-52 ade1 yck3::LEU2*	*YCK3* disrupted in 21RB
NRK2B	*MATα leu2-3,112 ura3-52 ade1 glo3Δ yck3Δ::LEU2*	*YCK3* and *GLO3* deleted in 21RB
YX6	*MATa/MATα leu2-3,112/leu2-3,112 ura3-52/ura3-52 ade1/ade1 GCS1/gcs1Δ::URA3 YCK3/yck3::LEU2*	GRK4-7 x NRK1B
YX7	*MATa/MATα leu2-3,112/leu2-3,112 ura3-52/ura3-52 ade1/ade1 GCS1/gcs1Δ::URA3 YCK3/yck3::LEU2 GLO3/glo3Δ*	GRK4-7 x NRK2B
FY56	*MATα his4-912δ lys2-128δ ura3-52*	Malone *et al.*, 1991
YTH11	*MATa his4 leu2 ura3 lys2 ypt32Δ::HIS4*	D. Gallwitz
YTH12	*MATa his4 leu2 ura3 lys2 ypt32Δ::HIS4 ypt31cs-LEU2*	D. Gallwitz

Table 1, continued

YTH12B	*MATα his4 leu2 ura3 lys2 ypt32Δ::HIS4 ypt31cs-LEU2*	MATα version of YTH12
PP444	*MAT***a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 gcs1Δ::LEU2*	Poon *et al.*, 1996
PP482	*MAT***a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 arf1Δ::URA3*	Poon *et al.*, 1996
tpd3.1	*MATα leu2 his3 ade2-1 ura3-52 tpd3-1 hml::SUP40 hmr::SUC2 sir4-1*	van Zyl *et al.*, 1992
AHY86	*MAT***a** *leu2 ura3 his3 cdc55::LEU2*	Healy *et al.*, 1991
Y1378	*MATα leu2 PPH21 pph22Δ::URA3 ura3*	van Zyl *et al.*, 1992
Y1379	*MATα leu2 pph21Δ::LEU2 PPH22 ura3 his3 trp1 ade2*	van Zyl *et al.*, 1992
MIS1	*MATα his6 met1 rme sst2-1*	D. Evans
RRC631	*MAT***a** *ade2 his6 met1 ura1 rme sst2-1*	D. Evans
YXW7	*MAT***a***/MATα lys2-128δ/LYS2 his4-912δ/HIS4 ura3/ura3 leu2/LEU2 gcs1Δ::URA3/GCS1*	GRK4-7 x Fy56
YXW8	*MATα his4 ura3 leu2 gcs1Δ::URA3*	segregant of YXW7
YXW9	*MAT***a***/MATα his4/his4 leu2/leu2 ura3/ura3 lys2/LYS2 gcs1Δ::URA3/GCS1 ypt31*cs*-LEU2/YPT31 ypt32::HIS4/YPT32*	YXW8 x YTH12
YXW11	*MAT***a***/MATα his4-912δ/HIS4 lys2-128δ/LYS2 ura3-52/ura3-1 leu2-3,112/LEU2 his3-11,15/HIS3 trp1-1/TRP1ade2-1/ADE2 arf1Δ::URA3/ARF1*	PP482 x FY56
10b	*MAT***a** *leu2-3,112 his4-912δ ura3 arf1Δ::URA3*	segregant of YXW11

Table 2. Plasmids used or constructed in this study

PLASMID	DESCRIPTION
pXW100	A multicopy *GCS1* plasmid, isolated from a YEp213-based yeast genomic library (from D. Thomas) as a multicopy *gcs1*-suppressing plasmid.
pXW120	A 5.2-kbp *YCK2* genomic insert in YEp213, isolated as a multicopy *gcs1*-suppressing plasmid; the same as pXW190.
pXW121	A subclone of the pXW120 insert, constructed by digesting pXW120 with *Hin*dIII and then purifying and religating the largest fragment, that contains a 2.3-kbp insert in YEp213.
pXW122	A subclone of the pXW120 insert, constructed by inserting a 3.4-kbp *Hin*dIII fragment, containing 2.9-kbp of genomic insert, into YEp351 (Hill *et al.*, 1986).
pXW123	A subclone of the pXW120 insert, constructed by inserting a 2.7-kbp *Xba*I/*Bgl*II fragment, which contains the entire *YCK2* ORF, into the *Xba*I/*Bam*HI sites of YEp351.
pXW124	The same 2.7-kbp *Xba*I/*Bgl*II fragment used to construct pXW123, inserted into pRS315 (Sikorski and Hieter, 1989).
pXW126	The same 2.7-kbp *Xba*I/*Bgl*II fragment used to construct pXW123, inserted into pRS313 (Sikorski and Hieter, 1989).
pB65R7	A 5-kbp genomic insert containing the *YCK1* gene in YEp24 (Robinson *et al.*, 1992; from J. Kuret).
YEpYCK1	A 2.3-kbp *Sma*I yeast genomic fragment from pB65R7 inserted into the *Sma*I site of YEp351.
pUCYCK1	The 2.3-kbp *Sma*I fragment from YEpYCK1 inserted into pUC18 (Sambrook *et al.*, 1989).

Table 2, continued

pXW222	Contains a modified *YCK1* gene that has the sequence between two internal *Bgl*II sites deleted and religated in frame; constructed by digesting YEpYCK1 with *Bgl*II and religating the largest fragment.
pXW224	Contains an internally deleted *YCK1* gene lacking the sequence between an internal *Fsp*I site and an internal *Bsa*AI site; constructed by digesting pUCYCK1 with *Hpa*I and *Bsa*AI and replacing this *Hpa*I/*Bsa*AI internal fragment with an internal *Hpa*I/*Fsp*I fragment of the same gene. The resultant internally deleted *YCK1* gene was then cloned into YEp351.
pXW223	Contains a C-terminally deleted *YCK1* gene lacking the coding sequence downstream of an internal *Bsa*AI site; constructed by transferring a *Sph*I/*Bsa*AI fragment from pUCYCK1, that contains the C-terminally truncated *YCK1* gene, into the *Sph*I and *Sma*I sites of YEp351.
pKSHRR25	A 3-kbp fragment containing the *HRR25* gene in pBluescript KS^+(Stratagene Cloning Systems, La Jolla, CA; from M. Hoekstra).
pGALHRR25	Contains the *HRR25* gene under the control of *GAL1-10* promoter in pRS316 (from M. Hoekstra; pRS316, see Sikorski and Hieter, 1989).
pXW321	A 3-kbp *Sal*I fragment from pKSHRR25, containing the *HRR25* gene, cloned into YEp351.
pHRR25-CC	Contains a chimeric gene with the C-terminal coding sequence of *YCK1* fused to the C-terminal sequences of *HRR25*; *YCK1* coding sequences upstream of a *Bsa*AI site were deleted by digesting pUCYCK1 with *Sph*I and *Bsa*AI, and replacing the *Sph*I/*Bsa*AI fragment with a *Sph*I/*Pvu*II fragment containing most of the *HRR25* coding sequences from pKSHRR25. The resultant chimeric gene was then cloned into YEp351.

Table 2, continued

pHRR25-YCK2	Contains a chimeric gene with the N-terminal coding sequences of *YCK2*, upstream of an *Nco*I site, replaced by the N-terminal coding sequences of *HRR25*. (see pYCK2-HRR25)
pYCK2-HRR25	Contains a chimeric gene with the N-terminal coding sequences of *HRR25*, upstream of an *Nco*I site, replaced by the N-terminal coding sequences of *YCK2*. An *Xba*I/*Nco*I fragment containing the N-terminal portion of *HRR25* from pXW321 and an *Xba*I/*Nco*I fragment containing the N-terminal of *YCK2* from pXW123 were exchanged to generate plasmids pHRR25-YCK2 and pYCK2-HRR25.
pHRR25-YCK1	Contains a chimeric gene with the N-terminal coding sequences of *YCK1*, upstream of an *Eco*RV site, replaced by the N-terminal coding sequences of *HRR25*. (see pYCK1-HRR25)
pYCK1-HRR25	Contains a chimeric gene with the N-terminal coding sequences of *HRR25*, upstream of an *Eco*RV site, replaced by the N- terminal coding sequences of *YCK1*. An *Sph*I/*Eco*RV fragment containing the N-terminal portion of *HRR25* from pKSHRR25 and an *Sph*I/*Eco*RV fragment containing the N-terminal portion of *YCK1* from pUCYCK1 were exchanged to generate these two chimeric genes, which were subsequently cloned into YEp351.
pXW150	A 4-kbp genomic insert containing *YCK3* and part of the *GLO3* gene in YEp213, isolated as a multicopy *gcs1*-suppressing plasmid; the same as pXW160.
pXW151	A subclone of the pXW150 insert, constructed by transferring a 2.5-kbp *Bam*HI/*Hin*dIII fragment to YEp351.

Table 2, continued

pXW152	A subclone of the pXW150 insert, constructed by transferring a 1.6-kbp *Bam*HI/*Hin*dIII fragment to YEp351.
YEpCDC55	The *CDC55* gene in YEp352 (Healy *et al.*, 1991).
YEpTPD3	The *TPD3* gene in YEp24 (van Zyl *et al.*, 1992).
phhp1$^+$	Contains an *HRR25* homologue from *S. pombe* driven by the *ADH1* promoter in a multicopy *URA3* vector (from M. Hoekstra).
phhp2$^+$	Contains another *HRR25* homologue from *S. pombe* driven by the *ADH1* promoter in a multicopy *URA3* vector (from M. Hoekstra).
phCKI	Contains the human α3-isoform of casein kinase I driven by the *GAL1-10* promoter in a *LEU2* based plasmid (from M. Hoekstra).
pNUF1Δ	A *YCK3* disruption plasmid, constructed as follows: a 4-kbp *Hin*dIII fragment containing *YCK3* was cloned into pHSS19 (Hoekstra *et al.*, 1991a), then an *Eco*RI partial digestion and religation was performed to eliminate the *Eco*RI fragment encompassing the *YCK3* translation initiation codon, and the resultant plasmid was subjected to mini-Tn3 shuttle mutagenesis using mTn3(*LEU2*) (Hoekstra *et al.*, 1991a; from M. Hoekstra).
pNKΔ-1	A *YCK3* disruption plasmid, constructed as follows: a 4-kbp *Hin*dIII fragment was cloned into pBluescript KS$^+$, and in the resultant plasmid an internal *Bam*HI/*Bgl*II fragment encompassing the *YCK3* translation initiation codon was replaced by a 1.7-kbp *HIS3 Bam*HI fragment (from M. Hoekstra).
pXW130	A 4.3-kbp genomic insert containing *YPT32* in YEp213, isolated as a multicopy *gcs1*-suppressing plasmid.

Table 2, continued

pXW170	A different 4.3-kbp genomic insert containing *YPT32* in YEp213, isolated as a multicopy *gcs1*-suppressing plasmid.
pXW180	A 3.9-kbp genomic insert containing *YPT32* in YEp213, isolated as a multicopy *gcs1*-suppressing plasmid.
pXW131	A subclone of the pXW130 insert, constructed by transferring a *Bgl*II/*Hin*dIII fragment containing 1.3-kbp of insert DNA to YEp351.
pXW132	A subclone of the pXW130 insert, constructed by transferring a *Sal*I/*Bgl*II fragment containing 3-kbp of insert DNA to YEp351.
pXW171	A subclone of the pXW170 insert, constructed by transferring a 3.5-kbp *Hin*dIII fragment containing the entire *YPT32* gene to YEp351.
pXW172	A *YPT32* disruption plasmid, constructed by inserting a 1.2-kbp *URA3* *Bam*HI fragment into the *Bgl*II site in pXW171.
pXW173	Another *YPT32* disruption plasmid, constructed by inserting a 1.7-kbp *HIS3* *Bam*HI fragment into the *Bgl*II site in pXW171.
pRS326-YPT32	Contains the *YPT32* gene in the multicopy vector pRS326 (from D. Gallwitz; for pRS326, see Sikorski and Hieter, 1989).
pRS326-YPT31	Contains the *YPT31* gene in the multicopy vector pRS326 (from D. Gallwitz).
pXW140	A 5.2-kbp genomic insert containing *YPT31* in YEp213, isolated as a multicopy *gcs1*-suppressing plasmid.
pXW141	A subclone of the pXW140 insert, constructed by inserting an *Xba*I fragment containing an approximately 2.1-kbp of insert DNA into the *Xba*I site of YEp351.

Table 2. continued

pXW142	A subclone of the pXW140 insert, constructed by inserting a *Sal*I fragment containing an approximately 2.7-kbp of insert DNA into the *Sal*I site of YEp351.
pXW143	A subclone of the pXW142 insert, constructed by inserting a 1.7-kbp *Pst*I fragment into the *Pst*I site of YEp351.
pXW144	A subclone of the pXW142 insert, constructed by digesting pXW142 with *Pst*I and religating the bigger fragment.
pUC143	A 1.7-kbp *Pst*I fragment from pXW143 cloned into pUC118 (Sambrook *et al.*, 1989).
pXW146	A 1.7-kbp *Pst*I fragment from pXW143 cloned into pRS315.
pXW147	A 1.7-kbp *Pst*I fragment from pXW143 cloned into pRS313.
pBR1.129	Contains the *YPT1* gene in the vector pBR322 (Neff *et al.*, 1983).
YEpYPT1	A 1.6-kbp fragment containing the *YPT1* gene in YEp351, constructed by transferring a 1.6-kbp *ClaI*/*Bam*HI fragment from pBR1.129 to pRS314 (Sikorski and Hieter, 1989) to acquire additional restriction sites, and then using those sites to transferr the same fragment to YEp351.
pTrcYPT31	A plasmid used to express N-terminally His_6-tagged Ypt31 recombinant protein in *E. coli*, constructed as follows: a 711-bp blunt-ended *Bsa*HI/*Fsp*I fragment, containing the entire *YPT31* ORF including 20 basepairs upstream of the ATG translation initiation codon, was inserted into the Klenow-filled-in blunt-ended *Bam*HI site of pTrc-HisC (InvitroGen). This procedure regenerates a *Bam*HI site at the *Bam*HI/*Bsa*HI junction.

Table 2, continued

pGBTYPT31	Contains a fusion gene with the entire *YPT31* coding sequence fused to the coding sequence of the Gal4 DNA-binding domain. A *Bam*HI/*Pst*I fragment from pTrcYPT31, containing the entire *YPT31* ORF, was cloned into pGBT9 (Chien *et al.*, 1991).
pGADYPT31	Contains a fusion gene with the entire *YPT31* coding sequence fused to the coding sequence of the Gal4 transactivation domain. A *Bam*HI/*Pst*I fragment from pTrcYPT31, containing the entire *YPT31* ORF, was cloned into pGAD424 (Chien *et al.*, 1991).
pUCYPT31	A 720-bp *Bam*HI/*Pst*I fragment from pTrcYPT31 cloned into pUC118; used in PCR reactions to generate two mutant *YPT31* genes, *ypt31-Q72L* and *ypt31-S27A*.
pGalQ72L	A 720-bp *Bam*HI/*Pst*I fragment containing the *ypt31-Q72L* gene cloned into the *Bam*HI/*Pst*I sites of the pPP389, and under the control of the *GAL1-10* promoter. pPP389 is a modified version of pEMBLyex4: instead of the d-*LEU2* gene, pPP389 has the *LEU2* gene (Poon *et al.*, 1996).
pGalS27A	A 720-bp *Bam*HI/*Pst*I fragment containing the *ypt31-S27A* gene cloned into the *Bam*HI/*Pst*I sites of pPP389.
pGADQ72L	A 720-bp *Bam*HI/*Pst*I fragment containing the *ypt31-Q72L* gene cloned into the plasmid pGAD424.
pGADS27A	A 720-bp *Bam*HI/*Pst*I fragment containing the *ypt31-S27A* gene cloned into pGAD424.
pXW110	A 4.1-kbp genomic insert containing the *CSG1* gene in YEp213, isolated as a multicopy *gcs1*-suppressing plasmid.

Table 2, continued

pXW111	A subclone of the pXW110 insert, constructed by transferring an *XhoI/SalI* fragment containing 1.5-kbp of insert DNA to YEp351.
pXW112	A subclone of the pXW110 insert, constructed by transferring an *XbaI/XhoI* fragment containing 2.6-kbp of insert DNA to pRS315.
pXW113	The same *XbaI/XhoI* fragment as that of pXW112 cloned into YEp351.
pXW114	A subclone of the pXW113 insert, constructed by transferring a *Hin*dIII fragment containing 1.1-kbp of insert DNA to YEp351.
pXW115	A subclone of the pXW113 insert, constructed by transferring a *Hin*dIII fragment containing 1.5-kbp of insert DNA to YEp351.
pUCCSG1	A 3.2-kbp *SphI/Bsa*A1 fragment (from pXW110) containing the entire *IMH1* gene cloned into pUC118.
YEpCSG1	A 3.2-kbp *SphI/Kpn*I fragment from pUCCSG1, containing *IMH1*, cloned into YEp352.
pCSG::HIS3	A *IMH1* deletion plasmid, constructed from pUCCSG1 by replacing the *IMH1* coding sequences between an internal *Xho*I site and an internal *Hpa*I site with a 1.7-kbp *HIS3 SalI/Sma*I fragment.
pETARF1H	Expresses a protein with LEHHHHHH fused to the C terminus of yeast Arf1. PCR was used to amplify *ARF1* using primers P11 (5'-ATTACATATGGGTTTGTTTGCC) and P14 (5'-AGAACTCGAGAGT TGAGTTTTTC); the resulting fragment was digested with *Nde*I and *Xho*I and inserted into pET21b (NovaGen).
pETARF1	Expresses yeast Arf1. PCR was used to amplify *ARF1* using primers P11 and P15 (5'-CCGCTCGAGAATTTTTAAGTTGAG); the resulting fragment was digested with *Nde*I and *Xho*I and inserted into pET21b.

Table 2, continued

pETARF2H	Expresses a protein with WINSSSVDKLAAALEHHHHHH fused to the C terminus of yeast Arf2. PCR was used to amplify *ARF2* using primers P21 (5'-GCGGATCCGATGGGTCTATACGC) and P22 (5'-GCGGATCC AGGATTGATTCTTCAAG); the resulting fragment was digested with *Bam*HI, blunt ended and inserted into blunt-ended *NdeI/Eco*RI sites of pET21b.
pETARF3	Expresses yeast Arf3. A *NdeI/Bam*HI fragment containing *ARF3* from plasmid pT7ARF3 (Lee *et al.*, 1994) was cloned into pET21b.
pETARF3H	Expresses C-terminally His_{10}-tagged yeast Arf3. Primers P31 (5'-TAGCC ATGGGCAATTCAATTT) and P3 (5'-ATGCCATGGTCTTTGGAACG TTTG) were used to PCR-amplify *ARF3*, and the resulting fragment was digested with *Nco*I and inserted into the *Nco*I site of pET16b (NovaGen).
pRB1297	Contains the yeast *ARF1* gene (Stearns *et al.*, 1990a); used as template DNA for PCR.
pRB1306	Contains the yeast *ARF2* gene (Stearns *et al.*, 1990a); used as template DNA for PCR.
pRArfGAP	Expresses rat ARF1-GAP in yeast. A *NcoI/Xho*I fragment containing the gene encoding rat ARF1-GAP from plasmid Z6 (Cukierman *et al.*, 1995), with the *Nco*I site blunt-ended, was cloned into the *SmaI/Sal*I sites of pEMBLyex4 so that expression is driven by the *GAL1-10* promoter.
pBNΔ4	Used to generate the *gcs1Δ* null mutation (Ireland *et al.*, 1994).
YIp-IF	Used to generate the *gcs1-2::LEU2* mutation; constructed by inserting a *GCS1* internal *BglII/Pst*I fragment to the *Bam*HI/*Pst*I sites of YIp351.

Table 2, continued

pB23	Contains the *GCS1* gene in YEp24 (from L. Ireland).
pXW303	Used to generate the *gcs1-2::HIS3* mutation; constructed by transferring the *GCS1* internal *Bgl*III/*Pst*I fragment from YIp-IF to pRS303 (Sikorski and Hieter, 1989).

Cell concentrations were determined as follows (Johnston *et al.*, 1979): 0.5-ml samples of cell culture were added to 4.5 ml fixing solution (0.15 M NaCl, 3.7% formaldehyde) and the fixed cells were sonicated briefly (Hartwell *et al.*, 1970) to disrupt cell clumps using a Model W140 Sonifier Cell Disrupter (Heat Systems Ultrasonics Inc., Long Island, NY). Sonicated cells were diluted 10- or 100-fold in Isotonic Saline Solution (Fisher Scientific, Nepean, Ont.) and counted using a Coulter Particle Counter (Model ZM, Coulter Electronics, Hialeah, FL). Cell morphology was assessed by direct microscopic examination.

E. coli cells were grown on solid or liquid 2xYT medium (Messing, 1983) at 37°C. 2xYT medium containing ampicillin (100 μg/ml) was used to select for cells containing plasmids that conferred ampicillin resistance. 2xYT medium supplemented with 0.5% casamino acids, 0.4% glucose and 100 μg/ml ampicillin was used for the expression of recombinant yeast proteins. *E. coli* strains were maintained for long-term storage as frozen stocks in 15% glycerol at -70°C.

3. Yeast genetic techniques

A) Diploid construction

Haploid cells of opposite mating type were mixed and incubated on YEPD solid medium 5-8 h at a temperature permissive for both types of cells (Sherman *et al.*, 1979). Diploid cells were then selected from this mating mixture by transferring cells from the mating mixture to a nutritional selection medium or an appropriate temperature for further incubation so that neither haploid cells could grow, but the resultant diploid cells could because of complementation of mutations. In cases where no nutritional markers or temperature-sensitivity phenotypes could be used to select the resultant diploids, cells from the mating mixture were transferred to an agar slab and zygotes, identified by their typical "dumbbell" shape (Sherman *et al.*, 1979), were picked out by micromanipulation using a Singer MK. III Micromanipulator (Singer Instruments Co., Ltd., Watchet,

England) and a phase-contrast microscope (Carl Zeiss) and incubated at appropriate conditions to form colonies, which were subjected to further analysis.

B) Mating-type determination

To test the mating type of haploid cells or to confirm the diploid status of some diploid strains, the *sst2* mutant strains MIS1 (*MAT*α) and RRC631 (*MAT***a**) were used as tester strains in the halo assay described by Fink and Styles (1972). For this assay, MIS1 cells or RRC631 cells were first grown in YM1 medium to a cell concentration of about 2 x 10^6 /ml, and 300 µl of the cell culture were spread on YEPD medium to form a lawn of tester cells. Cells with unknown mating type were patched on to the surface of this lawn of tester cells by replica plating and incubated at a permissive temperature overnight. Cells that have a mating type opposite to that of the tester cells in the lawn secrete a pheromone inhibiting the growth of tester cells surrounding the patch but leaving tester cells elsewhere uninhibited. As a result, a clear zone or "halo" forms surrounding a patch of cells that have an opposite mating type to that of the tester cells. Diploid cells are not able to form halos on either tester strain.

C) Tetrad analysis

Tetrad analysis was used to construct some strains, and to study the genetic interactions between mutated genes of interest.

Diploid cells from a log-phase culture were washed once with sporulation medium and incubated in sporulation medium at 23°C for 4-7 days to induce sporulation. Sporulation efficiency was determined by microscopic examination. Asci from 1 ml of the sporulated culture were collected and resuspended in 1 ml of PBS (containing, per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4, adjusted to pH 7.4 with concentrated HCl). Asci walls were digested by adding 50 µl β-glucuronidase (Sigma Chemical Co., St. Louis, MO) and incubated for 1-2 h (the time varies among strains). β-

glucuronidase was then removed by washing the cells twice with PBS, the cells were resuspended in 2 ml PBS, and a portion of the mixture was spread onto an agar slab.

The four meiotic spores from each individual ascus were separated and arranged on the agar slab using a Singer MK III Micromanipulator and a phase-contrast microscope. The agar slab was then incubated at a permissive temperature of 23°C or 30°C until spores gave rise to colonies, which were subjected to further analysis to assess their genotypes and novel phenotypes.

4. Routine DNA techniques

A) Isolation of plasmid DNA from *E. coli*

Preparation of plasmid DNA was performed by the alkaline lysis protocol described in Sambrook *et al.* (1989).

B) Isolation of plasmid DNA from yeast

Plasmid DNA was extracted from yeast by vortexing with glass beads as described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1989).

C) Restriction endonuclease digestion of DNA

Restriction endonucleases were purchased from Bethesda Research Laboratories (Gaitherburg, MD; BRL) and New England Biolabs (Beverly, MA), and used with the buffers supplied according to the supplier's recommendations.

D) Agarose-gel electrophoresis and DNA-fragment purification

Agarose-gel electrophoresis was performed as described by Sambrook *et al.* (1989) using either TAE (40 mM Tris-acetate, 1 mM EDTA) or TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) buffers and molecular-biology-grade agarose (BRL). Ethidium bromide (0.5 μg/ml) was added to the gel before casting. Concentrated loading buffer

consisted of 25% Ficoll, 0.01% bromophenol blue, and 0.01% xylene cyanol. *Hin*dIII-digested lambda DNA, 1-kb ladder or 100-bp ladder (BRL) were used as size markers. DNA fragments excised from agarose gels were purified using a GeneClean Kit (Bio101, La Jolla, CA) according to the manufacturer's instructions.

E) DNA ligations for plasmid constructions

T4 DNA ligase was purchased from BRL, and ligations were performed as described by King and Blakesley (1986) using the ligation buffer supplied by the manufacturer.

F) Transformation of *E. coli*

Electroporation-competent *E. coli* cells were prepared as described (Ausubel *et al.*, 1989) and stored at -70°C. For use, frozen cells were thawed and 40 - 80 µl of cells were used per transformation. Usually, 0.5 µl from a 20-µl ligation mixture was added to the thawed cells, and cells plus DNA were transferred to electroporation cuvettes (Bio-Rad Laboratories, Mississauga, Ont.). The cells were electroporated using a Bio-Rad Gene Pulser (1.4 kV, 200 ohms for 1 mm-gapped cuvettes or 2.5 kV, 200 ohms for 2 mm-gapped cuvettes, with a time constant between 4.2-4.9 msec). Cells were then resuspended in 1 ml 2xYT and portions were spread onto YT + ampicillin solid medium and incubated 37°C overnight for colony formation.

G) Yeast transformations

Yeast cells were transformed by the method of Schiestl and Gietz (1989) using the lithium acetate transformation protocol as described (Ausubel *et al.*, 1989). Briefly, cells were inoculated into 50 ml YM1 + Glucose medium (adenine was added to 20 µg/ml for *ade* mutants) and incubated overnight to 1-2 x 10^7 cells/ml, and were then diluted to 2 x 10^6 cells/ml using fresh medium and incubated for another 4 - 5 h before they were collected by centrifugation (IEC Universal model UV, International Equipment Co.,

Needham Hts., MA) at 2000 rpm for 5 min in 50-ml sterile Falcon tubes. Cells were then washed twice with 50 ml H_2O and once with 10 ml H_2O, then resuspended in 0.5 ml lithium acetate solution (0.1 M lithium acetate, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). Transforming DNA (0.1-5 μg) plus 5 μl carrier DNA (10 mg salmon sperm DNA/ml, sheared and sonicated) and 1.2 ml PEG solution (40% PEG 3350, 0.1 M lithium acetate, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) were added to 200 μl cell suspension in microfuge tubes. Cells were incubated at 30°C for 60 min, and then heat-shocked at 42°C for 15 min. Finally, cells were pelleted by microcentrifugation for 5 sec, resuspended in PBS, and spread on selective medium for further incubation. Usually 1 μg of DNA can give rise to about 2-5 x 10^5 transformant colonies. This method was used for library screening and for constructing gene-deletion mutant strains.

For routine introduction of plasmids into yeast cells, a rapid method described by Chen *et al.* (1992) was used with some modifications. 300 μl of fresh stationary-phase yeast culture (per transformation) were transferred to a microfuge tube and cells were pelleted by microcentrifugation, washed once with 300 μl of lithium acetate solution (0.1 M lithium acetate, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and resuspended in 100 μl PEG solution (40% PEG 3350, 0.2 M lithium acetate, 0.1 M DTT). 5 μl of transforming DNA plus 5 μl of carrier DNA were added to the cells, and the cells-plus-DNA mixture was incubated at 45°C for 30 min before transfer to nutritional selective medium for further incubation to select transformants.

5. Southern analysis

Genomic DNA was extracted from yeast by vortexing with glass beads (Ausubel *et al.*, 1989). Usually, DNA extracted from a 5-ml fresh stationary-phase yeast culture was dissolved in 100 μl TE buffer, and 15 μl of the sample was subjected to restriction endonuclease digestion and resolved by agarose gel electrophoresis. DNA was then transferred to nylon membranes (Amersham Canada Ltd., Oakville, Ont.) by the alkaline-

transfer method of Reed and Mann (1985) as described (Ausubel *et al.*, 1989). After transfer, the membrane was washed with 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate) and the DNA was cross-linked to the membrane by UV irradiation using a model 2400 UV crosslinker (Stratagene, La Jolla, CA) according to manufacturer's instructions.

DNA blots were incubated in hybridization buffer (5 x SSC, 0.05 M $Na_2H_2PO_4$, pH 6.5, 1 x Denhardt's solution [50 x Denhardt's consists of 5 g Ficoll, 5 g polyvinylpyrolidine, and 5 g bovine serum albumin per liter], 50% formamide, 0.2 µg salmon sperm DNA/ml, 1% SDS) for 6 h at 42°C before radiolabeled probes were added, and the blots were then incubated at 42°C overnight. Blots were washed 4 times in 2 x SSC, 0.1% SDS at room temperature for 5-min periods, and twice in 0.1 x SSC, 0.1% SDS at 50°C for 20-min periods. Autoradiography was performed by exposing blots to Kodak X-OMAT AR film using Dupont Cronex Lightning-Plus intensifying screens (Dupont Co., Wilmington, DE).

6. Northern analysis

RNA was extracted from yeast using the method of Penn *et al.* (1984). Briefly, cells from a 50-ml culture were pelleted, resuspended in 300 µl of LETS buffer without SDS (0.1 M LiCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.0), and rapidly frozen in liquid nitrogen. Cells were then thawed, pelleted, resuspended in 300 µl of LETS plus 1% SDS, and lysed using glass beads and phenol:chloroform:isoamyl alcohol. RNA was precipitated with 2 volumes of 95% ethanol, dissolved in 15 µl H_2O, and quantitated spectrophotometrically (Sambrook *et al.*, 1989). The separation of RNA on formaldehyde agarose gels and the transfer of RNAs to nylon membranes (Amersham) were performed as described by Sambrook *et al.* (1989). After transfer, RNA was crosslinked to the membrane by UV irradiation using a model 2400 UV crosslinker (Stratagene). Filter hybridization and autoradiography used procedures similar to those

for Southern analysis.

To detect the mRNAs of various genes, the *ACT1* probe was a 1-kbp *Hin*dIII/*Xho*I fragment from pRS208 (a gift from R. Storms), the *YCK2* probe was a 2.7-kbp *Xba*I/*Bgl*II fragment encompassing the entire ORF of *YCK2* from pXW120, the *YCK1* probe was a 0.8-kbp internal *Bgl*II fragment of *YCK1* from pXW221, the *YCK3* probe was a 1.5-kbp *Bam*HI/*Hin*dIII fragment containing part of *YCK3* ORF from pXW152, the *HRR25* probe was a 3-kbp *Sal*I-*Sau*3A fragment containing the entire ORF of *HRR25* from pKSHRR25, and the *IMH1* probe was a 3.3-kbp *Xba*I/*Sph*I fragment containing the C-terminal coding sequence of the *IMH1* gene.

7. Radiolabeled probes

For Southern and Northern hybridizations, DNA fragments were purified from agarose gel sections using a GeneClean Kit (Bio101), and radiolabeled with [α-^{32}P]dCTP (Amersham) using a Random Primed DNA Labeling Kit (BRL) according to the manufacturer's instructions. Unincorporated [α-^{32}P]dCTP was separated from the DNA using NICK columns purchased from Pharmacia (Baie d'Urfe, Quebec).

To confirm disruption of the *GCS1* gene by Southern analysis, 500-bp *Bgl*II/*Pst*I *GCS1* internal fragment was used for making the probe. To confirm disruption of the *YCK3* gene by Southern analysis, a 3.5-kbp *Sal*I fragment from pXW150, containing the entire *YCK3* gene and some coding sequence of the neighboring *GLO3* gene, was used to make the probe.

8. DNA sequencing analysis

The chain-termination DNA-sequencing method (Sanger *et al.*, 1977) was used to determine various DNA sequences. Double-stranded DNA was sequenced as described by Mierendorf and Pfeffer (1987) with minor modifications. Briefly, 2 µl 2 M NaOH, 1 mM EDTA was added to 20 µl plasmid DNA, and the DNA was denatured by incubation

at 37°C for 30 min. The denatured DNA was then precipitated with 2 volumes of 100% ethanol, washed with 70% ethanol, and dissolved in up to 14 µl deionized water. Sequencing reactions were carried out using a Sequenase Version 2.0 Kit (USB, United States Biochemical Company, Cleveland, OH) according to the manufacturer's instructions. [α-^{35}S]dATP was purchased from Amersham. Sequencing reactions were resolved on 6% polyacrylamide denaturing buffer-gradient gels (Biggin, 1983) using a model S2 Sequencing Gel Electrophoresis System (BRL). After electrophoresis, gels were incubated in fixing buffer (10% acetic acid, 10% methanol) for 1 h before they were dried on a Model 583 gel dryer (Bio-Rad). Autoradiography was performed using Kodak XAR-5 film.

Sequencing analysis was performed on the following occasions: 1) Partial DNA sequence was determined for *YCK2, YCK3, YPT32*, and *YPT31* to identify them as multicopy suppressors of the *gcs1* cold sensitivity. 2) The N-terminal coding sequence of *IMH1* and the entire coding sequence for *YPT31* were determined from one strand using *Exo*III-generated sequentially deleted clones. 3) Sequence analysis confirmed the fidelity of the PCR-amplified *ARF1*, *ARF2*, *ypt31-S29A*, and *ypt31-Q71L* genes. 4) Sequence analysis also confirmed the structures of plasmids pTrcYPT31 and pRArfGAP.

Sequentially deleted plasmids were generated using the procedure of Henikoff (1987), with some modifications. Briefly, 2-5 µg of plasmid DNA was digested with one restriction enzyme, and the resulting DNA fragment was purified from agarose gel sections using the GeneClean Kit (Bio101) and digested with a second restriction enzyme. The resulting DNA fragment was again purified from an agarose gel and incubated at 37°C in 1 x *Exo*III buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol) containing 2 µl of *Exo*III (BRL). Portions (2.5-µl) of the digest mixture were removed at 30-sec intervals, added to 7.5 µl Mung Bean Nuclease (MBN) Mix (75 µl MBN Mix was made by mixing 10 µl of 10 x MBN Buffer [500 mM sodium acetate, pH 5.0, 300 mM NaCl, 10 mM $ZnSO_4$], 4.4 µl [200 U] MBN, and 60.6 µl H_2O)

and incubated at 37°C for 10 min. DNA samples were then resolved by electrophoresis through an agarose gel, and sequentially deleted DNA fragments were purified using a GeneClean Kit, circularized by ligation using T4 DNA ligase (BRL), and transformed into *E. coli* cells for amplification and sequence determination. To determine the N-terminal coding sequence for *IMH1*, plasmid pXW111 was digested with *Bam*HI and *Sac*I, and then subjected to *Exo*III deletion. Sequentially deleted plasmids pXW111, d1-3, d2-1, d3-2, d4-1, d5-1, d6-1, and d3-3 were used for DNA sequencing. To determine the coding sequence for *YPT31*, plasmid pUC143 was digested with *Bam*HI and *Kpn*I and subjected to *Exo*III digestion to generate the sequentially deleted plasmids D1-1, D2-4, D3-1, D4-6, D5-9, D6-11, D7-5, D8-8, D9-13, and D9-14 for sequencing.

9. Expression and purification of recombinant proteins in *E. coli*

To express recombinant Ypt31 protein in *E. coli*, a blunt-ended 711-bp *Bsa*HI/*Fsp*I fragment containing the *YPT31* gene was cloned into the blunt-ended *Bam*HI site of the pTrcHisC vector (Invitrogen Co., San Diego, CA), which regenerated a *Bam*HI site at the *Bam*HI/*Bsa*HI junction. The resultant plasmid pTrcYPT31 was transformed into *E. coli* strain JF1754 (McNeil *et al.*, 1980) to express an N-terminally His-tagged Ypt31 protein. As a result, 39 residues (MGGSHHHHHHGMASMTGGQQMGRDLYDDDDRWIRLGI ST) were added to the intact Ypt31 protein at the N terminus. The expression of this His-tagged Ypt31 protein was induced as follows: 1) 1 ml of a fresh overnight culture of JF1745 cells containing pTrcYPT31 was added to 100 ml of expression medium (2 x YT, 0.5% casamino acids, 0.4% glucose, 100 μg ampicillin/ml) and incubated 37°C. 2) When the A_{600} reached 0.30, IPTG was added to a final concentration of 1 mM and cells were incubated for another 5-7 h before they were pelleted by centrifugation and frozen at -70°C.

As a pilot experiment, 1-ml samples of culture were taken at 0, 1.5, 3, 5, and 7 h

to determine the optimum time of induction, and whether the expressed protein was soluble or insoluble (in inclusion bodies). Cells were thawed, washed with 200 µl 10 mM Tris-HCl, pH 7.5, resuspended in 200 µ lysis buffer (50 mM-Tris-HCl, pH 7.5, 5 mM EDTA, 3 mg lysozyme, freshly added, per ml), and incubated on ice for 1 h. 14 µl of 5 M NaCl and 15 µl 0.1% Nonident P-40 were added to the cell suspension, which was then sonicated using a Model W140 Sonifier Cell Disrupter for 2 min in 30-sec bursts. The lysates were cleared by microfuge centrifugation at 14,000 rpm for 15 min. The supernatants, containing soluble proteins, were transferred to new tubes, and the pellets, containing cell debris and insoluble proteins, were washed with 200 µl 10 mM Tris-HCl, pH 7.5 and resuspended in 200 µl 10 mM Tris-HCl, pH 7.5, 1 M NaCl. The protein concentrations of those fractions were determined using a Bio-Rad Kit (Bio-Rad). Proteins were resolved by 1-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) as described (Laemmli, 1970) using a Bio-Rad Mini-Protein Electrophoresis System (Bio-Rad) and Wide-Range SDS-PAGE Standards (Bio-Rad) as molecular weight markers.

For purification of His_6-tagged Ypt31 protein from the soluble fraction of an *E. coli* extract, pellets of cells from a 100-ml induced cell culture were thawed and resuspended in 4 ml Sonication Buffer (50 mM Na-PO_4, pH 8.0, 300 mM NaCl), and lysozyme was added to 2 mg/ml. After incubation on ice for 30 min, cells were sonicated for 2 min at 4°C. The lysate was transferred to microfuge tubes and centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatant was collected. 2 ml of a 50% slurry of Ni-NTA resin (Qiagen Inc., Chatsworth, CA), previously equilibrated in Sonication Buffer, was added to the supernatant. The mixture was incubated at 0°C with gentle stirring for at least 1 h, then loaded into a Bio-Rad mini-column and washed with 20 ml Sonication Buffer followed with 30 ml Wash Buffer (50 mM Na-PO_4, 300 mM NaCl, 10% glycerol, pH 6.0). To elute proteins bound to the resin, 8-ml fractions of Wash Buffer containing increased concentrations of imidazole (0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 M) were used to

wash the resin, and the elutes were collected in fractions and subjected to SDS-polyacrylamide gel electrophoresis analysis. Wash Buffer containing 0.05 M imidazole was able to elute most proteins bound non-specifically to the Ni-NTA resin, and Wash Buffer with 0.1 M imidazole was sufficient to elute the His-tagged Ypt31 protein. Protein purified using this protocol was typically >85% pure.

To express N-terminally myristoylated yeast Arf proteins in *E. coli* for ARF-GAP studies (Poon et al., 1996), genes encoding various yeast Arf proteins were amplified using PCR to incorporate suitable restriction sites for cloning into the vector pET21b (Novagen). Plasmids pETARF1H, pETARF2H, pETARF3H, pETARF1, and pETARF3 (Table 2) were generated in this fashion, and express His-tagged versions of Arf1, Arf2, and Arf3, and non-tagged Arf1, Arf3 proteins, respectively. For expression of these recombinant Arf proteins in *E. coli*, each plasmid was transformed into DE3 cells containing an additional plasmid, pBB131, which expresses a yeast N-terminal myristoyl transferase. The induction of *ARF* gene expression was performed in similar fashion to that of *YPT31*, except that Kanamysin (50 μg/ml) and 60 μM myristic acid were added to the expression medium (Randazzo *et al.*, 1992). I found that the optimum time for induction was 3-5 h.

10. Assessing the GTP-binding and GTPase activities of His-tagged Ypt31 protein

Most GTP-binding proteins, when expressed and purified in *E. coli*, are in the GDP-bound form. In the absence of Mg^{++} and in the presence of GTP, these GDP-bound proteins can release the GDP and, in exchange, bind GTP (Randazzo *et al.*, 1992). To assess the GTP-binding activities of Ypt31, in a total 50 μl of reaction mix, 0 to 0.75 μg of partially purified His-tagged Ypt31 protein was added to GTP Binding Buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1 mM dithiothreitol, 15 μCi [α-^{32}P]GTP, 0.5 μM GTP, 25 μg BSA). After 40 min of incubation at 30°C, the reaction mix was rapidly passed

through a nitrocellulose filter, which was subsequently washed 4 times with 2 ml cold Buffer B (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 M KCl, 1 mM β-mercaptoethanol) and air dried. The radioactivity bound to proteins that are bound to the filters was assessed by counting the filters using a Wallac 1410 Liquid Scintillation Counter (Pharmacia, Wallac Oy, Finland).

To assess the GTPase activity of His-tagged Ypt31 protein, the recombinant protein was first loaded with [γ-^{32}P]GTP in a reaction similar to that for GTP binding, except that the incubation time was 15 min at 30°C instead of 40 min. After the incubation, $MgCl_2$ was added to 5 mM and the loading mix was put on ice. GTP hydrolysis was carried out by mixing 130 µl Buffer A (50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol), 10 µl BSA (10 mg/ml), 20 µl 10 mM ATP, and 40 µl loading mix from the previous step, and incubating at 30°C. Samples were removed at different times and rapidly passed through nitrocellulose filters, which were washed, dried, and counted as described above. To assess whether other proteins might activate the GTPase activity of His-tagged Ypt31 protein, *E. coli* or yeast extract (3 to 4 µg of total protein) was also added to the GTP-hydrolysis reaction mix.

11. Polymerase Chain Reaction (PCR) techniques

A) PCR amplification of yeast ARF genes

To generate suitable restriction sites that can be used to clone yeast *ARF* genes into expression vectors, primers (Table 2) 22 to 25 nucleotides long were designed. Restriction sites were placed 2 - 3 nucleotides away from the 5' ends of primers, with the remaining portions of the primers forming perfect matches with the wild-type genes. PCR amplification reactions were carried out as described (Ausubel *et al.*, 1989) using a Model 110S COY TempCycler (DiaMed Lab Supplies Inc., Mississauga, ON) according to manufacturer's instructions. Generally a reaction mix contained 500 ng primer 1, 500 ng primer 2, 10 ng template plasmid, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1 x *Taq* polymerase

buffer (BRL), and 2.5 U *Taq* polymerase. Primers were purchased from BRL.

B) PCR-based site-directed mutagenesis of *YPT31*

To generate the *ypt31-S29A* and *ypt31-Q71L* mutant forms of the *YPT31* gene a PCR-based site-directed mutagenesis approach was used (Deng and Nickloff, 1992). Mutagenesis primer P_{SA} (5'-GACAATAGATTGGCTTTCCCTACC) contained a GGATT to GGCTT change, which results in a change in amino acid sequence from Ser^{29} to Ala, and mutagenesis primer P_{QL} (5'-TGTAACGTTCCAGGCCCGCAGTA) contains a CCTGG to CCAGG change, which results in a change in amino acid sequence from Gln^{71} to Leu. The mutagenesis was performed as follows: Firstly, a *Bam*HI/*Pst*I fragment from pTrcYpt31, containing the *YPT31* gene, was cloned into pUC118 to generate a template plasmid for PCR reactions. Secondly, PCR reactions using the Reverse Sequencing Primer (BRL) and one of the two mutagenesis primers were carried out to amplify parts of the *YPT31* gene, and as a result, the reaction using P_{SA} generated a 180-bp fragment, while the reaction using P_{QL} generated a 300-bp fragment. The two DNA fragments containing the desired coding-sequence changes were then purified and used as "mega-primers". PCR reactions using M13 -20 Sequencing Primer (BRL) and each of the two "mega-primers" were carried out to amplify the entire *YPT31* gene, which resulted in the *ypt31-S29A* and *ypt31-Q71L* mutant genes. PCR reactions were performed as described previously, except that about 2 μg of each "mega-primer" was used in the second-round PCR reactions.

C) Verification of PCR-amplified sequences

To confirmed that above PCR-amplified fragments contain the desired coding sequence changes and do not have errors resulted from PCR reactions, these PCR-amplified fragments were cloned into pUC118. Double-stranded DNA sequencing using the Reverse and M13 -20 sequencing primers confirmed that both mutant forms of the *YPT31* genes were generated.

III. RESULTS

1. Identification of multicopy plasmids that suppress *gcs1Δ* cold sensitivity

Suppression of a deletion mutation can occur by increased expression of a gene that has related function; the suppression caused by overexpression of such a suppressor gene suggests that the suppressor-gene product either replaces the product of the deleted gene in that same pathway or facilitates a pathway that bypasses the need for the deleted gene. Multicopy suppressor analysis has been very useful for dissecting the activities for many proteins (Robinson *et al.*, 1992). The same arguments apply for the *GCS1* gene and gene product. To investigate the functions of the Gcs1 protein, I isolated genes that, in increased dosage, bypass the need for Gcs1 during the resumption of cell proliferation from stationary phase.

To isolate multicopy suppressors of the cold sensitivity caused by *gcs1Δ*, I transformed *gcs1Δ* mutant cells (strain Grk4-7) with a multicopy YEp213-based yeast genomic library and allowed transformants to form colonies at a permissive temperature of 29°C. The resultant transformants were transferred to fresh solid medium by replicating plating, and selected for their ability to grow at a restrictive temperature of 15°C. Since most of the cells on the surface of a yeast colony are in stationary phase, this replicating procedure will transfer mostly stationary-phase cells to fresh medium, which is important for assessing suppression of the *gcs1Δ* mutant phenotype, as log-phase *gcs1Δ* cells will continue to proliferate at the restrictive temperature (Ireland *et al.*, 1994). Of the 23,000 transformants screened in this way, 24 transformants were able to grow at the restrictive temperature. To determine whether the cold resistance of these 24 transformants was plasmid-mediated, I grew each transformant in a non-selective nutritient medium at the permissive temperature 29°C to allow cells to grow even in the absence of plasmids. Cells that had lost their plasmids were then tested to see whether they retained cold resistance at the restrictive temperature of 15°C. Of the 24

transformants originally isolated, 14 (Table 3) were still able to grow at the restrictive temperature of 15°C even after loss of plasmids. Therefore, the cold resistance of each of these 14 transformants was due to genomic mutations affecting Gcs1 functions (mutational suppressors). To assess whether the mutational suppressors in these 14 transformants were recessive, each transformant was crossed with strain GCS1-2L9 (which contains the *gcs1-2* mutant allele in a W303-1B genetic background), and the resultant *gcs1Δ/gcs1-2* diploid cells, heterozygous for the suppressor mutation, were tested for growth at the restrictive temperature of 15°C. From this analysis, it appeared that the mutational suppressor(s) in 13 of the transformants could suppress the *gcs1Δ/gcs1-2* mutations, while the mutational suppressor(s) in one transformant, No. 6A, could not. Thus, the mutational suppressors in those 13 transformants are dominant, and the mutational suppressor(s) in transformant No. 6A might be recessive. Since I was concentrating on isolating multicopy suppressors, none of the 14 transformants were studied any further.

Of the 24 cold-resistant transformants isolated, 10 transformants were no longer able to grow at the restrictive temperature after plasmid loss. Therefore, the cold resistance of these 10 transformants was due to the presence of a plasmid (Table 3). Plasmids were isolated from each of these 10 transformants and subjected to partial restriction mapping to assess the pattern of plasmids able to allow growth. Since a yeast cell can take up several different plasmids at the same time, each different plasmid isolated from each individual transformant was retransformed into Grk4-7 cells to check its ability to confer cold resistance to these *gcs1Δ* mutant cells. Individual plasmids that suppress the cold sensitivity of the *gcs1Δ* mutation were thus identified and subjected to further investigation (Table 3).

The *GCS1* gene has a distinctive 500-bp *Bgl*II restriction fragment and a 800-bp *Hin*dIII restriction fragment (Ireland *et al.*, 1994), and these structural characteristics were exploited to test whether a suppressing plasmid might contain the *GCS1* gene itself.

Table 3. Suppressors of *gcs1* cold sensitivity in 24 different cold-resistant cell colonies.

Colony No.	Type of Suppressor	Plasmid contained	Suppressor gene
8A	Mutational, dominant	Not needed	Not determined
8C	Mutational, dominant	Not needed	Not determined
12A	Mutational, dominant	Not needed	Not determined
18A	Mutational, dominant	Not needed	Not determined
19A	Mutational, dominant	Not needed	Not determined
21A	Mutational, dominant	Not needed	Not determined
22A	Mutational, dominant	Not needed	Not determined
23A	Mutational, dominant	Not needed	Not determined
27A	Mutational, dominant	Not needed	Not determined
29A	Mutational, dominant	Not needed	Not determined
30A	Mutational, dominant	Not needed	Not determined
36A	Mutational, dominant	Not needed	Not determined
37A	Mutational, dominant	Not needed	Not determined
6A	Mutational, recessive	Not needed	Not determined
8B	Multicopy suppressor	pXW100	*GCS1*
7A	Multicopy suppressor	pXW110	*IMH1*
28B	Multicopy suppressor	pXW120	*YCK2*
35A	Multicopy suppressor	pXW130	*YPT32*
37B	Multicopy suppressor	pXW140	*YPT31*
7B	Multicopy suppressor	pXW150	*YCK3*
11A	Multicopy suppressor	pXW160	*YCK3*
13A	Multicopy suppressor	pXW170	*YPT32*
15A	Multicopy suppressor	pXW180	*YPT32*
17A	Multicopy suppressor	pXW190	*YCK2*

Thus, each suppressing plasmid was subjected to *Bgl*II and *Hin*dIII restriction digestions to test this possibility. Consistent with the finding that pXW100 conferred the strongest suppression of *gcs1Δ* cold sensitivity, pXW100 gave the diagnostic 500-bp *Bgl*II restriction fragment and the 800-bp *Hin*dIII restriction fragment (data not shown). Thus, pXW100 probably contains the wild-type *GCS1* gene. On the other hand, none of the other suppressing plasmids showed those two diagnostic restriction fragments; thus none of the other suppressing plasmids contains *GCS1*.

All these multicopy suppressors were isolated by their ability to suppress the cold sensitivity of strain Grk4-7, which is a *gcs1Δ* mutant strain constructed in a 21R genetic background. Much of the previous work done on *GCS1* in our lab was carried out using a W303 genetic background (Ireland *et al.*, 1994). Since strain W303 (Archambault *et al.* 1992) is widely used by many groups and has more useful genetic markers than does strain 21R, I decided to construct my own *gcs1Δ* strain in a W303 background. W303-1A (*MAT***a**) and W303-1B (*MAT*α) cells were transformed with linearized *GCS1* deletion plasmid pBN-Δ4 to generate *gcs1Δ* strains Gwk9A (*MAT***a**) and Gwk9B (*MAT*α). Both Gwk9A and Gwk9B cells show the cold-sensitive defect in resumption of cell proliferation from stationary phase (data not shown), and this mutant phenotype was suppressed when the cells were transformed with each of my multicopy suppressors. Thus, the Gwk9A and Gwk9B *gcs1Δ* mutant cells were used in future genetic studies, since the multicopy suppressors I isolated also function in this W303 genetic background.

2. Identification of *YCK2* and *YCK1* as multicopy suppressor genes

Since I found 9 plasmids (other than *GCS1* itself) that suppress the *gcs1* mutant phenotype, I set out to determine whether these 9 plasmids contained the same genomic insert. For this analysis, restriction maps were constructed for these 9 plasmids. Comparison of the restriction maps for two plasmids, pXW120 and pXW190, revealed that these two plasmids are the same (Figure 1; Table 3). To localize the suppressing

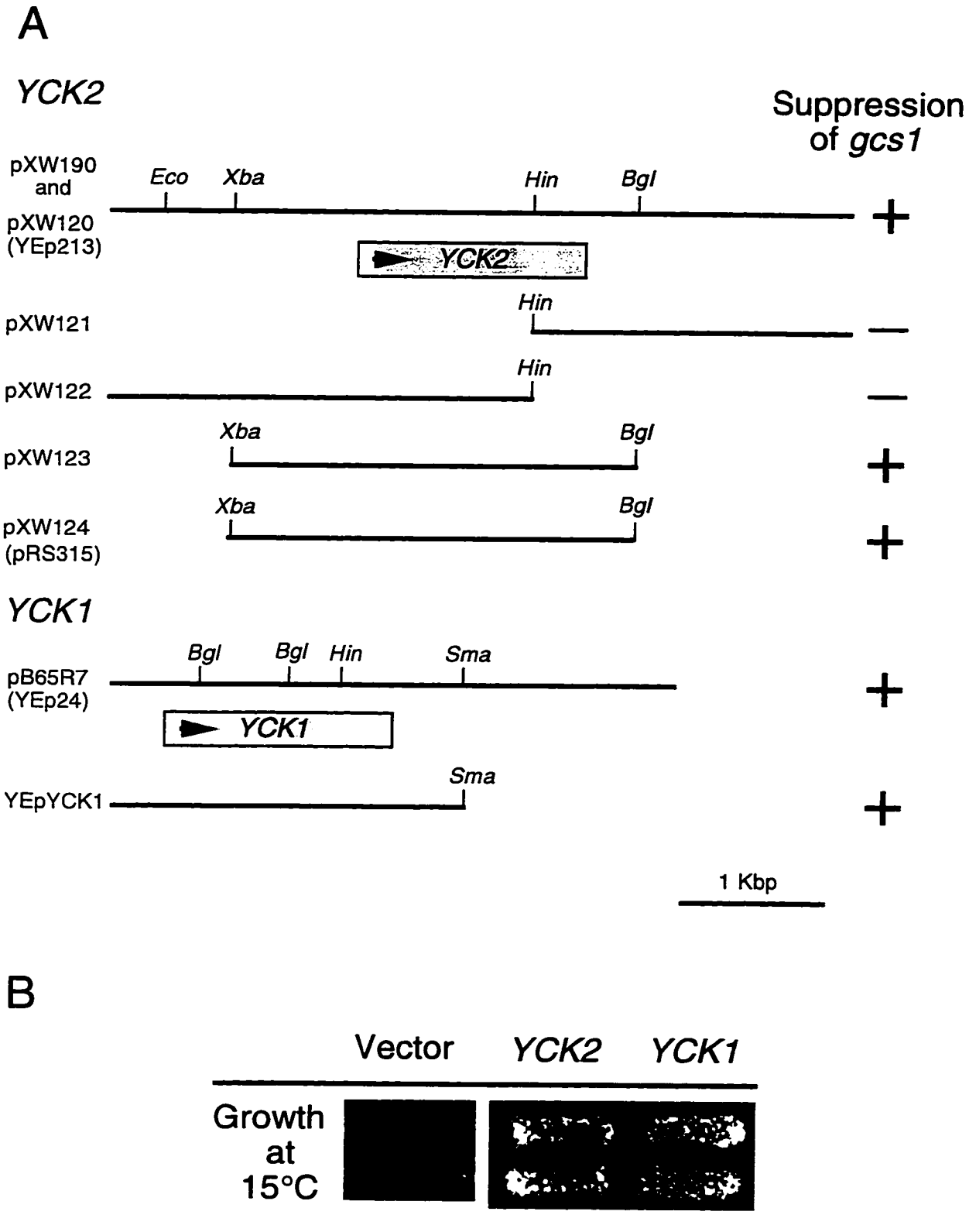
sequence within the 5.3-kbp insert on plasmid pXW120 (or pXW190), I constructed two non-overlapping subclone plasmids utilizing an internal *Hin*dIII site, and tested each for suppression of the *gcs1* cold sensitivity. This analysis showed that neither subclone plasmid, pXW121 or pXW122, suppresses the *gcs1* cold sensitivity as well as does pXW120. Thus, it is likely that the Open Reading Frame (ORF) of suppressor gene spans the *Hin*dIII site within the 5.3-kbp insert. I then determined the nucleotide sequence from the region surrounding the *Hin*dIII site. The approximately 300-bp sequence generated from this analysis, when searched against the Genbank, was identical to the published sequence of the *YCK2* (also called *CKI1*) gene (Robinson *et al.*, 1992; Wang *et al.*, 1992), which encodes a plasma-membrane-associated isoform of yeast casein kinase I (Vancura *et al.*, 1994). Consistent with this finding, the restriction map of the *YCK2* gene is clearly similar to the one I constructed for pXW120. I then generated a third subclone, pXW123, predicted to contain the entire ORF of *YCK2*, and found that it suppresses the cold sensitivity of *gcs1Δ* mutant cells. Thus, the *YCK2* casein kinase I gene is the multicopy-suppressor gene in pXW120 and pXW190. A fourth subclone plasmid, pXW124, containing the entire *YCK2* gene on the low-copy *CEN* plasmid pRS315, was also found to suppress *gcs1* cold sensitivity. Thus even a few extra copies of the *YCK2* gene suppress the cold sensitivity of *gcs1Δ* mutant cells.

YCK2 forms an essential gene pair with the closely related casein kinase I gene *YCK1* (also called *CKI2*) (Robinson *et al.*, 1992; Wang *et al.*, 1992), and these protein products show high sequence identity (~75%). While yeast cells lacking one of these *YCK* genes behave just like wild-type cells, cells lacking both *YCK1* and *YCK2* are dead (Robinson *et al.*, 1992; Wang *et al.*, 1992). To test whether these genes also have overlapping functions in suppressing the *gcs1* mutant phenotype, I obtained the *YCK1* gene (Robinson *et al.*, 1992) from others, generated a multicopy plasmid YEp*YCK1* containing the entire *YCK1* ORF in plasmid YEp351, and found that *YCK1* in multiple copies also suppresses the *gcs1* cold sensitivity (Figure 1). Therefore either *YCK2* or

Figure 1: Suppression of *gcs1Δ* cold sensitivity by increased dosage of *YCK1* or *YCK2*.

(A) Subclone analysis of *YCK2* and *YCK1*. Only yeast sequences are shown, and the vector is YEp351 or otherwise as indicated; boxes and arrows indicate the position and 5' → 3' direction of the open reading frames. Restriction sites are *Bgl*II (*Bgl*), *Eco*RI (*Eco*), *Hin*dIII (*Hin*), *Sma*I (*Sma*), and *Xba*I (*Xba*). To assess the suppression of *gcs1Δ* cold sensitivity, *gcs1Δ* mutant cells (strain Gwk9A) transformed with different plasmids were incubated on leucine-free medium at the permissive temperature of 29°C for several days and then replica-plated and incubated at 15°C for 7 days. The vector control was YEp351.

(B) Suppression of *gcs1Δ* cold sensitivity by increased dosage of *YCK2* and *YCK1*. *gcs1Δ* cells (strain Gwk9A) transformed with *YCK2* multicopy plasmid pXW123, *YCK1* multicopy plasmid YEpYCK1, and vector YEp351 were assessed as described for Panel A.


A
YCK2
Suppression of gcs1
pXW190 and pXW120 (YEp213)
Eco
Xba
Hin
Bgl
YCK2
pXW121
Hin
pXW122
Hin
pXW123
Xba
Bgl
pXW124 (pRS315)
Xba
Bgl
YCK1
pB65R7 (YEp24)
Bgl
Bgl
Hin
Sma
YCK1
YEpYCK1
Sma
1 Kbp
B
Vector
YCK2
YCK1
Growth at 15°C

YCK1 in multiple copies suppresses the *gcs1* mutant phenotype.

3. Genetic interaction between *gcs1* and a *yck2-2ts* mutation

Two mutations affecting a common process, when combined, can sometimes generate a new phenotype that is more severe than caused by either one alone, and this is termed synthetic enhancement of impairment. Synthetic enhancement between mutant genes is a useful genetic tool to identify genes with related functions and to dissect genetic pathways (Guarente, 1993). To address the issue of whether the normal functions of Yck2 and Yck1 are related to Gcs1 activity, I also looked for genetic interactions between *gcs1Δ* and *yck* mutations. Because *YCK2* and *YCK1* genes are functionally redundant, I assumed that any genetic interactions shown among *gcs1* and *yck* mutations would only be revealed if both *YCK2* and *YCK1* were mutated. I therefore obtained the haploid strain YI228, which harbors *yck2Δ* and *yck1Δ* mutations and is kept alive by a plasmid-borne temperature-sensitive *yck2-2*ts mutant allele (Vancura *et al.*, 1994). Strain YI228 was first made diploid using a method based on mating-type switching (Materials and Methods), and then one copy of the *GCS1* gene in the diploid was disrupted by transformation with linearized *GCS1* deletion plasmid pBN-Δ4 (Ireland *et al.*, 1994). The resulting diploid transformant, heterozygous for the *gcs1Δ* allele but homozygous for the *yck2Δ* and *yck1Δ* mutations, retained the *yck2-2*ts temperature-sensitivity phenotype. This diploid was sporulated and the asci were dissected to obtain *gcs1Δ yck1Δ yck2Δ yck2-2*ts segregants, which were allowed to germinate at a temperature permissive for both *gcs1* and *yck1Δ yck2-2*ts. Of the 25 tetrads analyzed, no more than two of the four spores from each tetrad were able to form colonies: 18 tetrads showed a 2:2 segregation, 5 showed a 1:3 segregation, and 2 showed 0:4 segregation for viability. Judging by the nutritional markers at the *gcs1Δ* and *yckΔ* loci and by the *gcs1Δ*cs and *yck2-2*ts phenotypes, all the viable spores had the genotype *GCS1 yck2Δ yck1Δ yck2-2*ts, and none of them had the *gcs1Δ yck2Δ yck1Δ yck2-2*ts genotype. Since *gcs1Δ* spores germinate at

the permissive temperature (Ireland *et al.*, 1994), the combination of *gcs1Δ* and the *yck2Δ yck1Δ yck2*-2ts mutations must be lethal for spore germination. Thus, either Gcs1 activity or adequate Yck2 + Yck1 casein kinase I activity is needed for spore germination.

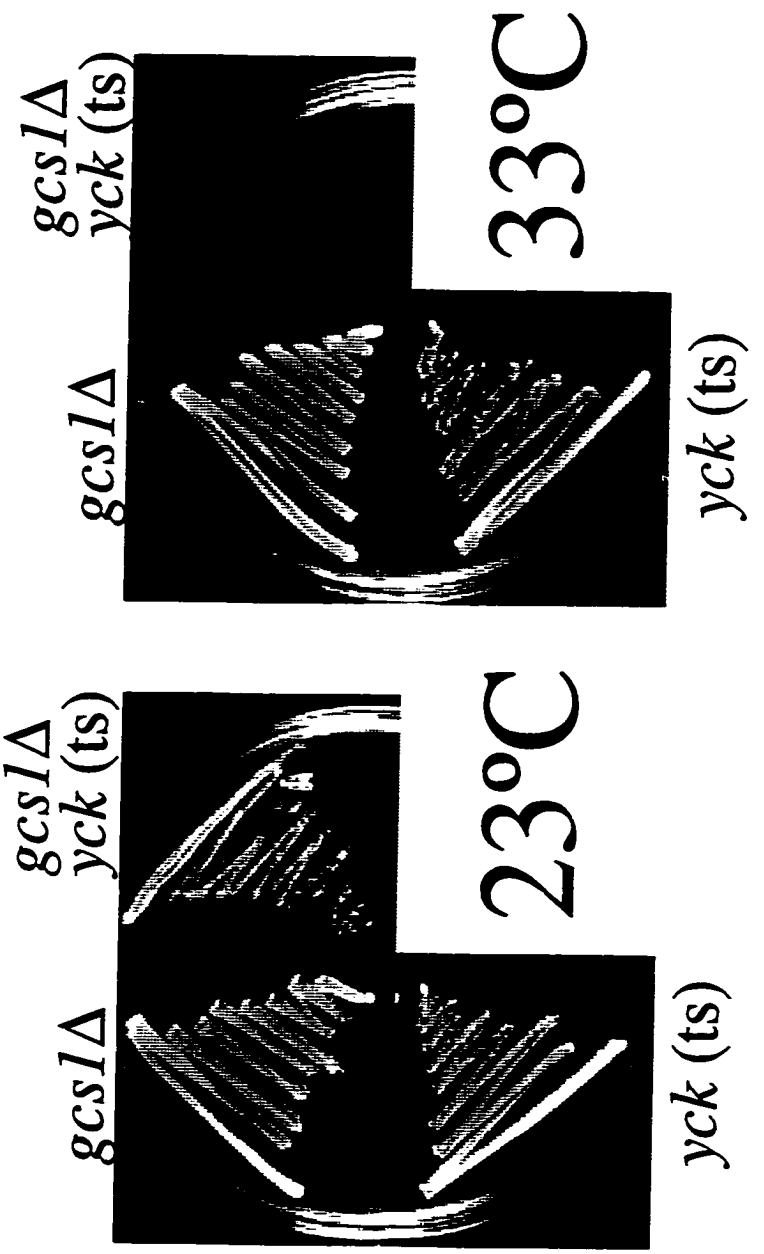
To test whether the combination of *gcs1Δ* and the *yck2Δ yck1Δ yck2*-2ts mutations is lethal only for spore germination, I also tried to inactivate the *GCS1* gene in YI228 (*yck2Δ yck1Δ yck2*-2ts) haploid cells. Since deleting *GCS1* could be lethal in a YI228 genetic background, YI228 cells were first transformed with pXW124, which is a low-copy wild-type *YCK2* plasmid, and then the genomic copy of *GCS1* was deleted by transforming the cells with linearized pBN-Δ4 plasmid. Interestingly, I was able to lose the pXW124 plasmid from these transformants and get viable haploid *gcs1Δ yck2Δ yck1Δ yck2*-2ts mutant cells that could grow at a permissive temperature of 23°C. Therefore, the combination of *gcs1Δ yck2Δ yck1Δ* and *yck2*-2ts mutations is lethal only for spore germination.

As expected, those quadruple-mutant cells could not grow at the restrictive temperature of 37°C because of the *yck2*-2ts mutation, and they showed the cold-sensitivity phenotype of the *gcs1Δ* mutation at the low temperature of 15°C. Furthermore, these mutant cells failed to grow at 33°C, a temperature at which both *gcs1Δ* mutant cells and parental *yck2Δ yck1Δ yck2*-2ts mutant cells do grow (Figure 2). Thus, in mitotic cells the loss of Gcs1 function also enhances the impairment caused by the decreased activities of Yck2 + Yck1, indicating Gcs1 activity and the Yck1 + Yck2 casein kinase I activity are probably involved in a common process for mitotic cell growth. This observation also indicates that Gcs1 protein has certain functions for cells at higher growth temperatures like 33°C, even though a single *gcs1* mutation does not obviously inhibit cell growth.

Since Gcs1 is not structurally similar to Yck1 and Yck2 and has no apparent protein kinase motifs, it is unlikely that increased Yck2 or Yck1 protein kinase activity

Figure 2: Synthetic enhancement between *gcs1Δ* and the *yck2Δ yck1Δ yck2-2*ts mutant situations.

gcs1Δ mutant cells (strain Gwk9A; shown as *gcs1Δ*), *yck2Δ yck1Δ yck2-2*ts mutant cells [strain YI228; shown as *yck*(ts)], and *gcs1Δ yck2Δ yck1Δ yck2-2*ts mutant cells [strain YX5; shown as *gcs1Δ yck*(ts)] were incubated on YEPD medium for 3 days at the indicated temperatures.


gcs1Δ
yck (ts)
gcs1Δ
yck (ts)
33°C
gcs1Δ
yck (ts)
gcs1Δ
yck (ts)
23°C

replaces Gcs1 activity. It is possible, however, that increased casein kinase I activity facilitates a parallel pathway that bypasses the need for Gcs1. The synthetic enhancement of impairment seen between the *gcs1Δ* null mutation and a *yck2Δ yck1Δ yck2*-2ts situation indicates those two parallel pathways could affect a common process. Therefore, like Gcs1, the Yck1 and Yck2 casein kinase I activity might also have a role in intracellular membrane transport.

4. Identification of *YCK3* as a multicopy suppressor gene

Restriction maps generated for plasmids pXW150 and pXW160 showed that these two plasmids are identical, and contain a common insert of about 4 kbp (Figure 3A). To localize the suppressing sequence on plasmid pXW150 I generated two non-overlapping subclones, pXW151 and pXW152, using an internal *Bam*HI restriction site, and tested these subclones for suppression of the cold sensitivity of *gcs1Δ* mutant cells. Neither subclone was able to suppress the *gcs1Δ* mutant phenotype; thus the ORF of this suppressor gene must span the *Bam*HI restriction site. To identify this suppressor gene I determined nucleotide sequence in the region surrounding the *Bam*HI site. An approximately 300-bp DNA sequences generated from this analysis showed that the suppressor gene on plasmid pXW150 encodes a protein similar in sequence to Yck1 and Yck2. Since the sequence of this gene was not in the databases at the time this work was done, I concluded that this suppressor gene encodes a novel isoform of yeast casein kinase I.

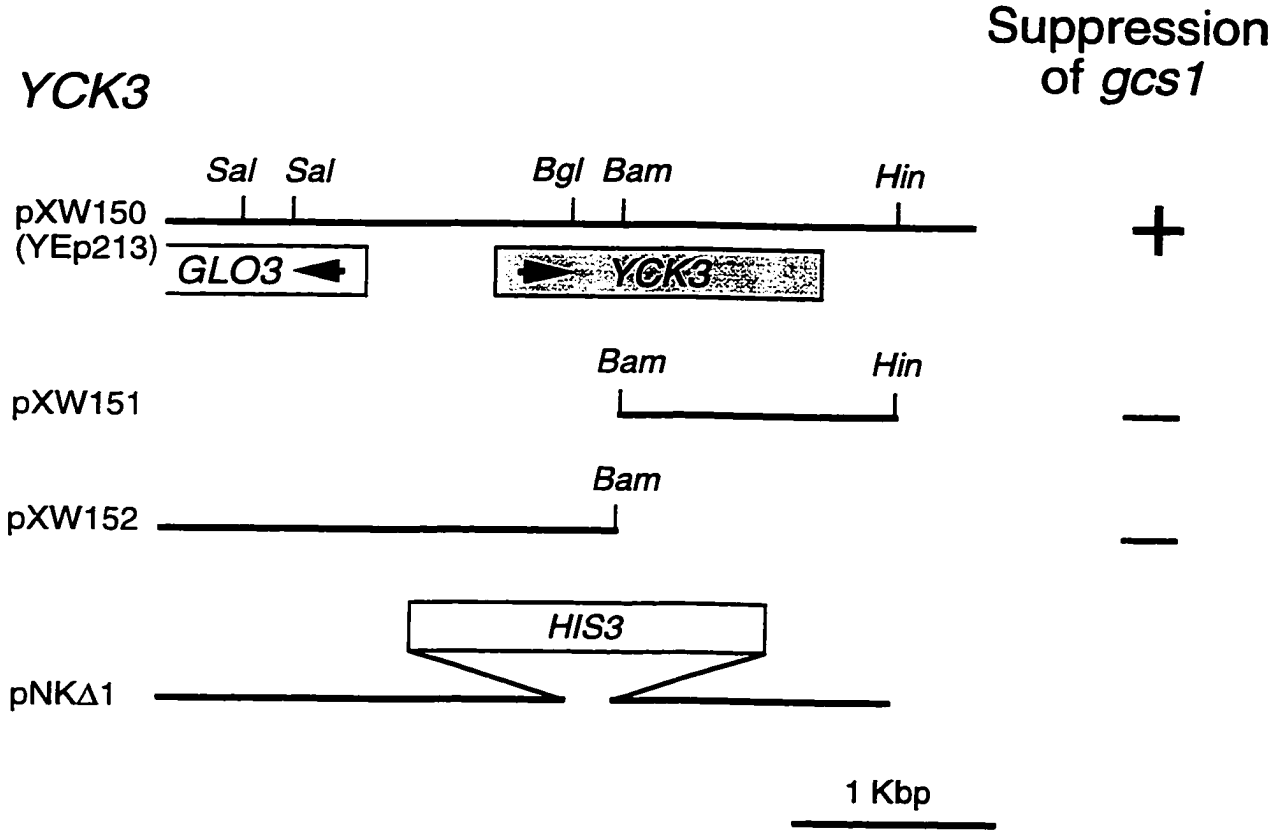
At the same time, Merl Hoekstra's group at ICOS Corporation (Bothell, WA) also cloned a new casein kinase I gene when they were isolating multicopy suppressors of an *hrr25* mutation; the *HRR25* gene encodes yet another yeast casein kinase I isoform (Hoekstra *et al.*, 1991). When we compared the restriction maps and nucleotide sequences of our genes, we realized that both groups had cloned the same gene, which we named *YCK3*. The entire coding sequence of *YCK3* was determined at ICOS

Figure 3: Suppression of *gcs1Δ* cold sensitivity by increased dosage of *YCK3*.

(A) Subclone analysis of *YCK3*. Only yeast sequences are shown, and the vector is YEp351 or otherwise as indicated; boxes and arrows indicate the position and 5' → 3' direction of the open reading frames. Restriction sites are *Bam*HI (*Bam*) *Bgl*II (*Bgl*), *Hin*dIII (*Hin*), and *Sal*I (*Sal*). To assess the suppression of *gcs1Δ* cold sensitivity by different *YCK3* genomic clones, *gcs1Δ* mutant cells (strain Gwk9A) transformed with different plasmids were incubated on leucine-free medium at the permissive temperature of 29°C for several days and then replica-plated and incubated at 15°C for 7 days. The vector control was YEp351. pNKΔ1 is a *YCK3* gene-deletion plasmid.

(B) Suppression of *gcs1Δ* cold sensitivity by increased dosage of *YCK3*. *gcs1Δ* mutant cells (strain Gwk9A) transformed with multicopy *YCK3* plasmid pXW150, multicopy *HRR25* plasmid pXW231, and vector YEp351 were assessed as described for Panel A. Two individual transformants were tested for each plasmid.

A


YCK3
Suppression of gcs1
Sal Sal
Bgl Bam
Hin
pXW150
(YEp213)
GLO3
YCK3
+
Bam
Hin
pXW151
—
Bam
pXW152
—
HIS3
pNKΔ1
1 Kbp

B

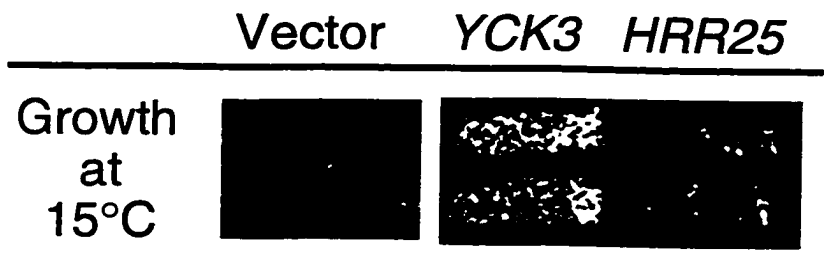

Vector
YCK3
HRR25
Growth at 15°C

Figure 4: The *YCK3* gene and flanking sequences.

The nucleotide sequence of the *YCK3* gene is numbered from the presumptive translation initiation codon ATG (bolded) of the *YCK3* ORF. The predicted amino-acid sequence is also numbered from the initiator methionine (bolded). Nucleotide and amino-acid numbering are shown on the left. A possible TATA box is underlined.

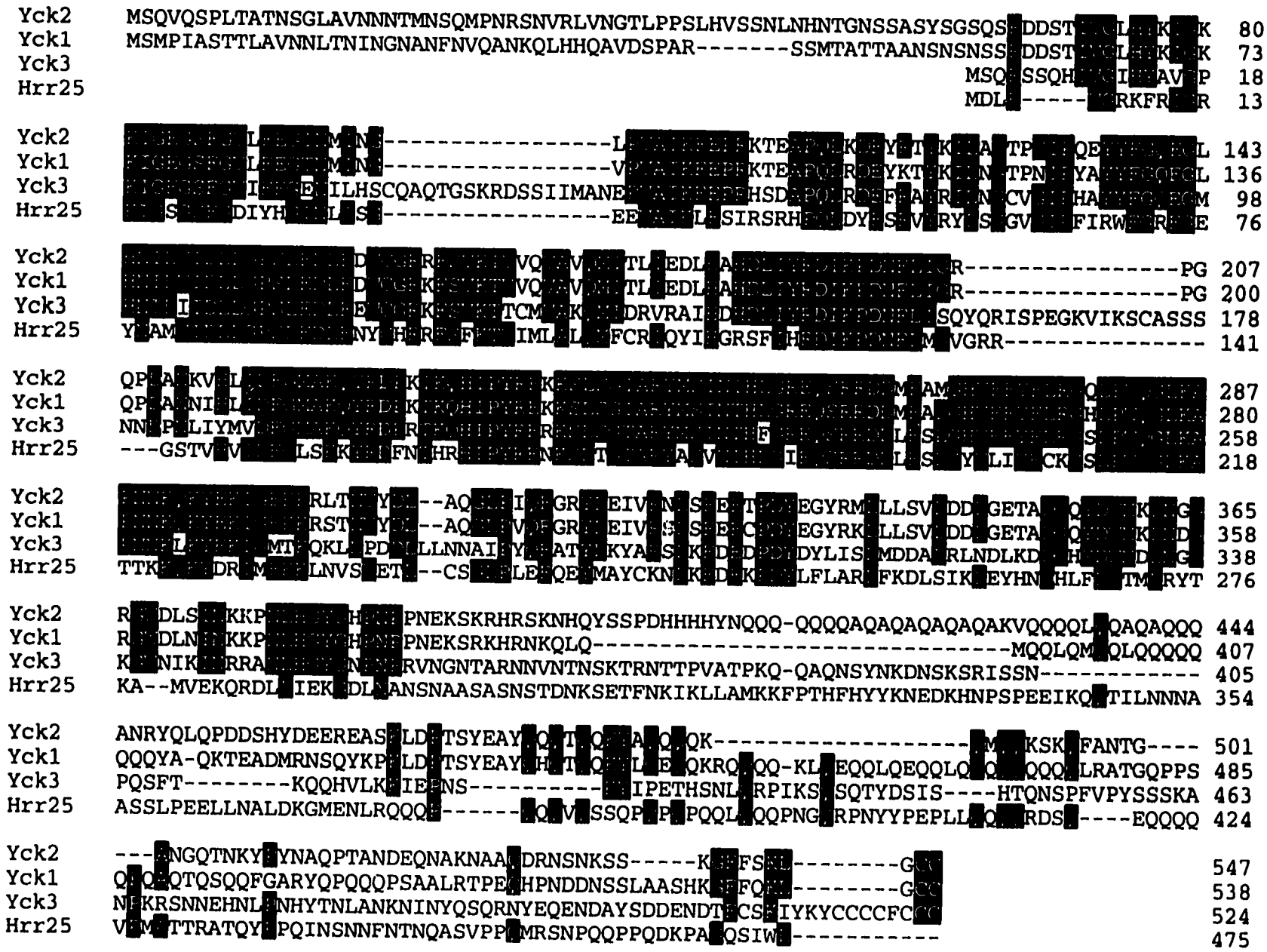
```
-132 CTG TTT TTT AAA AAA GAA GGA AAT CCG TTT TGC AAG AAT TGT CTG CTA TTT AAG GGT ATA

 -72 CGT GCT ACG GTC CAC TAA TCA AAA GTG GTA TCT CAT TCT GAA GAA AAA GTG TAA AAA GGA

 -12 CGA TAA GGA AAG ATG TCC CAA CGA TCT TCA CAA CAC ATT GTA GGT ATT CAT TAT GCT GTA
   1                 M   S   Q   R   S   S   Q   H   I   V   G   I   H   Y   A   V
  49 GGA CCT AAG ATT GGC GAA GGG TCT TTC GGA GTA ATA TTT GAG GGA GAG AAC ATT CTT CAT
  17  G   P   K   I   G   E   G   S   F   G   V   I   F   E   G   E   N   I   L   H
 109 TCT TGT CAA GCG CAG ACC GGT AGC AAG AGG GAC TCT AGT ATA ATA ATG GCG AAC GAG CCA
  37  S   C   Q   A   Q   T   G   S   K   R   D   S   S   I   I   M   A   N   E   P
 169 GTC GCA ATT AAA TTC GAA CCG CGA CAT TCG GAC GCA CCC CAG TTG CGT GAC GAA TTT AGA
  57  V   A   I   K   F   E   P   R   H   S   D   A   P   Q   L   R   D   E   F   R
 229 GCC TAT AGG ATA TTG AAT GGC TGC GTT GGA ATT CCC CAT GCT TAT TAT TTT GGT CAA GAA
  77  A   Y   R   I   L   N   G   C   V   G   I   P   H   A   Y   Y   F   G   Q   E
 289 GGT ATG CAC AAC ATC TTG ATT ATC GAT TTA CTA GGG CCA TCA TTG GAA GAT CTC TTT GAG
  97  G   M   H   N   I   L   I   I   D   L   L   G   P   S   L   E   D   L   F   E
 349 TGG TGT GGT AGA AAA TTT TCA GTG AAA ACA ACC TGT ATG GTT GCC AAG CAA ATG ATT GAT
 117  W   C   G   R   K   F   S   V   K   T   T   C   M   V   A   K   Q   M   I   D
 409 AGA GTT AGA GCA ATT CAT GAT CAC GAC TTA ATC TAT CGC GAT ATT AAA CCC GAT AAC TTT
 137  R   V   R   A   I   H   D   H   D   L   I   Y   R   D   I   K   P   D   N   F
 469 TTA ATT TCT CAA TAT CAA AGA ATT TCA CCT GAA GGA AAA GTC ATT AAA TCA TGT GCC TCC
 157  L   I   S   Q   Y   Q   R   I   S   P   E   G   K   V   I   K   S   C   A   S
 529 TCT TCT AAT AAT GAT CCC AAT TTA ATA TAC ATG GTT GAC TTT GGT ATG GCA AAA CAA TAT
 177  S   S   N   N   D   P   N   L   I   Y   M   V   D   F   G   M   A   K   Q   Y
 589 AGA GAT CCA AGA ACG AAA CAA CAT ATA CCA TAC CGT GAA CGA AAA TCA TTG AGC GGT ACC
 197  R   D   P   R   T   K   Q   H   I   P   Y   R   E   R   K   S   L   S   G   T
 649 GCC AGA TAT ATG TCT ATT AAT ACT CAT TTT GGA AGA GAA CAG TCA CGT AGG GAT GAT TTA
 217  A   R   Y   M   S   I   N   T   H   F   G   R   E   Q   S   R   R   D   D   L
 709 GAA TCG CTA GGT CAC GTT TTT TTT TAT TTC TTG AGG GGA TCC TTG CCA TGG CAA GGT TTG
 237  E   S   L   G   H   V   F   F   Y   F   L   R   G   S   L   P   W   Q   G   L
 769 AAA GCA CCA AAC AAC AAA CTG AAG TAT GAA AAG ATT GGT ATG ACT AAA CAG AAA TTG AAT
 257  K   A   P   N   N   K   L   K   Y   E   K   I   G   M   T   K   Q   K   L   N
 829 CCT GAT GAT CTT TTA TTG AAT AAT GCT ATT CCT TAT CAG TTT GCC ACA TAT TTA AAA TAT
 277  P   D   D   L   L   L   N   N   A   I   P   Y   Q   F   A   T   Y   L   K   Y
 889 GCA CGT TCC TTG AAG TTC GAC GAA GAT CCG GAT TAT GAC TAT TTA ATC TCG TTA ATG GAT
 297  A   R   S   L   K   F   D   E   D   P   D   Y   D   Y   L   I   S   L   M   D
 949 GAC GCT TTG AGA TTA AAC GAC TTA AAG GAT GAT GGA CAC TAT GAC TGG ATG GAT TTG AAT
 317  D   A   L   R   L   N   D   L   K   D   D   G   H   Y   D   W   M   D   L   N
1009 GGT GGT AAA GGC TGG AAT ATC AAG ATT AAT AGA AGA GCT AAC TTG CAT GGT TAC GGA AAT
 337  G   G   K   G   W   N   I   K   I   N   R   R   A   N   L   H   G   Y   G   N
1069 CCA AAT CCA AGA GTC AAT GGC AAT ACT GCA AGA AAC AAT GTG AAT ACG AAT TCA AAG ACA
 357  P   N   P   R   V   N   G   N   T   A   R   N   N   V   N   T   N   S   K   T
1129 CGA AAT ACA ACG CCA GTT GCG ACA CCT AAG CAA CAA GCT CAA AAC AGT TAT AAC AAG GAC
 377  R   N   T   T   P   V   A   T   P   K   Q   Q   A   Q   N   S   Y   N   K   D
1189 AAT TCG AAA TCC AGA ATT TCT TCG AAC CCG CAG AGC TTT ACT AAA CAA CAA CAC GTC TTG
 397  N   S   K   S   R   I   S   S   N   P   Q   S   F   T   K   Q   Q   H   V   L
1249 AAA AAA ATC GAA CCC AAT AGT AAA TAT ATT CCT GAA ACA CAT TCA AAT CTT CAA CGG CCA
 417  K   K   I   E   P   N   S   K   Y   I   P   E   T   H   S   N   L   Q   R   P
1309 ATT AAA AGT CAA AGT CAA ACG TAC GAC TCC ATC AGT CAT ACA CAA AAT TCA CCA TTT GTA
 437  I   K   S   Q   S   Q   T   Y   D   S   I   S   H   T   Q   N   S   P   F   V
1369 CCA TAT TCA AGT TCT AAA GCT AAC CCT AAA AGA AGT AAT AAT GAG CAC AAC TTA CCA AAC
 457  P   Y   S   S   S   K   A   N   P   K   R   S   N   N   E   H   N   L   P   N
1429 CAC TAC ACA AAC CTT GCA AAT AAG AAT ATC AAT TAT CAA AGT CAA CGA AAT TAC GAA CAA
 477  H   Y   T   N   L   A   N   K   N   I   N   Y   Q   S   Q   R   N   Y   E   Q
1489 GAA AAT GAT GCT TAT TCT GAT GAC GAG AAT GAT ACA TTT TGT TCT AAA ATA TAC AAA TAT
 497  E   N   D   A   Y   S   D   D   E   N   D   T   F   C   S   K   I   Y   K   Y
1549 TGT TGT TGC TGT TTT TGT TGC TGT TGA TAA AGC GAT TTT TAT ACT TTT CTC TTT TTC CTT
 517  C   C   C   C   F   C   C   C  OPA OCH
1609 TTT TTT TTT
```

Figure 5: Sequence similarities among the four yeast casein kinase I isoforms.

Alignment of Yck2, Yck1, Yck3 and Hrr25 predicted polypeptides using PileUp (Devereux *et al.*, 1984). White-on-black indicates where at least 3 proteins have the same amino acid. Amino acid positions are indicated on the right.


Yck2
Yck1
Yck3
Hrr25

Corporation. The predicted sequence of Yck3 has 524 amino acid residues (Figure 4), with an N-terminal region that is highly similar to the protein-kinase domains of Yck2, Yck1, and Hrr25. The C terminus of Yck3 contains a short stretch of cysteine residues that may be a prenylation signal similar to that of Yck2 and Yck1. The Yck3 protein also has a serine- and asparagine-rich region linking the protein-kinase domain and the C-terminal lipid-modification motif, which resembles the analogous proline- and glutamine-rich region of Hrr25 and the glutamine-rich regions of Yck2 and Yck1 (Figure 5; Robinson *et al.*, 1992; Wang *et al.*, 1992; Hoekstra *et al.*, 1991). Thus Yck3 is not only structurally similar to Yck2 and Yck1, but it also has similar activities in terms of its ability to suppress the *gcs1* mutant phenotype.

Though overexpression of each of the three casein kinase I isoforms Yck1, Yck2, and Yck3 can bypass the need for Gcs1 during the resumption of cell proliferation from stationary phase, when tested directly the increased expression of another isoform, Hrr25, either from a multicopy plasmid (pXW321) or from an inducible *GAL1-10* promoter (pGAL-HRR25; from M. Hoekstra), could not suppress the *gcs1* cold sensitivity (Figure 3B). Thus, more than simple casein kinase I activity must be involved in this suppression.

5. Lack of genetic interaction between *yck3Δ* and *gcs1Δ* mutations

Yck3 is a novel yeast casein kinase I isoform and increased activity of Yck3 also suppresses the *gcs1Δ* cold sensitivity, just like Yck1 and Yck2. To assess whether the normal activity of Yck3 is related to Gcs1 activity, it would be useful to determine whether a decrease in Yck3 activity, like the decrease in Yck1 + Yck2 activity, also enhances the impairment caused by the *gcs1Δ* mutation. For this analysis I obtained the *YCK3* disruption plasmid pNUF1Δ from Hoekstra (Figure 6) to generate *yck3Δ* mutant cells in the W303 genetic background. Interestingly, two types of transformants were obtained, one type cold-sensitive for growth at 15°C and the other not. Southern analysis

showed that in both types of transformant the genomic *YCK3* locus had been changed due to the peculiar features of plasmid pNUF1Δ. In the cold-sensitive transformant, not only was the *YCK3* gene disrupted but part of a neighboring gene, *GLO3* (Ireland *et al.*, 1994), was also deleted (Figure 6). In contrast, in the cold-resistant transformants only the *YCK3* gene was disrupted, with the *LEU2* gene from pNUF1Δ inserted in the middle of the *YCK3* ORF (Figure 6). The *YCK3* gene is therefore not essential, and cells bearing a *yck3::LEU2* disruption mutation do not appear to be very different from wild-type cells (data not shown). Similar results (data not shown) were obtained when the *YCK3* gene was deleted by using a different plasmid, pNKΔ-1, which replaces part of the *YCK3* ORF (Figure 3). Thus, the *YCK3* gene is not an essential gene, and cells lacking the *YCK3* gene have no apparent growth defects. The cold sensitivity of the *yck3Δ glo3Δ* transformants, therefore, is not caused by the *yck3Δ* mutation, but instead could be caused by the *glo3Δ* mutation.

Gwk9A cells harboring a *gcs1Δ* allele were crossed with cells harboring the *yck3::LEU2* allele, and the resultant diploid cells were sporulated and subjected to tetrad analysis. I found that spores harboring both *gcs1Δ* and *yck3::LEU2* germinated and formed colonies just like wild-type spores, and the resultant haploid cells did not exhibit impaired growth at various temperatures except for the *gcs1Δ* cold sensitivity (data not shown). Since Yck3 is not an essential protein, it is possible that Yck3 belongs to another essential family and the *yck3Δ* mutation does not decrease the activity of that casein kinase I family enough to enhance the impairment caused by *gcs1Δ*. As described above, *YCK3* was cloned by Hoekstra as a copy suppressor of the *hrr25* mutant phenotype. Thus, Yck3 and Hrr25 could have overlapping functions. This hypothesis was confirmed by the observation that the *YCK3* gene and the *HRR25* gene form an essential gene family. Cells with an *hrr25Δ* mutation display slow growth and a defect in DNA repair (Hoekstra *et al.*, 1991), and cells with both an *hrr25Δ* mutation and a *yck3Δ* mutation are dead (Wang *et al.*, 1996), even though cells with a single *yck3Δ* mutation

Figure 6: The *YCK3* disruption plasmid pNUF1Δ generates two different mutant situations.

Yeast chromosomal sequences ("Chrom.") are indicated as horizontal lines marked with restriction sites; boxes indicate the positions of open reading frames (ORF) as well as the Tn3(*LEU2*) mini transposon; small arrows within the boxes indicate the 5' → 3' direction of the ORFs; possible DNA recombination sites are indicated by big crosses. Possible recombination events that generated the *glo3Δ yck3Δ* mutant situation are diagrammed in (A), and possible recombination events that generated the *yck3::LEU2* mutant allele are diagrammed in (B). Southern analysis confirmed that those two different mutant situations were generated in different transformants.

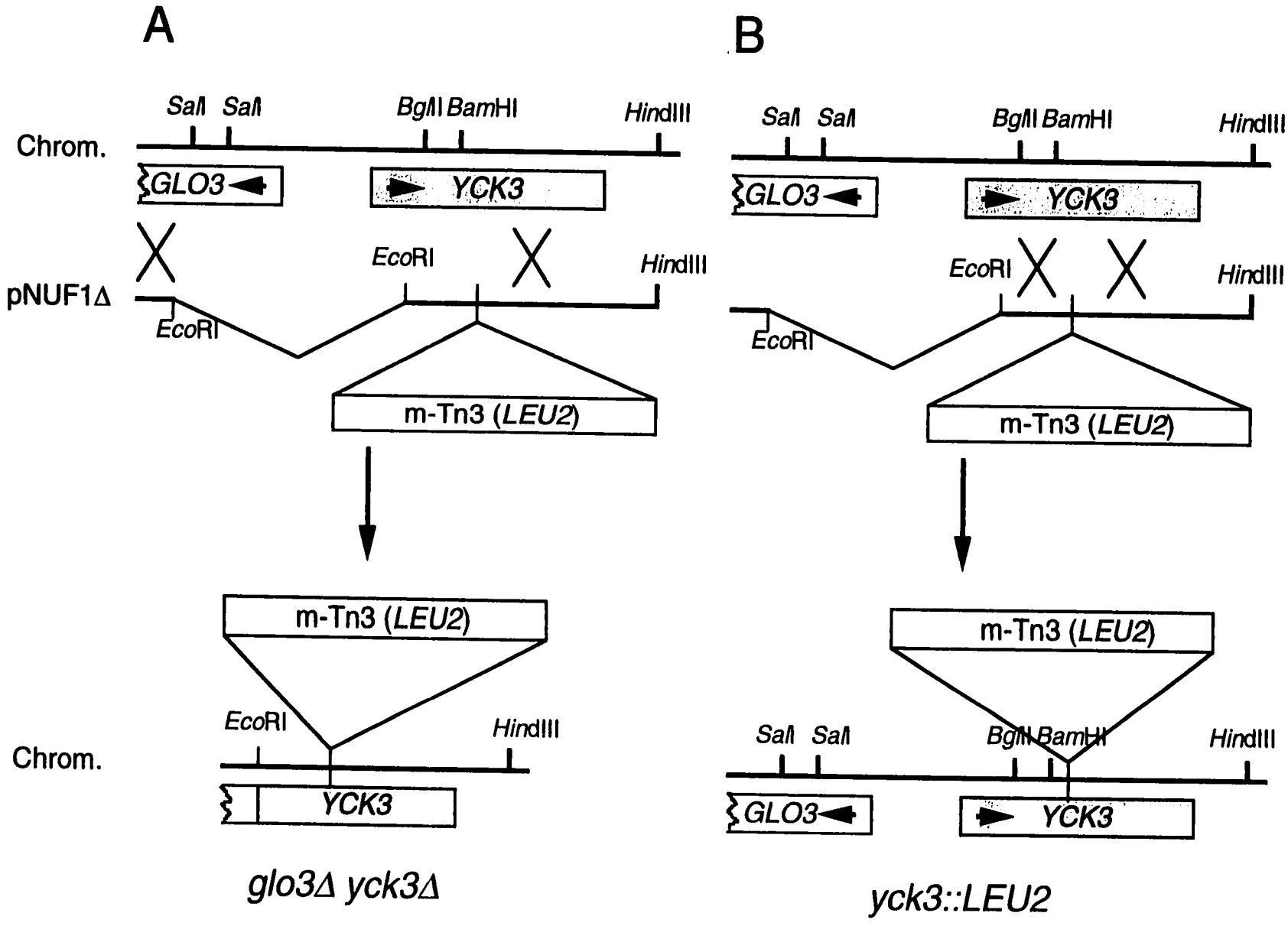
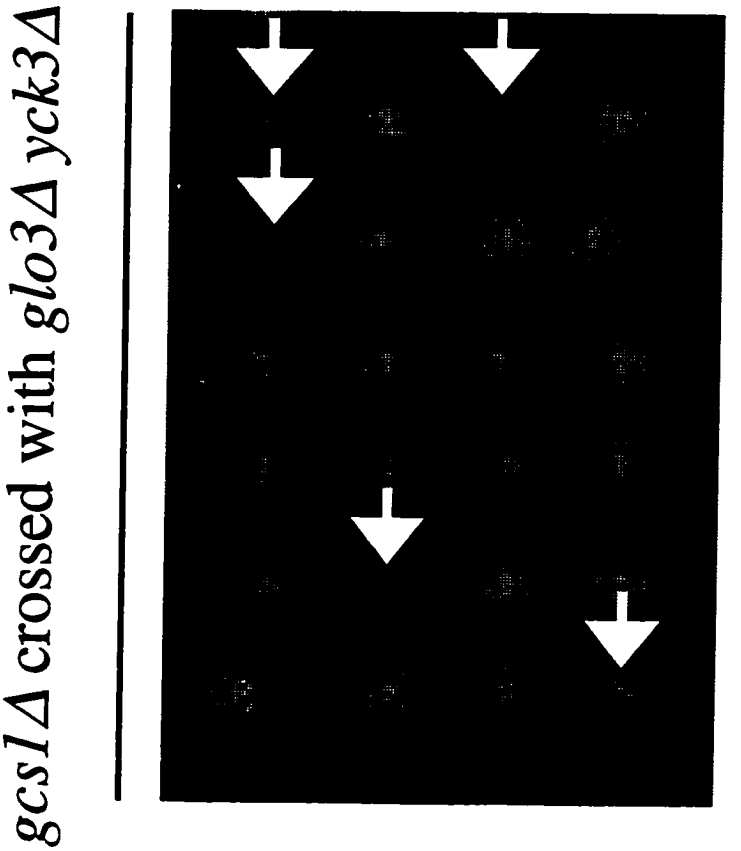

A
B
Chrom.
SalI SalI
BglII BamHI
HindIII
GLO3
YCK3
pNUF1Δ
EcoRI
m-Tn3 (LEU2)
Chrom.
glo3Δ yck3Δ
yck3::LEU2

Figure 7: Synthetic enhancement between *gcs1Δ* and the *glo3Δ yck3Δ* mutant situations.

Haploid *gcs1Δ* mutant cells (strain Grk4-7) were crossed with haploid *glo3Δ yck3Δ* mutant cells (strain NRK2B), and the resultant diploid cells were sporulated and subjected to tetrad analysis. Four spores from each ascus, separated and arranged at different positions (from top to bottom) on the surface of YEPD medium, were incubated at the permissive temperature of 29°C for 6 days to allow germination and colony formation. Colonies formed by spores bearing the *gcs1Δ glo3Δ yck3Δ* genotype, as indicated by nutritional markers, are indicated with white arrows.


gcs1Δ crossed with glo3Δ yck3Δ

have no apparent phenotype. Due to the lack of conditional *yck3::LEU2 hrr25* double-mutant cells, I was not able to determine whether decreased activities of both Hrr25 and Yck3 could impair *gcs1* mutant cells. This apparent lack of synthetic enhancement of *gcs1Δ* impairment may indicate that the Yck3 and Gcs1 activities are not involved in a common process or pathway. If so, then the suppression of the *gcs1* mutant phenotype by increased Yck3 may be due to increased Yck3 activity augmenting Yck2 and/or Yck1 activity.

Interestingly, when I crossed the cold-sensitive *yck3Δ glo3Δ* double-mutant cells discussed above with *gcs1Δ* cells to generate *yck3Δ glo3Δ gcs1Δ* cells, I found that spores bearing the triple *yck3Δ glo3Δ gcs1Δ* mutations formed only tiny colonies (Figure 7). In light of the absence of genetic interaction between *gcs1Δ* and *yck3Δ*, this observation may indicate a synthetic enhancement between a *gcs1Δ* and a *glo3Δ* mutation. Indeed, Glo3 is similar to Gcs1 in sequence and also contains the novel Zn-finger motif (Ireland *et al.*, 1994). The possible genetic interaction between a *gcs1Δ* and a *glo3Δ* indicates that Gcs1 and Glo3 may have related functions as well.

6. Increased expression of Yck3 suppresses a *yck2-2*ts mutant phenotype

The synthetic lethality between *yck3Δ* and *hrr25* mutation suggests that Yck3 is similar to Hrr25 in functions. In contrast, in my study, *YCK3* was isolated as a multicopy suppressor of the *gcs1* cold sensitivity, together with *YCK2* and *YCK1*, while *HRR25* was not a multicopy-suppressor of *gcs1* (Figure 3B). This implies that Yck3 might have overlapping activities with Yck1 and Yck2 as well. To test this hypothesis, pXW150, which contains the entire *YCK3* gene, was transformed into YI228 (*yck2Δ yck1Δ yck2-2*ts) cells. The resultant cells were tested for growth at the restrictive temperature of 37°C. As shown in Figure 8, when expressed from a multicopy plasmid, *YCK3* can partially suppress the temperature sensitivity of YI228 cells. Therefore, Yck3 does indeed have overlapping function with Yck1 and Yck2.

Figure 8: Suppression of *yck2Δ yck1Δ yck2-2*ts temperature sensitivity by increased dosage of *YCK3*.

*yck2Δ yck1Δ yck2-2*ts mutant cells (strain YI228) transformed with multicopy *YCK3* plasmid pXW150, multicopy *YCK2* plasmid pXW123, and vector YEp351 were incubated on YEPD medium for 3 days at the indicated temperatures.

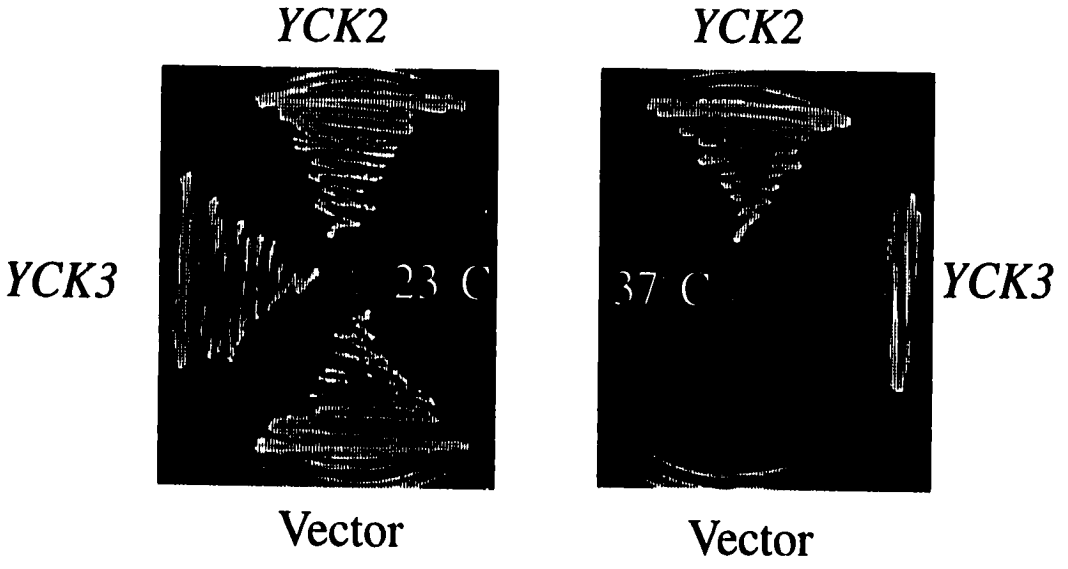
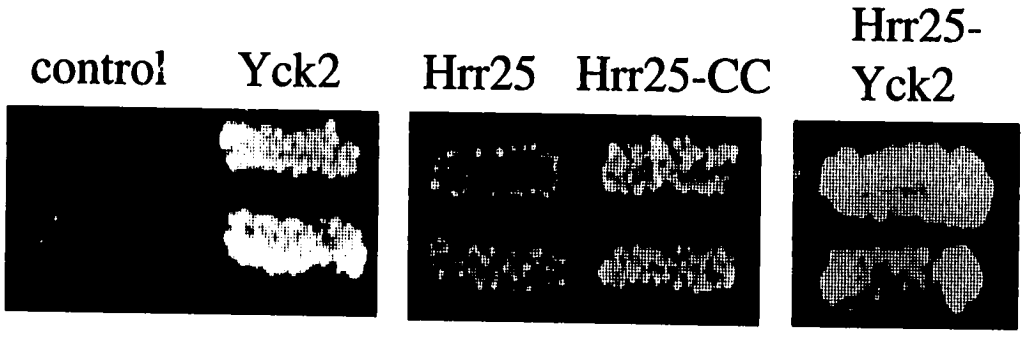

YCK2
YCK2
YCK3
23 C
37 C
YCK3
Vector
Vector

Figure 9: Suppression of *yck2Δ yck1Δ yck2-2*ts temperature sensitivity by increased expression of wild-type and modified Hrr25 proteins.

*yck2Δ yck1Δ yck2-2*ts mutant cells (strain YI228) transformed with multicopy plasmids pXW231 (expressing Hrr25), pXW232 (expressing Hrr25-CC), pHRR25-YCK2 (expressing Hrr25-Yck2), pXW123 (expressing Yck2) and control vector YEp351 were spread on YEPD medium and incubated at 23°C for several days before those cells were replica-plated and incubated at 37°C for 3 days.

casein kinase I isoforms



Similarly, I tested the effect of overexpressing *HRR25* in YI228 cells. Although overexpressing *HRR25* can not suppress the cold sensitivity caused by *gcs1*, overexpressing *HRR25* did partially suppress the temperature sensitivity of YI228 cells (Figure 9). Thus, elevated casein kinase I activity alone, regardless of isotype, is sufficient to maintain viability of a *yck2Δ yck1Δ yck2-2*ts triple-mutant cell. However, for suppression of the *gcs1* mutant phenotype more than simple casein kinase I activity is needed.

7. Both the protein-kinase domain and the C-terminal lipid-modification motif are important for suppression of *gcs1* cold sensitivity

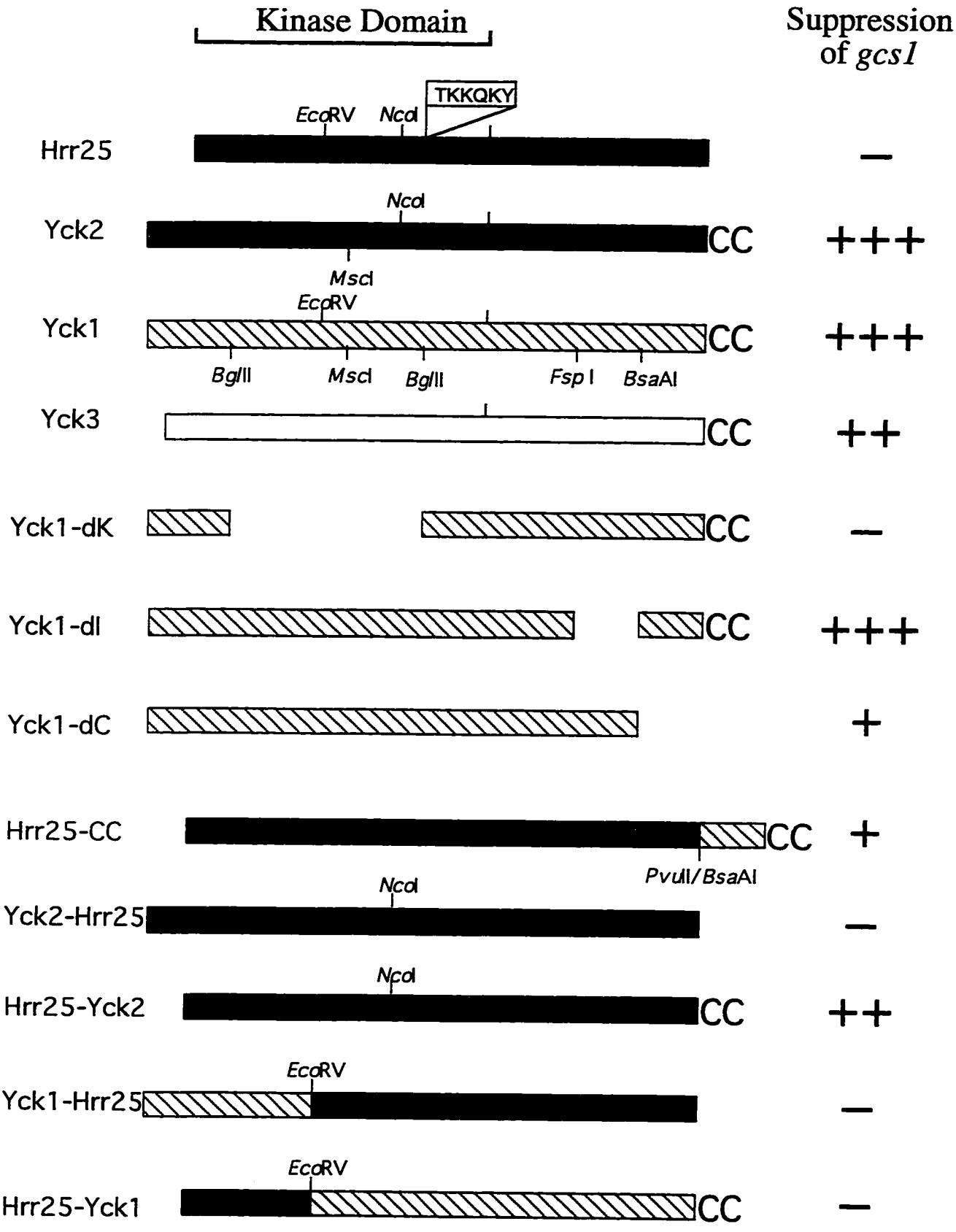
All three *gcs1*-suppressing casein kinase I isoforms have a similar structure, with a protein-kinase domain in the N-terminal region, a putative prenylation signal at the C terminus, and a potentially flexible region close to the C terminus. In Yck2 and Yck1, the flexible region is rich in glutamine, while this region in Yck3 is rich in serine and asparagine. To determine which of the three domains is important for suppression, I modified the casein kinase I genes and tested their encoded proteins for suppression of the *gcs1* cold sensitivity (Figure 10). Yck1 itself and Yck1-dI, a Yck1 variant comprising the protein-kinase domain and the C-terminal prenylation signal, but with part of the glutamine-rich region deleted, both suppressed the *gcs1* cold sensitivity with equal efficiency. On the other hand, Yck1-dK, a Yck1 variant lacking the protein-kinase domain, did not suppress the *gcs1* cold sensitivity, and Yck1-dC, a Yck1 derivative lacking the C-terminal prenylation signal, suppressed the *gcs1* mutant phenotype so poorly that suppression could only be seen after extended incubation at 15°C. These observations suggest that both the protein-kinase domain and C-terminal lipid-modification domains are necessary for suppression, while some of the flexible glutamine-rich domain can be deleted without affecting suppression (Figure 10). Lipid modification of proteins facilitates the association with membranes; the importance of the

C-terminal prenylation signal implies that membrane localization of casein kinase I is important for suppression of the *gcs1* mutant phenotype.

Since Hrr25 protein does not have a C-terminal prenylation signal, I assumed that the lack of suppression of the *gcs1* mutant phenotype by increased Hrr25 was due to the poor association of the enzyme with the proper membrane. To facilitate the localization of Hrr25 with membrane structures that usually associate with Yck2 and Yck1, I constructed a chimeric gene, *HRR25-CC*, encoding a protein that has the C-terminal 60 amino acid residues of Yck1 fused to the C terminus of Hrr25 (Figure 10). Overexpression of this chimeric gene from the multicopy plasmid pHRR25-CC was able to suppress the *gcs1* cold sensitivity weakly. Therefore, the C-terminal portion of Yck1 targets the protein-kinase domain of Hrr25 to where Yck1 usually functions and this localization suppresses the *gcs1* mutant phenotype. Hrr25 is implicated to have functions in DNA repair, and most of Hrr25 has shown to be associated with intact nuclei (Vancura *et al.*, 1994). On the other hand, most of Yck2 + Yck1 is associated with plasma membrane (Vancura *et al.*, 1994). Thus the presence of a nuclear-localization signal in the Hrr25 portion of the chimeric Hrr25-CC protein might still target most of the chimeric protein into nucleus and thus prevent effective suppression of *gcs1* mutations. The nuclear-localization signal of Hrr25 has not been directly identified, but at the C-terminal end of the protein-kinase domain is a TKKQKY sequence, resembling the nuclear-localization signal found in the SV40 large T antigen that functions in yeast (Nelson and Silver, 1989). To construct a chimeric protein that can better localize the Hrr25 protein kinase domain to the functional sites of Yck2 + Yck1, I used *Nco*I sites, which are present at analogous positions in both the *YCK2* gene and the *HRR25* gene immediately upstream of the sequence encoding the TKKQKY sequence, to exchange the protein-kinase domains of these two enzymes. Multicopy expression of the chimeric gene encoding the N-terminal portion of the Hrr25 protein-kinase domain (but not its TKKQKY sequence) and the rest of Yck2 (Hrr25-Yck2) suppressed *gcs1Δ* cold

Figure 10: Suppression of *gcs1* cold sensitivity needs a casein kinase I protein-kinase domain and a prenylation signal.

Open reading frames are indicted with boxes, and restriction sites used for gene construction are marked; "CC" indicates a C-terminal prenylation motif; the number of pluses indicates the extent to which *gcs1Δ* cold sensitivity was suppressed. For assessing suppression of *gcs1Δ* cold sensitivity, *gcs1Δ* mutant cells (strain Gwk9A) transformed with multicopy plasmids pXW321, pXW123, YEpYCK1, pXW150, pXW222, pXW224, pXW223, pHRR25-CC, pYCK2-HRR25, pHRR25-YCK2, pYCK1-HRR25, and pHRR25-YCK1 (expressing Hrr25, Yck2, Yck1, Yck3, Yck1-dK, Yck1-dI, Yck1-dC, Hrr25-CC, Yck2-Hrr25, Hrr25-Yck2, Yck1-Hrr25, and Hrr25-Yck1, respectively) were incubated on leucine-free medium at the permissive temperature of 29°C for several days and then replica-plated and incubated at 15°C for 7 days. The vector control was YEp351.


Kinase Domain
Suppression of gcs1
TKKQKY
EcoRV
NcoI
Hrr25
–
NcoI
Yck2
CC
+++
MscI
EcoRV
Yck1
CC
+++
BglII
MscI
BglII
FspI
BsaAI
Yck3
CC
++
Yck1-dK
CC
–
Yck1-dI
CC
+++
Yck1-dC
+
Hrr25-CC
CC
+
PvuII/BsaAI
NcoI
Yck2-Hrr25
–
NcoI
Hrr25-Yck2
CC
++
EcoRV
Yck1-Hrr25
–
EcoRV
Hrr25-Yck1
CC
–

sensitivity to a even better extent than did Hrr25-CC. On the other hand, multicopy expression of the chimeric gene encoding the N-terminal portion of the Yck2 protein-kinase domain and the rest of Hrr25, including the TKKQKY sequence, did not suppress a *gcs1Δ* mutation (Figure 10).

Consistent with the assumption that in Hrr25-CC and Hrr25-Yck2 the C-terminal prenylation signals of Yck2 and Yck1 help to localize the Hrr25 protein-kinase domain to the functional sites of Yck2 + Yck1, multicopy expression of those two chimeric genes encoding Hrr25-CC and Hrr25-Yck2 suppressed *yck1Δ yck2*-ts temperature sensitivity to a much better extent than did Hrr25 (Figure 9).

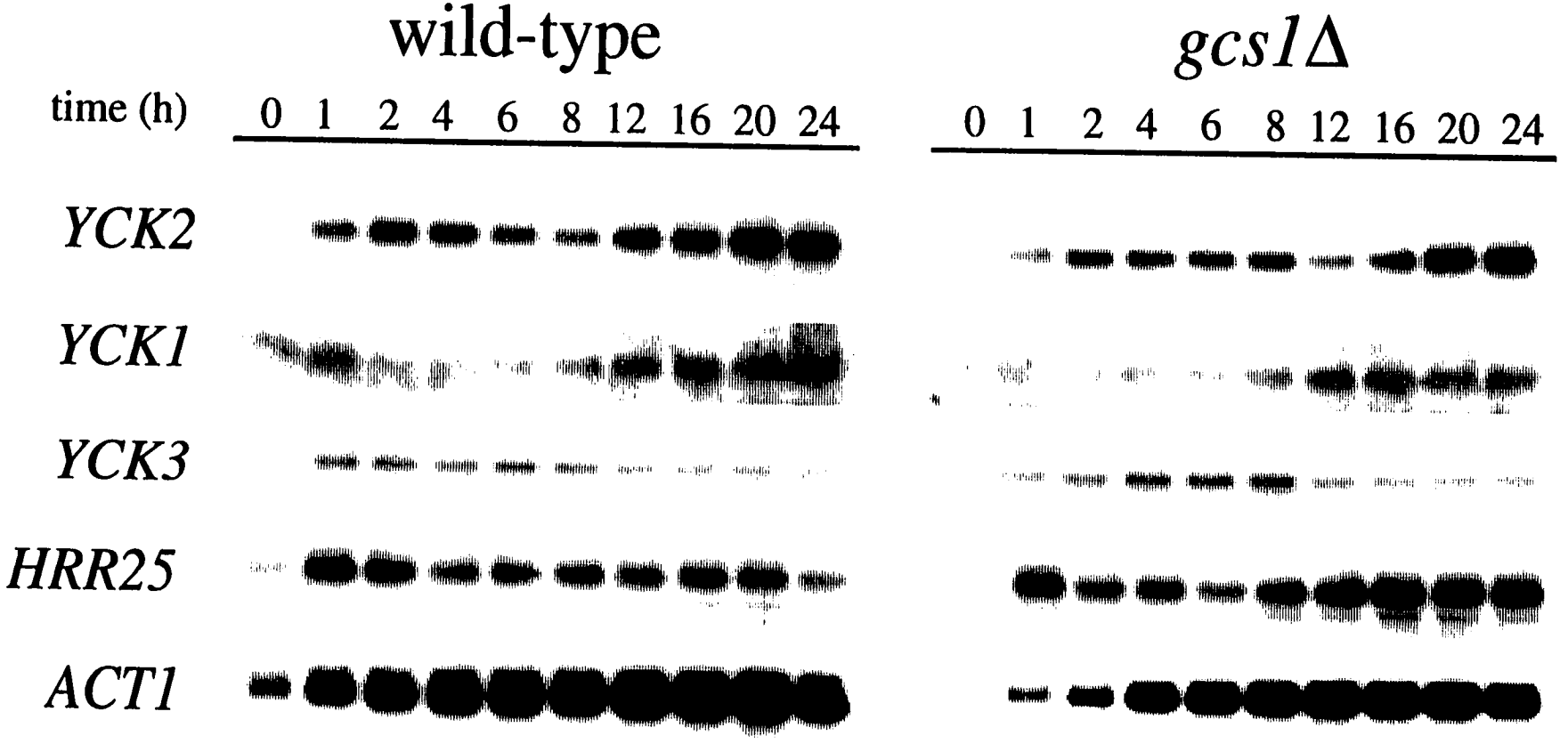
These observations have several implications. Firstly, it is the non-nuclear membrane-associated casein kinase I activity Yck2 + Yck1 that is important for suppression of *gcs1* cold sensitivity. The importance of this membrane association of casein kinase I is consistent with the function of Gcs1 in regulating membrane transport, suggesting that Yck1 and Yck2 suppress the absence of Gcs1 Arf1-GAP activity by modifying some membrane-associated protein. Secondly, the four isoforms of yeast casein kinase I may have overlapping functions. That is, the protein-kinase domains of these casein kinase I isoforms are exchangeable and can recognize common substrates, while the intracellular localization of each casein kinase I isoform determines its substrate specificity. Finally, Gcs1 does not have protein-kinase motifs and is not likely to be a casein kinase I isoform; thus increased casein kinase I activity is not simply replacing Gcs1, but instead may activate a pathway to bypass the need for Gcs1.

8. *YCK2*, *YCK1* and *YCK3* gene expression is not affected by a *gcs1Δ* mutation

One possibility for the activity of increased expression of casein kinase I isoforms to suppress the *gcs1* cold sensitivity is that a *gcs1Δ* mutation affects the expression of *YCK2*, *YCK1*, and *YCK3*, so that increased expression of these genes in a *gcs1* mutant cell simply restores casein kinase I activity to normal levels. To determine the impact of a

Figure 11: Transcription of yeast casein kinase I genes is not affected by a *gcs1Δ* mutation.

RNA isolated from wild-type or *gcs1Δ* stationary-phase cells (lane 0), and 1, 2, 4, 6, 8, 12, 16, 20, and 24 h after transfer of stationary-phase cells to fresh medium and incubation at 15°C (lanes 1, 2, 4, 6, 8, 12, 16, 20, and 24) was electrophoretically resolved on agarose gels and transferred to nylon membranes. RNA blots were probed separately with radiolabelled restriction fragments (see Materials and Methods) from five different genes: *ACT1*, *YCK2*, *YCK1*, *YCK3*, and *HRR25*.


wild-type
gcs1Δ
time (h)
0 1 2 4 6 8 12 16 20 24
0 1 2 4 6 8 12 16 20 24
YCK2
YCK1
YCK3
HRR25
ACT1

gcs1Δ mutation on casein kinase I gene expression, stationary-phase *gcs1Δ* cells and stationary-phase wild-type cells were simulated by addition of fresh medium and incubated at the restrictive temperature of 15°C. At intervals, cell samples were collected and the abundance of casein kinase I mRNAs was determined as these cells were responding to stimulation conditions. As shown in Figure 11, in wild-type cells the mRNA levels for *YCK2, YCK1* and *YCK3* were low in stationary phase and increased after the stimulation of fresh medium before the cells resumed cell proliferation. The same pattern occurred for *YCK2, YCK1* and *YCK3* mRNAs in *gcs1Δ* cells, even though those cells did not resume cell proliferation. Thus, a *gcs1* mutation has no effect on the expression of *YCK2, YCK1* and *YCK3* when stationary-phase cells are stimulated to resume cell proliferation. The suppression of the *gcs1* cold sensitivity by overexpression of casein kinase I genes is therefore not due to restoration of casein kinase I activities to normal levels; instead the suppression is due to additional casein kinase I activity.

9. Multicopy *CDC55* gene suppresses *gcs1* cold sensitivity

I have shown above that increased casein kinase I activity suppresses the *gcs1* cold sensitivity, and that the protein-kinase domain is important for this suppression. Thus, it is possible that suppression is mediated by increased phosphorylation of certain casein kinase I substrates (since casein kinase I suppresses a *gcs1Δ* mutation, a situation where Gcs1 protein is not present in the cell, Gcs1 is not a substrate of casein kinase I in this suppression). Protein phosphorylation plays an important role in regulating protein activities, and thus in regulating various cellular processes. While a kinase can phosphorylate a protein, the phosphorylated protein can be dephosphorylated by a protein phosphatase, so that another way to increase the phosphorylation of certain proteins is to decrease the corresponding protein-phosphatase activity. It has been reported that Yck2 and Yck1 activity might antagonize protein phosphatase type 2A (PP2A) activity (Robinson *et al.*, 1993). Yeast PP2A has three subunits: a catalytic subunit encoded by

several genes including *PPH21* and *PPH22* (Sneddon *et al.*, 1990; Ronne *et al.*, 1991), an accessory subunit encoded by the *TPD3* gene (van Zyl *et al.*, 1992), and a regulatory subunit encoded by the *CDC55* gene (Healy *et al.*, 1991). For some substrates of PP2A, Cdc55 seems to negatively regulate the phosphatase activity (DePaoli-Roach *et al.*, 1994). It has been shown that a mutation in the *CDC55* gene is synthetically lethal with the *yck1Δ yck2-2*ts mutant situation (Robinson *et al.*, 1993), suggesting that a decrease in Yck casein kinase I activity might have the same effect as an increase in PP2A activity. To learn whether a decrease in yeast PP2A activity might have the same effect as increasing Yck casein kinase I activity and thus suppress the *gcs1* mutant phenotype, I determined the effects of altered expression of genes encoding yeast PP2A subunits.

Multicopy expression of *TPD3*, encoding the accessary subunit of PP2A, had no effect on *gcs1* mutant cells and neither did a *tpd3-1* mutation. Therefore increased or decreased activity of the accessory subunit of PP2A has no effect on the *gcs1* cold sensitivity. Another way to decrease PP2A activity is to mutate the *PPH21* and *PPH22* genes. It has been shown by others that *PPH21* and *PPH22* have redundant functions: knocking out one gene has no obvious effects on cells, but knocking out both genes severely impairs cell growth (Ronne *et al.*, 1991). As tested directly, a single *pph21Δ* or a single *pph22Δ* mutation had no effect on the *gcs1Δ* cold sensitivity. (Due to the severe growth defect of *pph21Δ pph22Δ* double-mutant cells, I did not test whether the *pph21Δ pph22Δ* double-mutant situation can suppress the *gcs1* cold sensitivity.) Therefore, mutating one gene encoding the catalytic subunit of PP2A might not decrease PP2A activity enough to suppress the *gcs1Δ* cold sensitivity. On the other hand, expression of *CDC55* from a multicopy plasmid suppresses the *gcs1* cold sensitivity (data not shown; see Wang *et al.*, 1996), suggesting that altering PP2A activity through the regulatory subunit can indeed suppress the *gcs1* cold sensitivity. This observation, is consistent with those of others that yeast PP2A activity and Yck2 + Yck1 activity are related (Robinson *et al.*, 1994). Altering PP2A activity by overexpressing *CDC55* was able to mimic

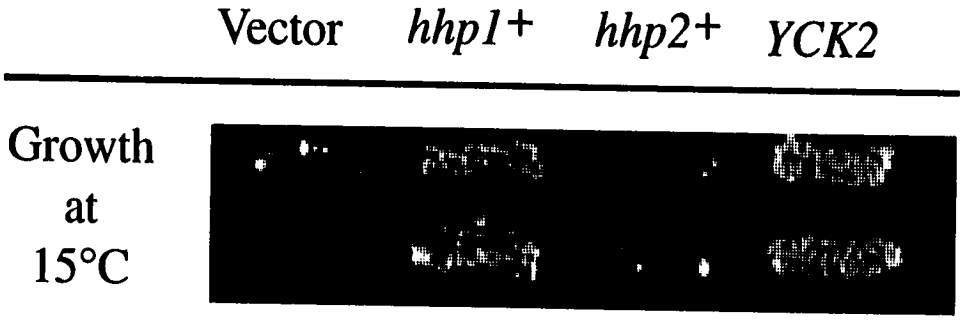
increased casein kinase I activity in terms of suppressing the *gcs1* mutant phenotype. Interestingly, though a *cdc55Δ* mutation is synthetically lethal with *yck1Δ yck2-2*ts mutations, a *cdc55Δ* mutation had no effect on a *gcs1Δ* mutation. The nature of genetic interactions observed among those genes is not known. Given the possibility that casein kinase I and PP2A could recognized more than one protein substrate, they could have multiple functions and *cdc55Δ yck1Δ yck2-2*ts triple-mutant cells could be dead for reasons not related to Gcs1 protein activity.

10. Suppression in a *gcs1-1* strain by casein kinase I from other species

From yeast to human, casein kinase I activity has been found in many eukaryotic systems, and the protein kinase domains of casein kinase I isolated from different systems are highly conserved (Dhillon and Hoekstra, 1994; Wang *et al.*, 1994; Rowles *et al.*, 1991). However, no C-terminally prenylated casein kinase I isoform has been isolated from any other system, even though certain forms of human casein kinase I have been shown to be associated with membrane structures (Gross *et al.*, 1995). Since casein kinase I isoforms from all eukaryotic systems are able to phosphorylate common substrates such as casein, I tested to see whether casein kinase I isoforms from other systems can also suppress the *gcs1* cold sensitivity. For this analysis I obtained a cDNA encoding one human casein kinase I isoform and cDNAs encoding two *Shizosaccharomyces pombe* casein kinase I isoforms from Hoekstra and tested directly their abilities to suppress *gcs1* cold sensitivity. The fission yeast *S. pombe* is distantly related to the budding yeast *Saccharomyces cerevisiae* used here, and the two *S. pombe* casein kinase I isoforms, Hhp1 and Hhp2, were isolated as multicopy suppressor of an *hrr25Δ* mutation; neither protein has a C-terminal prenylation signal (Dhillon and Hoekstra, 1994). To test the ability of these forms of casein kinase I to suppress *gcs1* cold sensitivity, yeast strain MDgcs1-3X (a *gcs1-1* mutant in the W303 genetic background) was transformed with plasmids containing either an *hhp1*$^+$ gene or an *hhp2*$^+$

Figure 12: Suppression of *gcs1-1* cold sensitivity by an *S. pombe* casein kinase I gene.

gcs1-1 mutant cells (strain MDgcs1-3X) transformed with plasmids phhp1$^+$ (containing the *S. pombe hhp1*$^+$ gene under the control of the *ADH1* promoter), phhp2$^+$ (containing the *S. pombe hhp2*$^+$ gene under the control of the *ADH1* promoter), pXW123 (a multicopy *YCK2* plasmid), and vector YEp351 were spread on YEPD medium and incubated at 29°C for several days before these cells were replica-plated and incubated at 15°C for 7 days. Two individual transformants were tested for each plasmid.


Vector
hhp1+
hhp2+
YCK2
Growth
at
15°C

gene, with each gene expressed from the *ADH1* promoter. The resultant transformants were tested for growth at the restrictive temperature of 15°C. As shown in Figure 12, while Hhp1 suppressed the *gcs1-1* mutation, Hhp2 did not. Since both the protein-kinase domain and proper membrane association of casein kinase I are important for the suppression of *gcs1* cold sensitivity, this result indicates that Hhp1 and Hhp2 might be localized to different parts of the cell or have different substrate specificities in the budding yeast *Saccharomyces cerevisiae*. When tested directly, the human casein kinase I isoform α3 (Hoekstra, personal communication), expressed from the yeast *GAL1-10* promoter, did not suppress the *gcs1-1* mutation (data not shown). Therefore, not all casein kinase I isoforms from other systems suppress the *gcs1* cold sensitivity. Since Hhp1, Hhp2 and the human casein kinase I α3 are suppressors of an *hrr25* mutation they might be homologues of Hrr25 (Dhillon and Hoekstra, 1994; Hoekstra, personal communication), which itself does not suppress the *gcs1* cold sensitivity. Thus, it would certainly be interesting to see whether there are human homologues of the Yck1 and Yck2 casein kinase I isoforms that might suppress the *gcs1* cold sensitivity when expressed in yeast. If so, it might indicate that, like their yeast counterpart, casein kinase I activity in other eukaryotic systems might also have a role in intracellular membrane trafficking.

11. Isolation of *YPT32* as a multicopy suppressor of *gcs1* cold sensitivity

I have shown that increased membrane-associated casein kinase I activity suppresses the *gcs1* cold sensitivity, indicating that Gcs1 may affect certain membrane-associated cellular activities. To further understand the functions of Gcs1, the other multicopy suppressors of the *gcs1Δ* mutation were identified and their relationships with Gcs1 and Yck isoforms of casein kinase I were established. From restriction analysis the *gcs1* suppressing plasmids pXW130, pXW170 and pXW180 appear to carry the same suppressor gene. Although each plasmid contains a different DNA insert, these plasmids

Figure 13: Subcloning analysis of *YPT32*.

Only yeast sequences are shown, and the vector is YEp351 or otherwise as indicated; the box and the arrow indicate the position and 5' → 3' direction of the *YPT32* open reading frame. To assess suppression of *gcs1Δ* cold sensitivity, *gcs1Δ* mutant cells (strain Gwk9A) transformed with different plasmids were incubated on leucine-free medium at the permissive temperature of 29°C for several days and then replica-plated and incubated at 15°C for 7 days. The vector control was YEp351.

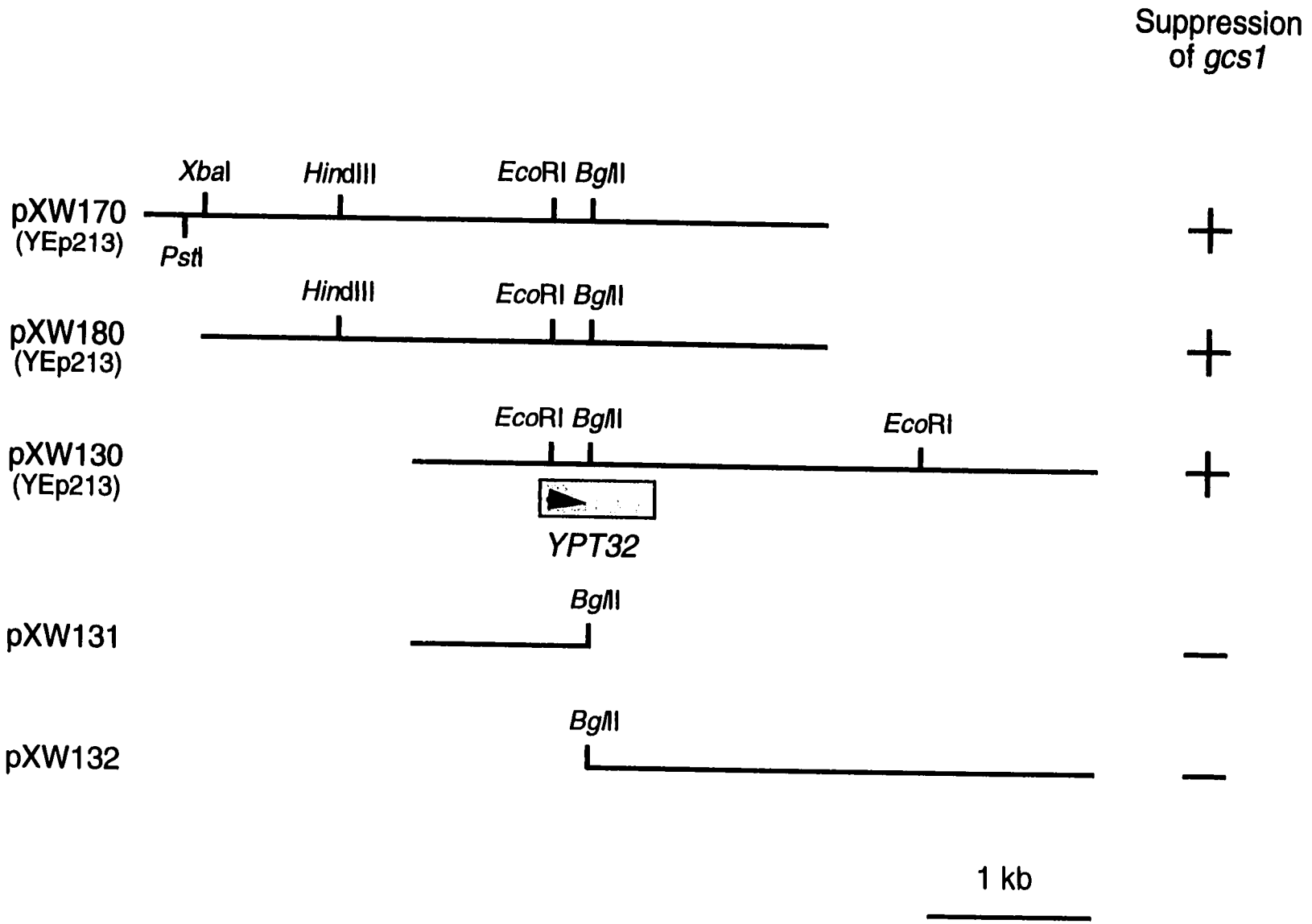
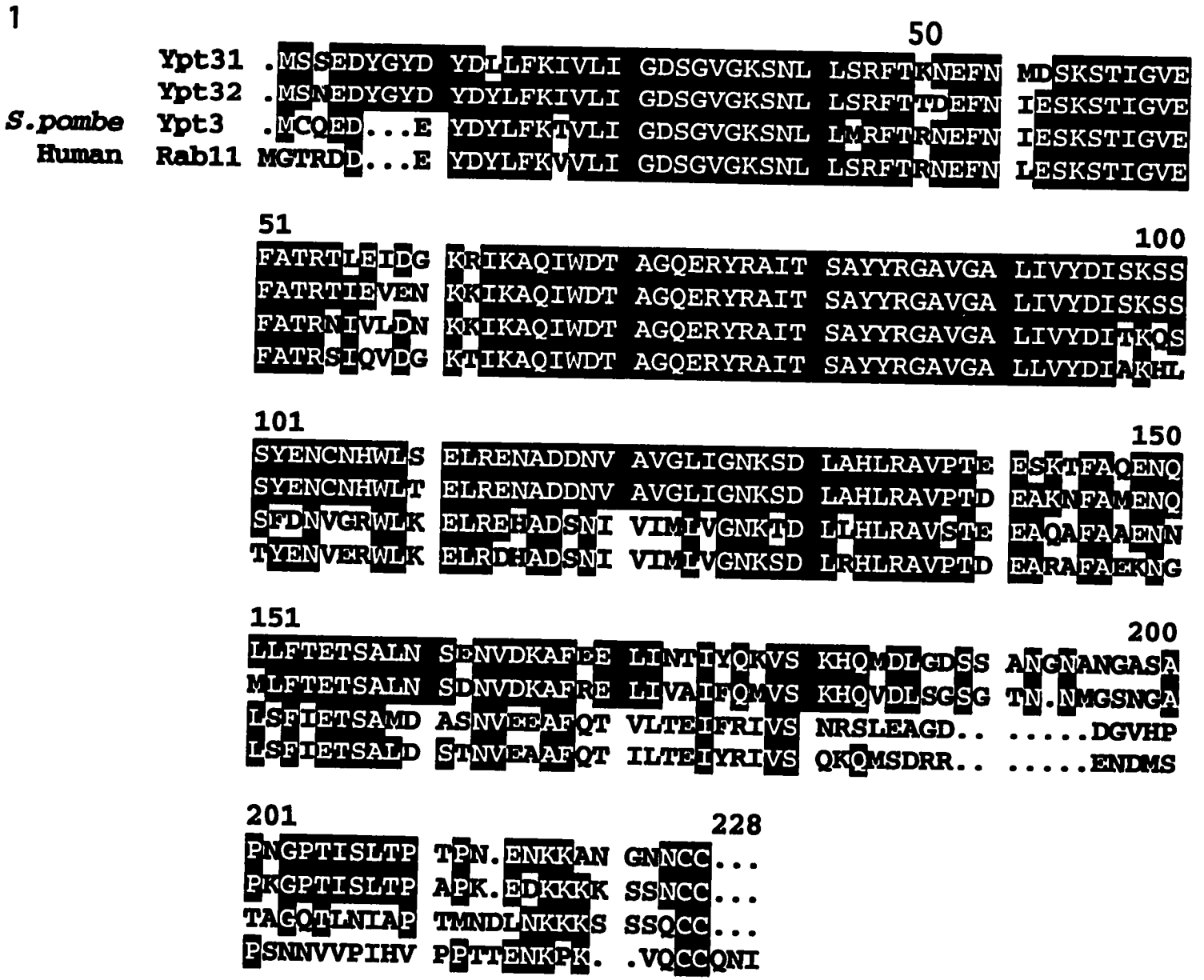

Suppression of gcs1
XbaI
HindIII
EcoRI BglII
pXW170
(YEp213)
PstI
HindIII
EcoRI BglII
pXW180
(YEp213)
EcoRI BglII
EcoRI
pXW130
(YEp213)
YPT32
BglII
pXW131
BglII
pXW132
1 kb

Figure 14: Ypt31 and Ypt32 are yeast homologues of the S. *pombe* Ypt3 and the human Rab11 proteins.

Alignment of Ypt31, Ypt32, Ypt3 and Rab11 predicted polypeptides using PileUp (Devereux *et al.*, 1984). White-on-black indicates where Ypt31 and Ypt32 have the same amino acid, or where at least 3 proteins have the same amino acid. Amino acid positions are indicated above the aligned sequences.


1
50
Ypt31 .MSSEDYGYD YDLLFKIVLI GDSGVGKSNL LSRFTKNEFN MDSKSTIGVE
Ypt32 .MSNEDYGYD YDYLFKIVLI GDSGVGKSNL LSRFTTDEFN IESKSTIGVE
S.pombe Ypt3 .MCQED...E YDYLFKTVLI GDSGVGKSNL LMRFTRNEFN IESKSTIGVE
Human Rab11 MGTRDD...E YDYLFKVVLI GDSGVGKSNL LSRFTRNEFN LESKSTIGVE
51
100
FATRTLEIDG KRIKAQIWDT AGQERYRAIT SAYYRGAVGA LIVYDISKSS
FATRTIEVEN KKIKAQIWDT AGQERYRAIT SAYYRGAVGA LIVYDISKSS
FATRNIVLDN KKIKAQIWDT AGQERYRAIT SAYYRGAVGA LIVYDITKQS
FATRSIQVDG KTIKAQIWDT AGQERYRAIT SAYYRGAVGA LLVYDIAKHL
101
150
SYENCNHWLS ELRENADDNV AVGLIGNKSD LAHLRAVPTE ESKTFAQENQ
SYENCNHWLT ELRENADDNV AVGLIGNKSD LAHLRAVPTD EAKNFAMENQ
SFDNVGRWLK ELREHADSNI VIMLVGNKTD LLHLRAVSTE EAQAFAAENN
TYENVERWLK ELRDHADSNI VIMLVGNKSD LRHLRAVPTD EARAFAEKNG
151
200
LLFTETSALN SENVDKAFEE LINTIYQKVS KHQMDLGDSS ANGNANGASA
MLFTETSALN SDNVDKAFRE LIVAIFQMVS KHQVDLSGSG TN.NMGSNGA
LSFIETSAMD ASNVEEAFQT VLTEIFRIVS NRSLEAGD.. .....DGVHP
LSFIETSALD STNVEAAFQT ILTEIYRIVS QKQMSDRR.. .....ENDMS
201
228
PNGPTISLTP TPN.ENKKAN GNNCC...
PKGPTISLTP APK.EDKKKK SSNCC...
TAGQTLNIAP TMNDLNKKKS SSQCC...
PSNNVVPIHV PPTTENKPK. .VQCCQNI

do have a common overlapping region of about 3 kbp. To localize the suppressing sequence I used a unique *Bgl*II site, which lies in the middle of the common 3-kbp region (Figure 13), to construct two non-overlapping subclones pXW131 and pXW132 using the multicopy vector YEp351. When transformed into Gwk9A cells, neither subclone plasmid suppressed *gcs1* cold sensitivity, while the original pXW130 was able to, indicating that the ORF of the suppressor gene on plasmids pXW170, pXW180 and pXW130 spans the *Bgl*II site.

To identify the suppressor gene on pXW130, I determined partial nucleotide sequence for pXW131 and pXW132. DNA sequences of about 300 bp surrounding the *Bgl*II site were determined and shown to encode a protein similar to many Ras-like small GTP-binding proteins, with high similarities (over 80% identity in this region) to the mammalian protein Rab11 (Chavrier *et al.*, 1990; Figure 14) and the fission yeast (*Shizosaccharomyces pombe*) protein Ypt3 (Miyake and Yamamoto, 1990; Figure 14). Thus, this suppressor gene is a structural homologue of Ypt3 and Rab11. Dieter Gallwitz's group in Germany had already characterized a yeast gene encoding a Ypt3-like protein, so I obtained the entire protein sequence and found that the deduced protein sequence of my suppressor is identical to Ypt32 (D. Gallwitz, personal communication).

To confirm that it is *YPT32* that suppresses the *gcs1* cold sensitivity, I also obtained the *YPT32* gene on a multicopy plasmid (pRS326-*YPT32)* from Gallwitz's lab, and found that it suppressed the *gcs1-2* mutation and a *gcs1-1* mutation (Figure 15). Thus *YPT32* is the suppressor gene on plasmids pXW170, pXW180, and pXW130.

12. Isolation of *YPT31* as a multicopy suppressor of *gcs1* cold sensitivity

Results from Gallwitz's lab showed that *YPT32* forms an essential gene family with *YPT31*, and that these two genes encode proteins that are over 85% identical in sequence. Though cells lacking either of these two genes do not show any impairment in growth, cells lacking both genes are not viable, indicating that, like the situation for Yck1 and

Yck2, Ypt31 and Ypt32 have essential overlapping functions (D. Gallwitz, personal communication). I obtained from Gallwitz the *YPT31* gene on a multicopy plasmid (pRS326-*YPT31*) and found that, like *YPT32*, multicopy *YPT31* also suppresses *gcs1* mutations (Figure 15). Since I was only provided the Ypt31 amino acid sequence by Gallwitz, I could not, at that time, determine whether one of my remaining two suppressing plasmids, pXW110 and pXW140, might carry the *YPT31* gene, which turned out to be the case.

One suppressing plasmid, pXW140, contains a 5.2-kpb yeast genomic insert (Figure 16). Subcloning localized the suppressing sequences to a 1.7-kpb region between *Sal*I and *Pst*I restriction sites, because one subclone, pXW143, containing this region was able to suppress the *gcs1* cold sensitivity. To determine nucleotide sequence for this suppressing gene, the 1.7-kbp DNA insert was cloned into pUC118 and subjected to Exonuclease III partial digestion to generate a series of sequentially deleted plasmids, and the resultant plasmids were subjected to DNA sequencing. DNA sequences of a 1.2-kbp region within the insert, generated from one strand of DNA, revealed that the predicted peptide sequence of an ORF in this region is identical to that of Ypt31. Thus *YPT31* is the multicopy suppressor gene in plasmid pXW140.

Ypt31 and Ypt32 are about 85% identical in sequence, and they are about 60% identical in sequence to the human Rab11 protein (Chavrier *et al.*, 1990; Figure 14) and to the *S. pombe* Ypt3 protein (Miyake and Yamamoto, 1990; Figure 14). While the function of the *S. pombe* Ypt3 is not characterized yet, the human Rab11 protein has been shown to be associated with the *trans*-Golgi network (TGN) and with TGN-originated vesicles, suggesting a role in regulating vesicle transport (Urbe *et al.*, 1993). Unpublished results from Gallwitz's lab also indicate that the yeast Ypt31 and Ypt32 might have a role in regulating intra-Golgi vesicle transport (D. Gallwitz, personal communication). The finding that increased Ypt31 or Ypt32 activity suppresses the *gcs1* mutant phenotype is consistent with a role for Gcs1 in regulating activities at the

Figure 15: Suppression of *gcs1-1* cold sensitivity by increased dosage of *YPT31* or *YPT32*.

gcs1-1 mutant cells (strain MDgcs1-3X) transformed with multicopy plasmids pRS326-YPT31 (containing *YPT31*; provided by D. Gallwitz), pRS326-YPT32 (containing *YPT32*; provided by D. Gallwitz), and vector YEp351 were spread on YEPD medium and incubated at 29°C for several days before these cells were replica-plated and incubated at 15°C for 7 days. Two individual transformants were tested for each plasmid.

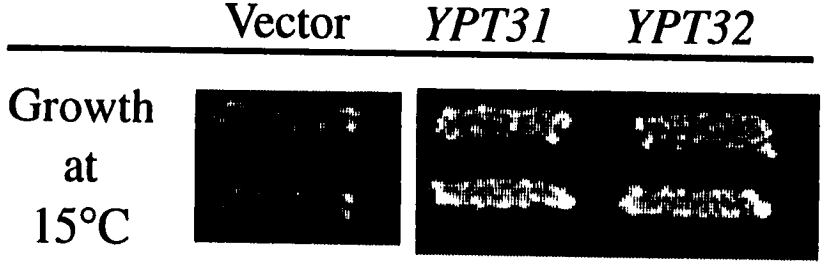
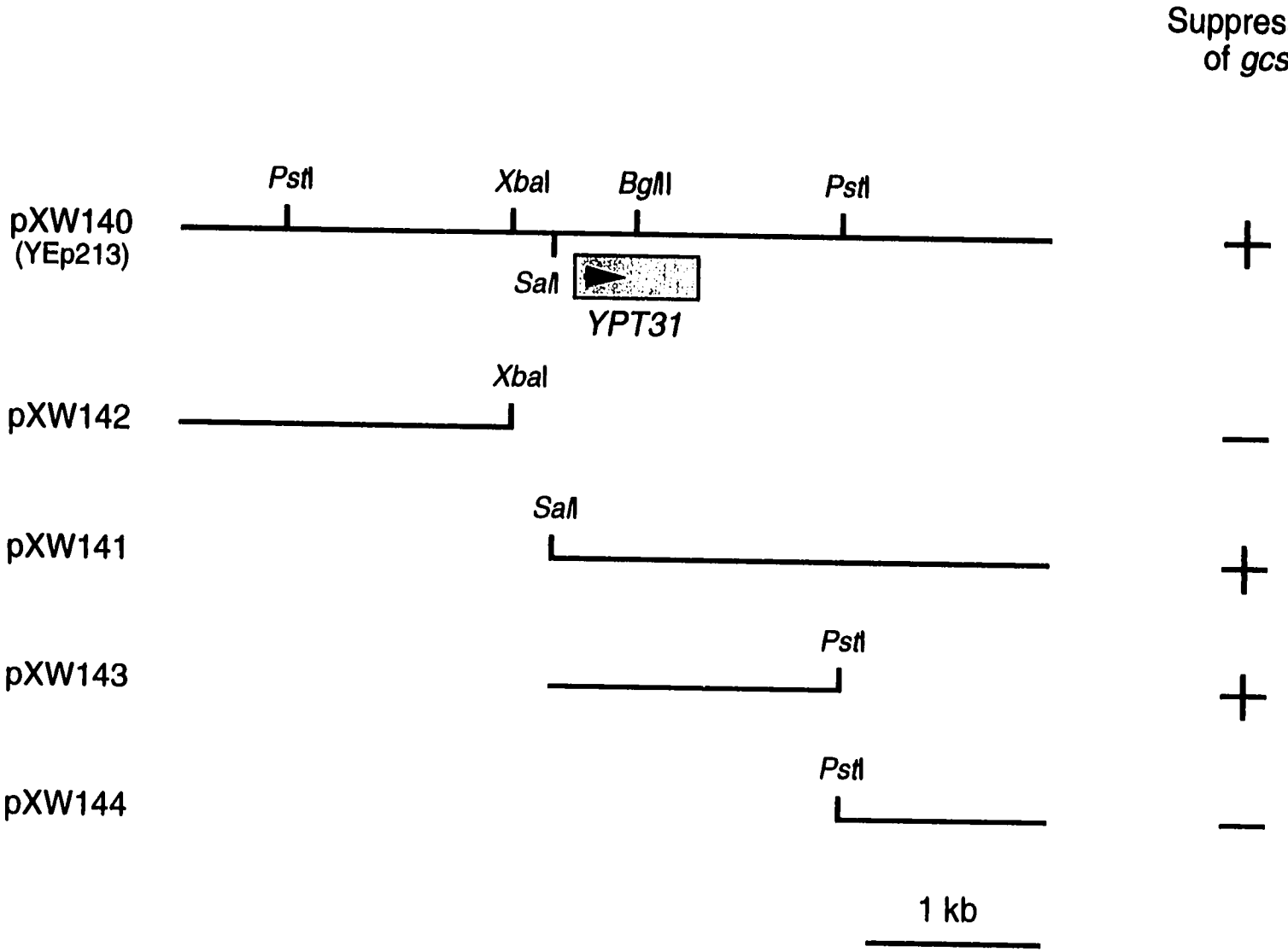

Vector
YPT31
YPT32
Growth
at
15°C

Figure 16: Subcloning analysis of *YPT31*.

Only yeast sequences are shown, and the vector is YEp351 or otherwise as indicated; the box and the arrow indicate the position and 5' → 3' direction of the *YPT31* open reading frame. To assess suppression of *gcs1Δ* cold sensitivity, *gcs1Δ* mutant cells (strain Gwk9A) transformed with different plasmids were incubated on leucine-free medium at the permissive temperature of 29°C for several days and then replica-plated and incubated at 15°C for 7 days. The vector control was YEp351.


Suppression of gcs1
pXW140 (YEp213)
PstI
XbaI
BglII
PstI
SalI
YPT31
+
pXW142
XbaI
−
pXW141
SalI
+
pXW143
PstI
+
pXW144
PstI
−
1 kb

membranes.

13. Not all *YPT* genes can suppress *gcs1* cold sensitivity

Ypt31 and Ypt32 belong to a group of small GTP-binding proteins called Ypt/Rab proteins (reviewed by Novick and Brennwald, 1993). Ypt/Rab proteins are about 25 kD in size, and share about 30% identity in sequence with Ras proteins. Ypt/Rab proteins are C-terminally prenylated (Pfeffer, 1992), and therefore are modified differently than Ras and Rho proteins, which are C-terminally farnesylated. In mammalian cells, there are more than a dozen Rab proteins ("Rab" stands for Ras-like proteins found in Rat brain). In the budding yeast *Saccharomyces cerevisiae*, nine Rab/Ypt proteins have been found, including Ypt1 (Segev *et al.*, 1988; Schmitt *et al.*, 1988), Sec4 (Salminen and Novick, 1987), Ypt51, Ypt52, Ypt53, Ypt6 and Ypt7, (Wichmann *et al.*, 1992) and they are called Ypt proteins, after the first member found. Ypt31 and Ypt32 share about 40% identity with some other yeast Ypt proteins (Figure 17). To test whether other *YPT* genes might also suppress *gcs1* mutations, a 1.6-kbp *Cla*I-*Bam*HI fragment containing the yeast *YPT1* gene was first cloned into pRS314 to exploit the multiple cloning sites of the vector and then transferred to YEp351. The resultant plasmid, YEpYPT1, was transformed into Gwk9A cells and tested for its ability to suppress *gcs1Δ* cold sensitivity. As shown in Figure 18, increased expression of *YPT1* did not suppress the cold sensitivity of Gwk9A cells. Thus not all *YPT* genes, when overexpressed, can suppress the *gcs1* cold sensitivity. In light of recent findings that different Rab/Ypt proteins function at different stages of intracellular membrane trafficking (Novick and Brennwald, 1993), it is possible that increased Ypt31 and Ypt32 activity only affects certain stages of membrane trafficking process and, in doing so, suppresses the *gcs1* cold sensitivity. One example of this specificity is provided by Sec4, which is important for the targeting and/or the fusion of secretion vesicles to the plasma membrane. When expressed at increased levels, Sec4 only suppresses certain late *sec* mutations (post-Golgi vesicle transport), and does not

Figure 17: Sequence similarities among different yeast Ypt/Rab proteins.

Alignment of Ypt31, Ypt32, Ypt1 and Sec4 predicted polypeptides using PileUp (Devereux *et al.*, 1984). White-on-black indicates where at least 3 proteins have the same amino acid.

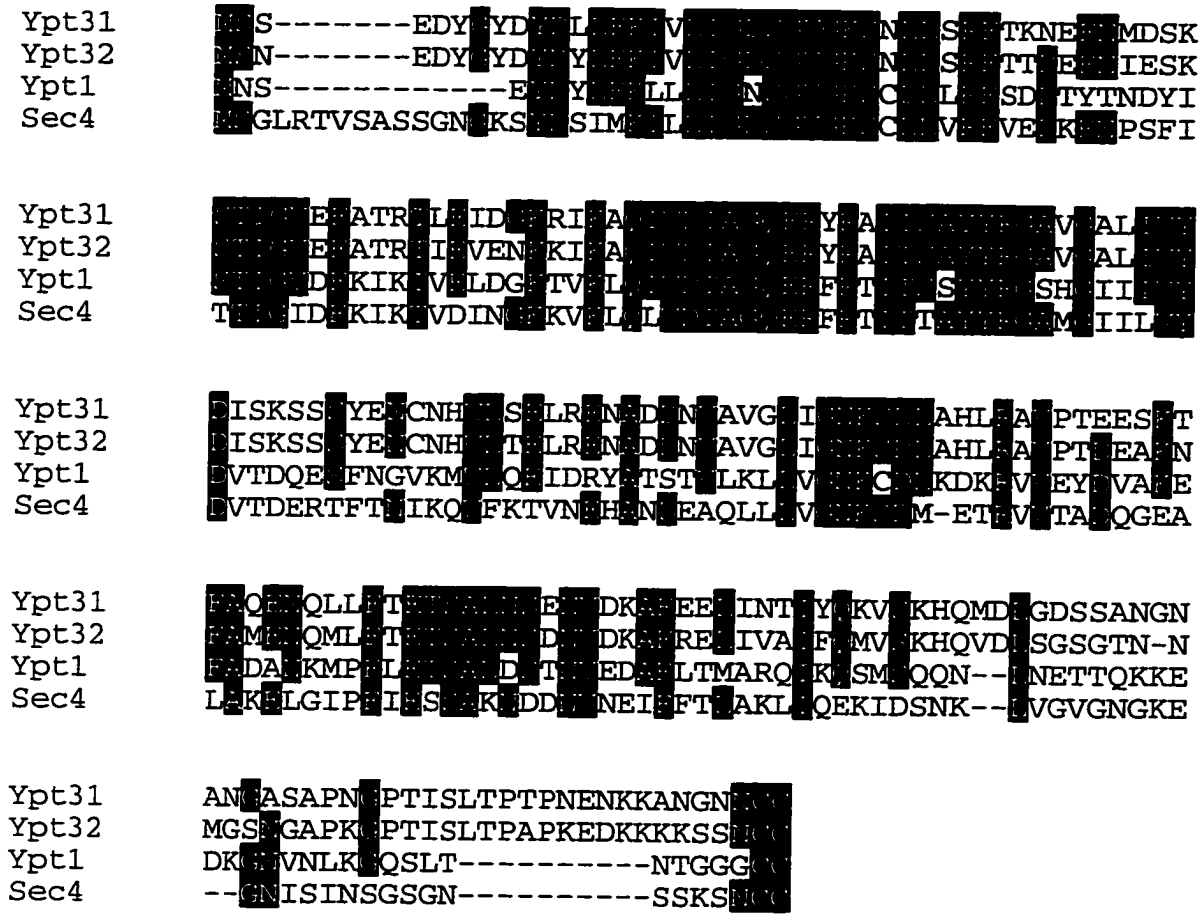
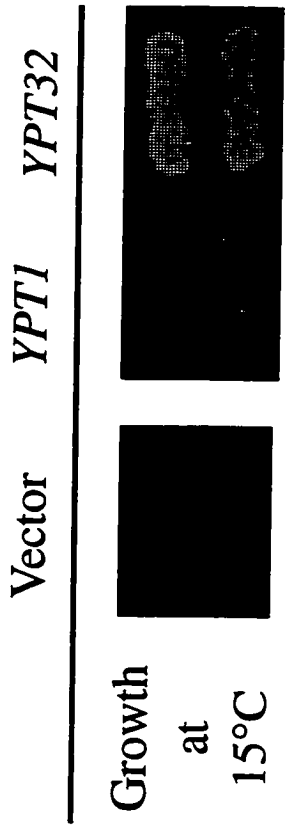

Ypt31
Ypt32
Ypt1
Sec4

Figure 18: Increased dosage of *YPT1* did not suppress *gcs1Δ* cold sensitivity.

gcs1Δ cells (strain Gwk9A) transformed with *YPT32* multicopy plasmid pXW130, *YPT1* multicopy plasmid YEpYPT1, and vector YEp351 were streaked out on leucine-free medium and incubated at 29°C for several days before those cells were replica-plated and incubated at 15°C for 7 days. Two individual transformants were tested for each plasmid.


Vector
YPT1
YPT32
Growth at 15°C

suppress mutations affecting the early steps in membrane trafficking (Salminen and Novick, 1987).

14. Genetic interactions between *gcs1* and the *ypt31*cs *ypt32Δ* situation

Though elevated Ypt31 or Ypt32 activity suppresses the *gcs1* cold sensitivity, this activity might not reflect the normal functions of these two proteins. To address the question of whether normal Ypt31 and Ypt32 activities are related to Gcs1 functions, I tested whether a decrease in Ypt31 + Ypt32 activity can exacerbate the *gcs1* mutant phenotype. The study was able to be carried out because Gallwitz's group kindly provided us with a *ypt32Δ ypt31*cs cold-sensitive haploid mutant yeast strain, YTH12. To construct a *ypt32Δ ypt31*cs *gcs1Δ* triple-mutant strain, I first made YTH12 cells diploid using a method based on yeast mating-type switching (Materials and Methods), in preparation for the deletion of one copy of the *GCS1* gene in the resultant diploid YTH12D cells. However, I found that the diploid YTH12D cells, homozygous for *ypt32Δ* and *ypt31*cs, were not able to sporulate (data not shown); thus I was unable to construct *ypt32Δ ypt31*cs *gcs1Δ* triple-mutant haploid cells through this approach. However, it is interesting to note that diploid cells homozygous for *gcs1* mutations also have a defect in sporulation (Drebot *et al.*, 1987), and this indicates that a mutation in the *GCS1* gene and mutations in the *YPT31* and *YPT32* genes could affect some common cellular processes required for sporulation.

As an alternative approach, classical genetic recombination was used in an effort to construct a *ypt32Δ ypt31*cs *gcs1Δ* triple-mutant cell. Grk4-7 cells, containing a *gcs1Δ* mutation, were first crossed with Fy56 cells, and the resultant YXW7 cells were sporulated and subjected to tetrad analysis. From this cross, haploid YXW8 cells, bearing the *gcs1Δ* and *his4* mutations, were identified and crossed with YTH12 cells, and the resultant diploid cells, YXW10, were sporulated and subjected to tetrad analysis. Of the 25 complete tetrads analyzed, the genotypes of spores from 19 complete tetrads could

Table 4 Viability of *gcs1Δ* x *ypt32Δ ypt31*cs segregants

Spore genotype	Total No.	Viable No.	Viable %
Wild type	11	11	100 %
gcs1Δ	7	6	86 %
ypt32Δ	11	10	91 %
*ypt31*cs	7	7	100 %
*gcs1Δ ypt31*cs	13	8	62 %
gcs1Δ ypt32Δ	9	6	67 %
*ypt31*cs *ypt32Δ*	9	9	100 %
*gcs1Δ ypt32Δ ypt31*cs	9	0	0 %

76 spores from 19 complete tetrad were analyzed.

be completely determined. As summarized in Table 4, while the viability of wild-type spores or spores with just one mutation in the *GCS1*, *YPT31*, or *YPT32* genes was approximately 100%, only about 60% of the spores bearing a *gcs1Δ* mutation and a mutation in either one of the *YPT31* and *YPT32* genes were viable. Furthermore, none of the nine spores shown genetically to have a *gcs1Δ ypt32Δ ypt31*cs genotype could form colonies. Thus, the combination of a *gcs1Δ* mutation and *ypt32Δ ypt31*cs mutations is lethal. A decrease in Ypt31 + Ypt32 activity enhances the impairment caused by the *gcs1Δ* mutation (or *vice versa*). These results indicate that Ypt31 + Ypt32 activity and Gcs1 activities are probably involved in a common process, and they could affect a common step in membrane trafficking. Since Gcs1 does not appear to have a GTP-binding motif, it is unlikely that increased Ypt31 or Ypt32 replaces Gcs1 function. It is, however, possible that increased Ypt31 + Ypt32 function activates a parallel pathway, and thus bypasses the requirement of Gcs1 during the resumption of cell proliferation from stationary phase.

15. Increased expression of *YCK1* suppresses the cold sensitivity of *ypt32Δ ypt31*cs cells

Ypt/Rab GTPases have been implicated in regulating various intracellular membrane transport processes. In yeast, Ypt1 has been implicated in regulating membrane transport from the endoplasmic reticulum (ER) to the Golgi apparatus (Segev *et al.*, 1988), and Sec4 has been implicated in regulating membrane transport from the Golgi to the plasma membrane (Salminen and Novick, 1987). In mammalian cells, Rab1 is involved in ER-to-Golgi transport (Peter *et al.*, 1994), and Rab4 (van der Sluijs *et al.*, 1992) and Rab5 (Bucci *et al.*, 1992) are associated with early endosome activities. Preliminary experiments have localized Rab11, a mammalian homologue of Ypt31 and Ypt32, to vesicles originated in the *trans*-Golgi network (Urbe *et al.*,1993). Thus it is likely that Ypt31 and Ypt32 are also involved in certain stages of membrane trafficking. Given the

Figure 19: Suppression of *ypt32Δ ypt31*cs cold sensitivity by increased dosage of *YCK1*.

*ypt32Δ ypt31*cs mutant cells (strain YTH12) transformed with *YCK1* multicopy plasmid pB65R7, *YPT32* multicopy plasmid pRS326-YPT32, *GCS1* multicopy plasmid pB23, and vector YEp352 were spread on uracil-free medium and incubated at 29°C for several days before these cells were replica-plated and incubated at 15°C for 7 days. Two individual transformants were tested for each plasmid.

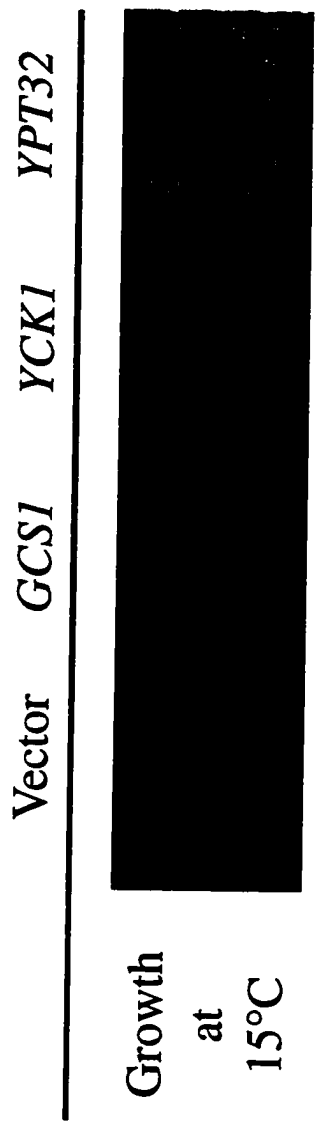

Vector
GCS1
YCK1
YPT32
Growth at 15°C

Table 5. Relationships among Gcs1, Ypt31 and Yck1

Suppressor genes	Mutant cells: *gcs1*cs	*ypt31*cs	*yck2-2*ts
GCS1	+	—	—
YPT31	+	+	—
YCK1	+	+	+

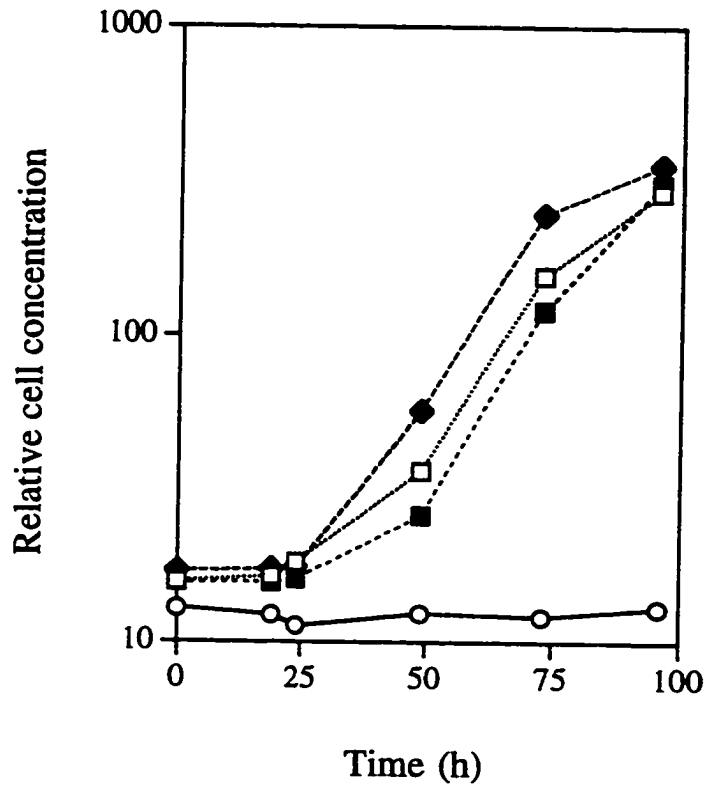
genetic interaction seen between a *gcs1* mutation and the *ypt32Δ ypt31*cs mutant situation, it is possible that Gcs1, Ypt31 and Ypt32 are involved in the regulation of certain common steps in membrane trafficking. Ypt/Rab proteins are C-terminally prenylated, and so far all yeast Ypt proteins have a C-terminal "CC" prenylation motif. This motif, as described above, is also found in three isoforms of the yeast casein kinase I that suppress the *gcs1* mutant phenotype. My previous finding that membrane association of casein kinase I activity is important for suppressing the *gcs1* cold sensitivity suggests that casein kinase I activity might also be involved.

To study the relationships among Gcs1, Yck1 +Yck2 and Ypt31 + Ypt32, I set out to test whether increased Gcs1 or Yck1 activity can suppress the *ypt32Δ ypt31*cs cold sensitivity. As shown in Figure 19, multicopy expression of *YCK1* from plasmid pB65R7 did suppress the cold sensitivity of *ypt32Δ ypt31*cs cells, whereas multicopy expression of *GCS1* (plasmid pB23) had no effect. Thus increased membrane-associated casein kinase I activity can suppress not only *gcs1* cold sensitivity, but also the cold sensitivity caused by the *ypt32Δ ypt31*cs mutant situation. In a separate assay I separately overexpressed *YPT31* and *GCS1* in YI228 cells, which have the genotype *yck1Δ yck2Δ yck2*-2ts, but suppression of the temperature sensitivity was not observed (data not shown). Therefore, while increased Yck casein kinase I activity suppresses the *gcs1* cold sensitivity and suppresses the *ypt32Δ ypt31*cs cold sensitivity, increased Gcs1 or Ypt31 activity does not compensate for the loss of Yck casein kinase I activity. This non-reciprocal suppression either indicates that Yck casein kinase I activity affects more cellular functions than do Ypt31 and Gcs1, or suggests that in a common cellular process the Yck casein kinase I activity functions downstream of Ypt31 and Gcs1. The suppression relationships of *GCS1*, *YCK1*, and *YPT31* genes are summarized in Table 5.

16. Additive effects of *YPT31* and *YCK2* genes on the suppression of *gcs1* cold sensitivity

Figure 20: Additive effect of *YPT31* and *YCK2* on the suppression of *gcs1Δ* cold sensitivity.

gcs1Δ mutant cells (strain Gwk9A) transformed with 2 low-copy *YPT31* plasmids (indicated by "□"; pXW146 and pXW147), 2 low-copy *YCK2* plasmids (indicated by "■"; pXW124 and pXW127), 1 low-copy *YPT31* plasmid plus 1 low-copy *YCK2* plasmid (indicated by "◆"; pXW124 and pXW147), and 2 low-copy vectors (indicated by "○"; pRS315 and pRS313) were inoculated in leucine-free and histidine-free liquid medium and incubated at 29°C for several days to reach stationary phase. Portions of those stationary-phase cultures were then transferred to fresh leucine-free and histidine-free liquid medium and incubated at 15°C. At 0, 20, 24, 48, 72, and 96 h, samples were removed from those cell cultures and assessed for cell concentration.


1000
100
10
Relative cell concentration
0
25
50
75
100
Time (h)

I have shown above that increased dosage of *YCK2* suppresses the *gcs1* cold sensitivity, and that increased dosage of *YPT31* suppresses the *gcs1* cold sensitivity as well. It was therefore interesting to see whether increased dosage of both *YCK2* and *YPT31* can suppress *gcs1* better than that of either gene alone. For this analysis, *YCK2* was cloned into the low-copy vectors pRS315 and pRS313 to generate plasmids pXW124 and pXW127 respectively; *YPT31* was similarly cloned into pRS315 and pRS313 to generate pXW146 and pXW147, respectively. Then *gcs1Δ* mutant cells (strain Gwk9A) were transformed with two low-copy plasmids carrying either *YCK2* (pXW124 and pXW127), or *YPT31* (pXW146 and pXW147), or *YCK2* plus *YPT31* (pXW124 and pXW147), and the resultant transformants were tested for the ability to resume cell proliferation from stationary phase at the restrictive temperature of 15°C. As shown in Figure 20, either *YCK2* or *YPT31*, when present in cells on two different low-copy plasmids, suppressed the *gcs1* mutant phenotype, but the presence of both a low-copy *YCK2* plasmid and a low-copy *YPT31* plasmid suppressed the *gcs1* mutant phenotype better than did either gene alone. Even though the difference in suppression is small among those situations, similar results were also observed when suppression was assessed using solid medium (data not shown). Thus increased dosage of *YCK2* and increased dosage of *YPT31* do have additive effects on a common process, as indicated by suppression of *gcs1* cold sensitivity. Together with the observation that increase dosage of *YCK2* suppresses the cold sensitivity of *ypt32Δ ypt31*cs mutant cells, it is likely that both the Yck1 + Yck2 activity and the Ypt31 + Ypt32 activity are involved in a common pathway, and that Yck1 + Yck2 casein kinase I might function downstream of Ypt31 + Ypt32.

17. Ypt31 is a GTP-binding protein

It is inferred from the derived polypeptide sequence that Ypt31 belongs to the Ypt/Rab family of small GTP-binding proteins; however, no one has provided biochemical evidence that Ypt31 actually binds GTP. Thus, I set out to study whether Ypt31 binds

Figure 21: Purification of N-terminally His_6-tagged recombinant Ypt31 protein.

The supernatant fraction (lane S) of an *E.coli* extract expressing His_6-tagged recombinant Ypt31 protein, the flow-through faction (lane F) after the supernatant fraction had been passed through a Ni-NTA column, column elute fractions 1, 2, 3, and 4 (lanes 1, 2, 3, and 4; using Wash Buffer [50 mM Na-PO_4, 300 mM NaCl, 10% glycerol, pH 6.0] containing 50 mM imidazole), fractions 5, 6, 7, and 8 (lanes 5, 6, 7, and 8; using Wash Buffer containing 100 mM Imidazole), fractions 9, 10, 11, and 12 (lanes 9, 10, 11, and 12; using Wash Buffer containing 200 mM imidazole), and molecular weight standards (lane M) were electrophoretically resolved on a SDS-polyacrylamide gel. Proteins were stained with Coomassie Blue. The arrow indicates the position of the His_6-tagged recombinant Ypt31 protein.

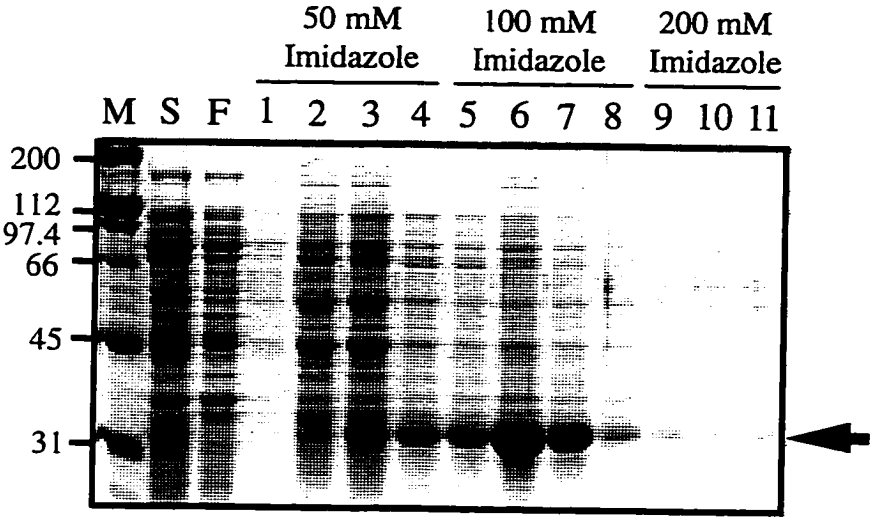
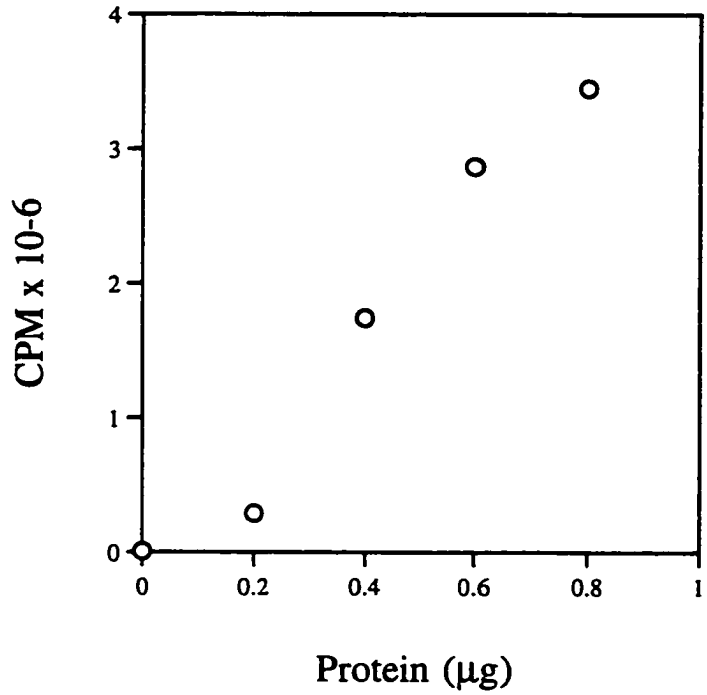

50 mM Imidazole
100 mM Imidazole
200 mM Imidazole
M S F 1 2 3 4 5 6 7 8 9 10 11
200
112
97.4
66
45
31

Figure 22: Ypt31 binds GTP *in vitro*.

Partially purified His_6-tagged recombinant Ypt31 protein was assayed for its ability to bind GTP. The amount of recombinant protein used is indicated on the abscissa, and the corresponding protein-bound [α-^{32}P]GTP is indicated on the ordinate.


CPM x 10-6
4
3
2
1
0
0
0.2
0.4
0.6
0.8
1
Protein (µg)

GTP *in vitro*. To generate enough purified Ypt31 protein for the assay I decided to express and purify recombinant Ypt31 protein from *E. coli*. Therefore, I cloned the *YPT31* gene into plasmid pTrc-HisC, which, when transformed into *E. coli* cells, allows the expression of N-terminally histidine-tagged Ypt31. The short stretch of histidine residues added at the N terminus allows the recombinant protein to bind Ni^{++}-NTA resin (Qiagen) and therefore to be easily purified from an *E. coli* cell extract. The addition of the His_6-tag raised the molecular weight of Ypt31 from 26 kD to 30 kD as predicted and as indicated electrophoretically (Figure 21). To partially purify the recombinant His-tagged Ypt31 protein, *E. coli* extract containing the His-tagged Ypt31 was loaded onto a Ni^{++}-NTA column under conditions that allowed the poly-histidine residues of the recombinant protein to bind to the Ni^{++}-NTA resin. To elute the recombinant protein from the column, buffers containing increasing concentrations of imidazole were used, as imidazole competes with the poly-histidine residues for binding to the Ni^{++}-NTA resin (using procedures provided by Qiagene). As shown in Figure 21, using this method I was able to partially purify His-tagged Ypt31 protein to >85% in purity.

It is reported by many that when small GTP-binding proteins are expressed and purified in *E. coli*, they are predominantly bound to GDP. In the presence of GTP and the absence of Mg^{++}, these *E. coli*-expressed GDP-bound proteins can release GDP and bind GTP in exchange. To test whether His-tagged Ypt31 can also bind GTP, the partially purified recombinant Ypt31 protein was tested for its ability to load [α-^{32}P]GTP in the absence of Mg^{++}. As shown in Figure 22, His-tagged Ypt31 did bind GTP in this assay. Therefore Ypt31 is a GTP-binding protein.

18. Generation of two additional *ypt31* mutants

Small GTP-binding proteins like Ras and Rab exist in cells in either GDP-bound or GTP-bound forms. While a Guanine Nucleotide Exchange Factor facilitates the exchange of GTP for GDP, and thus mediates the transition from the GDP-bound form to the GTP-

bound form, a GTPase activating protein (GAP) may be required to activate the hydrolysis of GTP to GDP, and in so doing changes the protein from the GTP-bound form to the GDP-bound form. The ability to cycle in this way between the GDP-bound form and the GTP-bound form is essential for these GTP-binding proteins to carry out their normal biological activities. Mutations that either prevent the hydrolysis of GTP or block the release of GDP cause malfunctions of these proteins and can generate detectable phenotypes (Pfeffer 1992). The same argument could also apply to Ypt31 and Ypt32. Mutant Ypt31 proteins that are unable to hydrolyze GTP or to release GDP might have detectable phenotypes and show some genetic interactions with *gcs1*. Therefore I used standard site-directed mutagenesis procedures (Materials and Methods) to generate two specific Ypt31 mutants. In one mutant form asparagine (Q) at position 72 was changed to leucine (L), and in the other, serine (S) at position 27 was changed to alanine (A). Sequence considerations suggest that the Q72L mutation is analogous to the Rab5Q79L mutation (Figure 23), which yields a Rab5 mutant protein that is deficient in GTP hydrolysis and thus is predominantly in the GTP-bound, active form (Stenmark *et al.*, 1995). In contrast, the S27A mutation is analogous to the Rab5S34N mutation, which yields a Rab5 mutant protein impaired in binding GTP, and thus predominantly in the inactive, GDP-bound form (Stenmark *et al.*, 1995).

To test the effects of these two mutant Ypt31 forms on *gcs1Δ* mutant and on wild-type cells, the *ypt31-Q72L* mutant gene and the *ypt31-S27A* mutant gene were each cloned into vector pPP389, placing each gene under the control of the *GAL1-10* promoter. Using the *GAL1-10* promoter I could control the expression of these mutant genes by growing transformants on media containing different carbon sources, which simplified the tests on cell growth. Plasmids carrying one of the mutant genes were then transformed into wild-type or *gcs1Δ* cells. The expression of these mutant genes was induced when yeast cells were grown on media containing galactose as sole carbon source. As shown in Figure 24, the wild-type *YPT31* gene suppressed *gcs1* cold

Figure 23: Two mutant forms of Ypt31.

Alignment of Ypt31, Rab5, and H-Ras predicted polypeptides. Boxes indicate positions of amino acid substitutions in the two mutant Ypt31 proteins (bolded), and that these substitutions in Ypt31 are analogous to those in two known mutant Rab5 proteins and one mutant H-Ras protein.

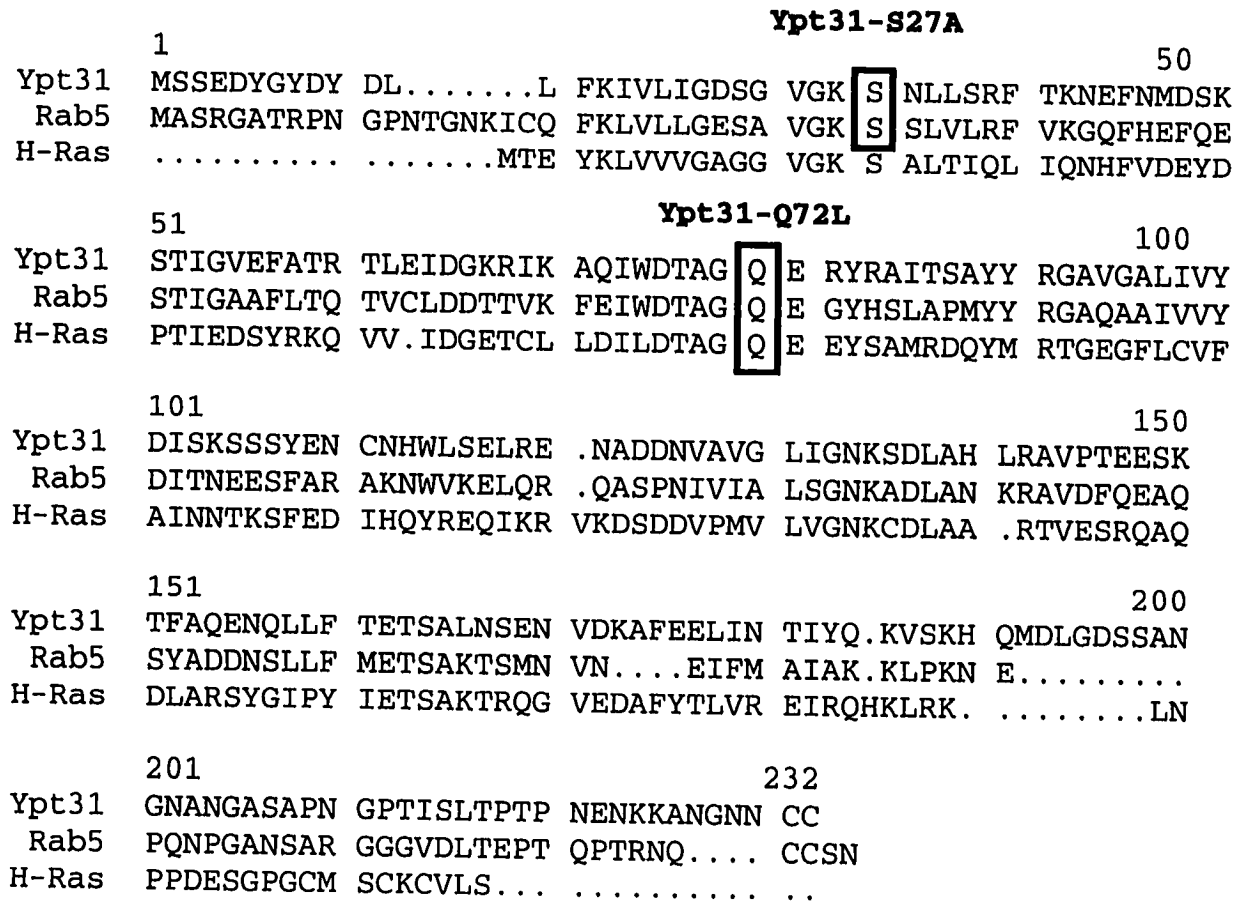
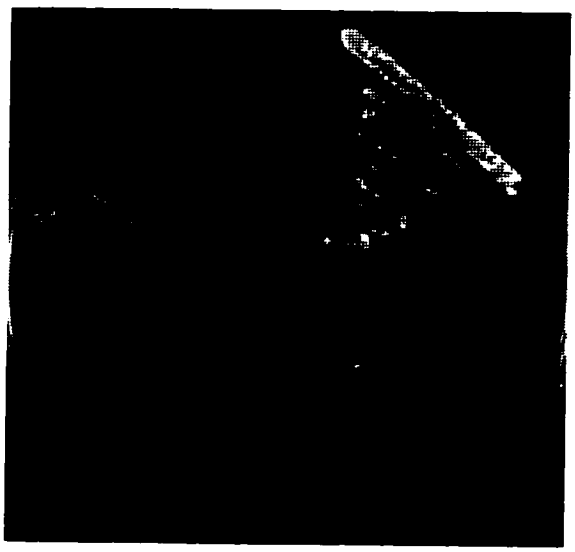

Ypt31-S27A
Ypt31-Q72L
1
50
Ypt31 MSSEDYGYDY DL.....L FKIVLIGDSG VGK S NLLSRF TKNEFNMDSK
Rab5 MASRGATRPN GPNTGNKICQ FKLVLLGESA VGK S SLVLRF VKGQFHEFQE
H-Ras ....MTE YKLVVVGAGG VGK S ALTIQL IQNHFVDEYD
51
100
Ypt31 STIGVEFATR TLEIDGKRIK AQIWDTAG Q E RYRAITSAYY RGAVGALIVY
Rab5 STIGAAFLTQ TVCLDDTTVK FEIWDTAG Q E GYHSLAPMYY RGAQAAIVVY
H-Ras PTIEDSYRKQ VV.IDGETCL LDILDTAG Q E EYSAMRDQYM RTGEGFLCVF
101
150
Ypt31 DISKSSSYEN CNHWLSELRE .NADDNVAVG LIGNKSDLAH LRAVPTEESK
Rab5 DITNEESFAR AKNWVKELQR .QASPNIVIA LSGNKADLAN KRAVDFQEAQ
H-Ras AINNTKSFED IHQYREQIKR VKDSDDVPMV LVGNKCDLAA .RTVESRQAQ
151
200
Ypt31 TFAQENQLLF TETSALNSEN VDKAFEELIN TIYQ.KVSKH QMDLGDSSAN
Rab5 SYADDNSLLF METSAKTSMN VN....EIFM AIAK.KLPKN E.....
H-Ras DLARSYGIPY IETSAKTRQG VEDAFYTLVR EIRQHKLRK. .....LN
201
232
Ypt31 GNANGASAPN GPTISLTPTP NENKKANGNN CC
Rab5 PQNPGANSAR GGGVDLTEPT QPTRNQ.... CCSN
H-Ras PPDESGPGCM SCKCVLS... ....

Figure 24: Suppression of *gcs1Δ* cold sensitivity by the Ypt31-Q72L mutant protein.

gcs1Δ mutant cells (strain Gwk9B) transformed with plasmids pGalYPT31, pGalQ72L, and pGalS27A (expressing wild-type Ypt31, Ypt31-Q72L and Ypt31-S27A, respectively), as well as control vector pPP389 were spread on leucine-free medium containing galactose as sole carbon source and incubated at 23°C for several days before those cells were replica-plated to similar medium and incubated at 15°C for 9 days.

ypt31-Q72L *YPT31*



ypt31-S27A vector

Growth at 15°C

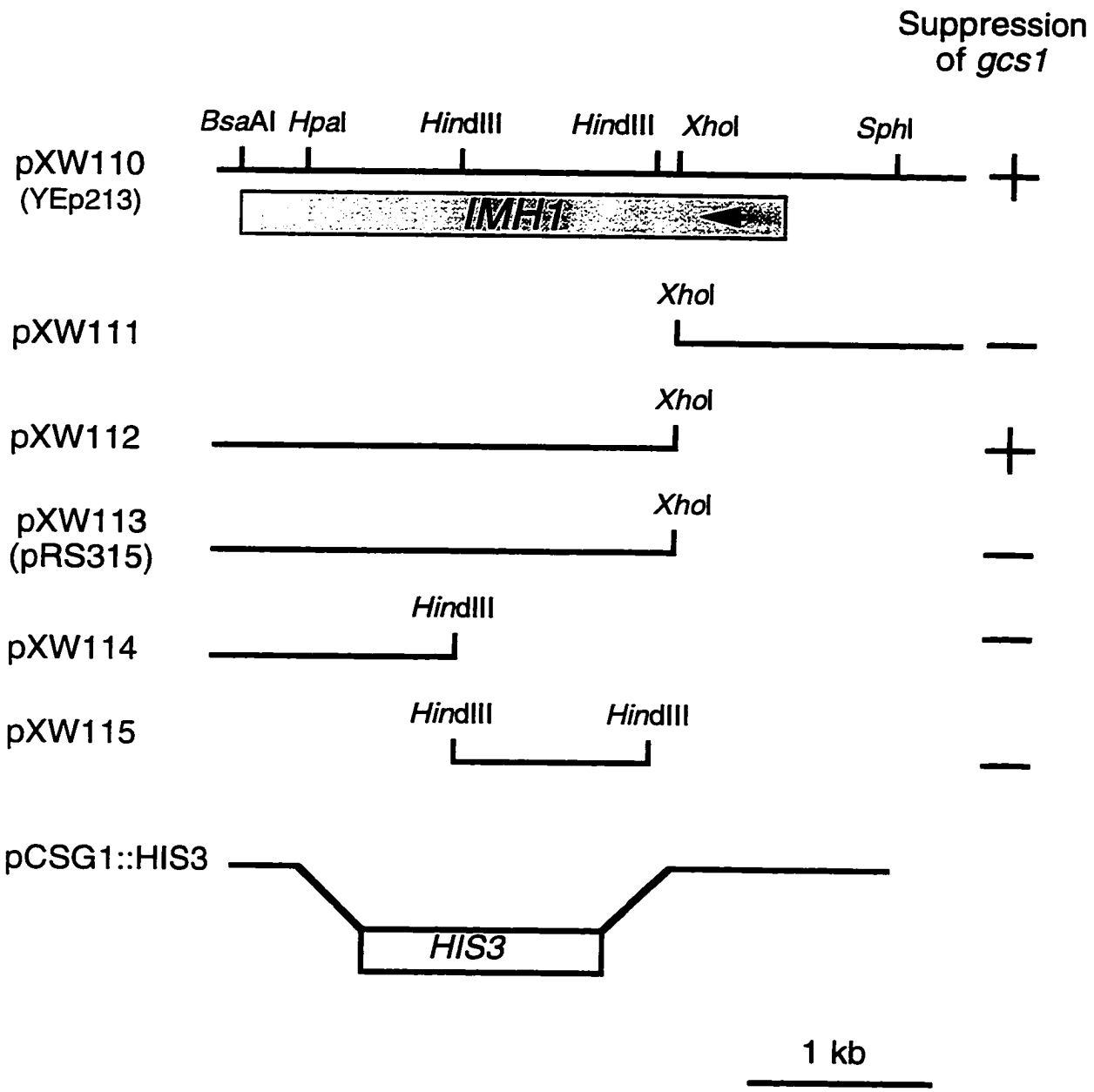
sensitivity, but the *ypt31-S27A* mutant gene failed to suppress the *gcs1* cold sensitivity. The Ypt31-S27A mutant protein, analogously to Rab5S34N, could have a defect in releasing GDP, resulting in a GDP-bound, inactive form. In contrast, the *ypt31-Q72L* mutant gene did suppress the *gcs1* cold sensitivity, but not as well as the wild-type *YPT31* gene did. The Ypt31-Q72L mutant protein, analogously to Rab5Q79L, could have deficient GTPase activity, resulting in a persistent GTP-bound, active form of the protein. Thus, the suppression of *gcs1* cold sensitivity by Ypt31-Q72L suggests that the active form (GTP-bound) of Ypt31 is needed for this suppression, yet the cycling between the GTP-bound and the GDP-bound forms of Ypt31 must also be important because the persistently active Ypt31-Q72L suppressed the *gcs1* cold sensitivity not as well as did the wild-type protein. As measured by growth rates, neither the *ypt31-S27A* gene nor the *ypt31-Q72L* gene had effects on the growth of wild-type cells at 15°C, 23°C, or 37°C (data not shown), indicating that neither mutant allele is dominant in a *YPT31 YPT32* genetic background, even though they were expressed from the strong *GAL1-10* promoter. These mutant Ypt31 forms do not seem to interfere with the normal function of endogenous wild-type Ypt31 and/or Ypt32 proteins; however, there may be enough wild-type Ypt31 and Ypt32 proteins in cells to override any deleterious effects. Therefore it would be interesting to see whether Ypt31-Q72L and Ypt31-S27A proteins have any dominant effects in cells with lower level of endogenous wild-type Ypt31 activity, such as in cells lacking the *YPT32* gene. It would also be interesting to see whether those two mutant proteins allow *ypt31 ypt32* double-null cells to be viable, a test that would indicate whether those two mutant proteins have any activity at all.

19. Isolation of *IMH1* as a multicopy suppressor of *gcs1* cold sensitivity

Restriction mapping of the suppressing plasmid pXW110 indicated that the plasmid has a 4.1-kbp yeast DNA insert (Figure 25). To localize the suppressing sequence within the insert, I first carried out a subcloning analysis. Using a unique *Xho*I site within the insert

Figure 25: Subcloning analysis of *IMH1*.

Only yeast sequences are shown, and the vector is YEp351 or otherwise as indicated; boxes and arrows indicate the position and 5' → 3' direction of open reading frames. To assess the suppression of *gcs1Δ* cold sensitivity, *gcs1Δ* mutant cells (strain Gwk9A) transformed with indicated plasmids were incubated on leucine-free medium at the permissive temperature of 29°C for several days and then replica-plated and incubated at 15°C for 7 days. The vector control was YEp351. pCSG1::HIS3 is a *IMH1* gene-deletion plasmid.


Suppression of gcs1
BsaAI
HpaI
HindIII
HindIII
XhoI
SphI
pXW110
(YEp213)
IMH1
XhoI
pXW111
XhoI
pXW112
XhoI
pXW113
(pRS315)
HindIII
pXW114
HindIII
HindIII
pXW115
pCSG1::HIS3
HIS3
1 kb

I generated two non-overlapping multicopy subclone plasmids, pXW111 containing a 1.6-kbp *Xho*I/*Sau*3A fragment, and pXW112 containing a 2.6-kbp *Sau*3A/*Xho*I fragment. pXW112 suppressed the cold sensitivity of *gcs1Δ* cells (strain Gwk9A), indicating that the suppressing gene lies within the 2.6-kbp insert.

To further localize suppressing sequences within the 2.6-kbp region, *Hin*dIII restriction sites were used to generate smaller non-overlapping subclones in plasmids pXW114 and pXW115; neither subclone suppressed *gcs1* cold sensitivity, indicating that the ORF of the suppressor gene lies across an internal *Hin*dIII site. To identify the suppressor gene, partial DNA sequence was determined, and partial DNA sequences generated from each end of the 1.5-kbp insert in pXW115 encoded peptide sequences similar to each other, suggesting they belong to the same protein. Since one *Hin*dIII site is close to the *Xho*I site of the original 4.1-kbp insert, it is possible that the start codon of the suppressor gene could be close to the *Xho*I site, and partial DNA sequence generated around the *Xho*I site showed that there is a large ORF lying across the *Xho*I site. It is possible that the suppression of *gcs1* cold sensitivity by the multicopy plasmid pXW112 is achieved by the expression of a truncated gene driven by a fortuitous promoter in the flanking YEp351 vector sequences. In fact, the same sequence on a low-copy plasmid failed to suppress *gcs1* cold sensitivity. To test this hypothesis and to estimate the size of the suppressor gene in this region, I used RNA blot analysis with the 2.6-kbp genomic fragment as a probe, and detected a 3.2-kb mRNA (data not shown). This result indicated that the suppressor gene on pXW110 is larger than 2.6-kbp in size, and that plasmid pXW112 contains only part of that gene.

Peptide sequences deduced from the DNA sequences determined above were used to search against Genbank (Release 78, 1993) for similar protein sequences using the BLASTp program. This search identified weak similarities to many proteins with coiled-coil motifs (reviewed in Adamson *et al.*, 1993), but no identical sequence was found. Thus this suppressor gene encodes a novel protein, and I named the gene *CSG1*, for copy-

suppressor of *gcs1*. However, just before submitting this thesis, I found that *CSG1* is the same as *IMH1*, which had just been reported as a low-copy suppressor of the *ypt6-169*ts mutation (Li and Warner, 1996), therefore I refer to this suppressor gene as *IMH1* in the following sections. (To avoid confusion within the lab, strain and plasmid names that had *CSG1* in them are not changed.) Since at that time the sequence of *IMH1* was not in Genbank or any other sequence databank, I decided to initiate DNA-sequence determination for the full-length gene. From partial DNA sequences I could predict that the promoter and N-terminal coding sequences were probably on pXW111, within the 1.5-kbp DNA insert. Plasmid pXW111 was subjected to Exonuclease III (ExoIII) digestion to generate a series of sequentially deleted plasmids. Sequence analysis of these deleted clones generated a continuous 900-bp sequence upstream of the *Xho*I site, which revealed that the start codon of the suppressor ORF was 591 bp upstream of the *Xho*I site. Moreover, sequences farther upstream were identical to published sequences of the yeast *CDC25* gene (Camonis *et al.*, 1986). Thus *IMH1* lies downstream of *CDC25*, which is located on chromosome XII, with only about 500 bp separating the ORFs of *CDC25* and *IMH1*. The partial *CDC25* gene in pXW110 was not responsible for suppressing the *gcs1* mutant phenotype because pXW111, containing the same *CDC25* gene fragment, did not suppress *gcs1*. When I sent the predicted N-terminal 198-residue peptide sequence of the Imh1 protein to Genbank to search for similar proteins, I found that the N-terminal region of Imh1 protein, like the internal segments discussed above, showed weak similarity to many proteins with coiled-coil motifs.

While I was sequencing the 2.5-kbp DNA insert on pXW112, I found that the nucleotide sequence of the region around *CDC25* had been determined by the yeast genome project and, to my delight, the entire sequence of *IMH1* had been determined. Imh1, named YRL018 by the genome project (SacchDB 4.5.B10, 1996), is a protein of 912 amino acid residues (Figure 26), with a calculated molecular weight of 105 kD and a predicted pI of 5.42. The protein is hydrophilic, and does not appear to have a signal

Figure 26: The *IMH1* gene product.

The nucleotide sequence of the *IMH1* gene is numbered from the presumptive translation initiation codon ATG (bolded) of the *IMH1* ORF. The predicted amino-acid sequence is also numbered from the initiator methionine (bolded). Nucleotide and amino-acid numberings are shown on the left. Typical heptad repeat sequences are underlined.

```
-96 AAT ATA AGA GAA CCA AAT GTA GCA GAA ACG TCA AAT AGT AAG CAA CAG GAG TTA CAT TTG

-36 AGG TGA TTT TAA CTT AGG GAA ACA TTT GCT GAT TCA ATG TTC AAA CAG CTG TCA CAA ATT
  1                                                 M   F   K   Q   L   S   Q   I

 25 GGT AAG AAT CTT ACC GAT GAA TTA GCG AAG GGC TTA GCC GAT GAT ATG AGC CCT ACC CCG
  9  G   K   N   L   T   D   E   L   A   K   G   L   A   D   D   M   S   P   T   P

 85 TCA GAA CAA CAA ATC GAA GAT GAT AAG AGT GGC TTG CCA AAA GAA ATA CAA GCT AAA TTA
 29  S   E   Q   Q   I   E   D   D   K   S   G   L   P   K   E   I   Q   A   K   L

145 AGA AAA TTT GAG AAA TAT GAA CAA AAA TAC CCT TTG CTA CTC TCC GCA TAC AAA AAT GAA
 49  R   K   F   E   K   Y   E   Q   K   Y   P   L   L   L   S   A   Y   K   N   E

205 AAA TTA AAG TCA GAG AAG TTA GAG GCT GTT GAA AAG ATT TTA GCG GAA AAT ACA CCC ATA
 69  K   L   K   S   E   K   L   E   A   V   E   K   I   L   A   E   N   T   P   I

265 TCT AAT ATT GAC GAC GCA GTG GAT ACG TTG CCA GCT TTT TTC CAG GAT TTA AAC AAC AAA
 89  S   N   I   D   D   A   V   D   T   L   P   A   F   F   Q   D   L   N   N   K

325 AAT AAC CTA TTG AAT GAT GAG ATC AAG AGA TTA ACT AAG CAG AAC TCG GAA ATT CCA GAA
109  N   N   L   L   N   D   E   I   K   R   L   T   K   Q   N   S   E   I   P   E

385 AGC GCC TCT AGT GAA ACT CTG AAG GAT AAA GAA GAG GAA TTT TTG AAA AAA GAG CAA AAT
129  S   A   S   S   E   T   L   K   D   K   E   E   E   F   L   K   K   E   Q   N

445 TAT AAA AAT GAC ATA GAC GAT CTA AAA AAA AAA ATG GAA GCT TTA AAC ATA GAA TTG GAT
149  Y   K   N   D   I   D   D   L   K   K   K   M   E   A   L   N   I   E   L   D

505 ACT GTA CAA AAA GAA AAA AAT GAT ACT GTT TCA GGT TTG AGA GAA AAA ATA GTT GCA CTG
169  T   V   Q   K   E   K   N   D   T   V   S   G   L   R   E   K   I   V   A   L

565 GAA AAT ATA CTA AAG GAA GAA AGG GAG GCC AAA AAA CAG AAA GAA GAA GTA TCT ATA TCC
189  E   N   I   L   K   E   E   R   E   A   K   K   Q   K   E   E   V   S   I   S

625 GAA CTG AAG GAA GAA TTG GCT ATA AAG AAC CAT TCT CTC GAG GAC AGT CGA ATG AAG ATA
209  E   L   K   E   E   L   A   I   K   N   H   S   L   E   D   S   R   M   K   I

685 ACC GAA TTG GAG CAA AAT TTG TCT TCG AAA AGT ACT ATA ATG GAG GAA AAG TCC TCA GAG
229  T   E   L   E   Q   N   L   S   S   K   S   T   I   M   E   E   K   S   S   E

745 TTG GCA GAA CTA AAT ATT ACT TTA AAA GAG AAA GAG CGC AAG CTG AGT GAA TTG GAA AAA
249  L   A   E   L   N   I   T   L   K   E   K   E   R   K   L   S   E   L   E   K

805 AAA ATG AAG GAG TTA CCG AAG GCG ATA TCT CAT CAA AAT GTA GGA AAC AAT AAC AGA AGG
269  K   M   K   E   L   P   K   A   I   S   H   Q   N   V   G   N   N   N   R   R

865 AAA AAG AAT AGA AAC AAG GGA AAG AAA AAT AAG GGA GGC ATA ACT ACG GGT GAT ATC AGT
289  K   K   N   R   N   K   G   K   K   N   K   G   G   I   T   T   G   D   I   S

925 GAA GAG GAA ACG GTC GAT AAC TCA ATC AAT ACT GAA GAA TAT GAT AAG CTT AAA GAA AAT
309  E   E   E   T   V   D   N   S   I   N   T   E   E   Y   D   K   L   K   E   N

985 TTG CAA GAA TTA CAA GAA AAA TAT AAA GAT TGT GAA GAT TGG AAG CAA AAG TAT GAA GAT
329  L   Q   E   L   Q   E   K   Y   K   D   C   E   D   W   K   Q   K   Y   E   D

1045 ATA GAA GCA GAA CTA AAA GAT GCT AAA GAA TTG GAA AAC TCA CAG CTC GAA AAA TCA GCA
349  I   E   A   E   L   K   D   A   K   E   L   E   N   S   Q   L   E   K   S   A

1105 AAG GAG CTG GAA ACC CTT AAC ACC GAG TTG ATC GAT ACC AAG AAG TCA TTG AAA GAA AAA
369  K   E   L   E   T   L   N   T   E   L   I   D   T   K   K   S   L   K   E   K

1165 AAT TCG GAG CTA GAG GAG GTG AGA GAT ATG CTG AGG ACT GTA GGC AAT GAG CTT GTG GAC
389  N   S   E   L   E   E   V   R   D   M   L   R   T   V   G   N   E   L   V   D

1225 GCA AAA GAT GAG ATT AAA GAG TCT TCG AGT AAA CAA AAT GAA GAA GTG AAA ACC GTT AAG
409  A   K   D   E   I   K   E   S   S   S   K   Q   N   E   E   V   K   T   V   K

1285 CTG GAG CTC GAT GAT TTA CGC CAT AAA AAT GCA ACG ATG ATC GAG GCC TAC GAA GCT AAA
429  L   E   L   D   D   L   R   H   K   N   A   T   M   I   E   A   Y   E   A   K
```

```
1345 AAT ACT GAG TTG AGA AGT AAG ATA GAG TTA TTG AGC AAG AAA GTA GAG CAT CTG AAG AAT
 449  N   T   E   L   R   S   K   I   E   L   L   S   K   K   V   E   H   L   K   N

1405 TTA TGT ACA GAA AAG GAG AAA GAG CAG ACT ACA TCG CAG AAC AAG GTA GCC AAA TTA AAT
 469  L   C   T   E   K   E   K   E   Q   T   T   S   Q   N   K   V   A   K   L   N

1465 GAG GAG ATA TCT CAA CTT ACC TAC GAA AAA TCA AAC ATA ACA AAG GAG CTT ACT TCT TTA
 489  E   E   I   S   N   L   T   Y   E   K   S   N   I   T   K   E   L   T   S   L

1525 AGA ACC TCT TAT AAA CAA AAG GAG AAA ACT GTG AGT TAC TTG GAG GAA CAA GTT AAA CAA
 509  R   T   S   Y   K   Q   K   E   K   T   V   S   Y   L   E   E   Q   V   K   Q

1585 TTT AGT GAG CAA AAG GAC GTG GCT GAA AAA TCC ACA GAA CAG CTG AGA AAA GAT CAT GCT
 529  F   S   E   Q   K   D   V   A   E   K   S   T   E   Q   L   R   K   D   H   A

1645 AAA ATT TCT AAC AGA TTA GAC TTA TTA AAA AAG GAA AAT GAG ACA CTG CAT AAT GAT ATC
 549  K   I   S   N   R   L   D   L   L   K   K   E   N   E   T   L   H   N   D   I

1705 GCA AAG AAT TCT AAT TCC TAC GAG GAG TAT TTG AAA GAA AAT GGT AAA TTA TCG GAA AGA
 569  A   K   N   S   N   S   Y   E   E   Y   L   K   E   N   G   K   L   S   E   R

1765 TTG AAT ATT TTG CAA GAA AAA TAC AAT ACC TTG CAA AAT GTA AAA AGT AAT TCG AAT GAA
 589  L   N   I   L   Q   E   K   Y   N   T   L   Q   N   V   K   S   N   S   N   E

1825 CAC ATA GAT TCT ATC AAA AGA CAA TGT GAG GAA CTA AAT GTC AAG TTG AAG GAA TCT ACA
 609  H   I   D   S   I   K   R   Q   C   E   E   L   N   V   K   L   K   E   S   T

1885 AAA AAA ATT TTA TCT TTA GAA GAT GAA CTA AAT GAA TAT GCT AAT ATT GTT CAA GAC AAA
 629  K   K   I   L   S   L   E   D   E   L   N   E   Y   A   N   I   V   Q   D   K

1945 ACC AGA GAA GCT AAC ACA TTG AGA AGG TTA GTT TCG GAC AGT CAG ACA GAT GAT TCG AGC
 649  T   R   E   A   N   T   L   R   R   L   V   S   D   S   Q   T   D   D   S   S

2005 AAA CAA AAA GAG TTG GAG AAT AAA TTG GCC TAT TTA ACG GAT GAA AAG AAT AAA TTG GAA
 669  K   N   K   E   L   E   N   K   L   A   Y   L   T   D   E   K   N   K   L   E

2065 GCA GAA TTA GAC TTA CAA ACA TCC AGA AAG GCC ACT GAA TTA CAA GAG TGG AAG CAT ACA
 689  A   E   L   D   L   Q   T   S   R   K   A   T   E   L   Q   E   W   K   H   T

2125 GTA ACT GAG CTG AAA TCG GAA ATA CAC GCT TTA AAG CTT CGT GAA GAG GGA CTA AAA TCA
 709  V   T   E   L   K   S   E   I   H   A   L   K   L   R   E   E   G   L   K   S

2185 GAG GTT GAC GCA TTG AAA CAT GTT AAC AAT GAC ATC AAA AGG AAG ACT CAA GCC ACT TCA
 729  E   V   D   A   L   K   H   V   N   N   D   I   K   R   K   T   Q   A   T   S

2245 GAT GAT TCC GAT CAG TTG GAA CAG ATC ACA TCT AAT TTA AAA CTC TCA TTG TCT AAG GCT
 749  D   D   S   D   Q   L   E   Q   I   T   S   N   L   K   L   S   L   S   K   A

2305 GAT GAA AAG AAT TTT GAG CTA CAG TCT GCC AAT GAG AAA CTT CTG AAT TTA AAT AAC GAA
 769  D   E   K   N   F   E   L   Q   S   A   N   E   K   L   L   N   L   N   N   E

2365 CTT AAC AAG AAA TTT GAT CGA TTA CTA AAA AAT TAT CGT TCA TTG TCC TCT CAA TTG AAT
 789  L   N   K   K   F   D   R   L   L   K   N   Y   R   S   L   S   S   Q   L   N

2425 GCT TTA AAG GAA AGA CAA TAC AGT GAC AAG TCA GGA AGA GTT AGT AGG TCT GGT TCT ATC
 809  A   L   K   E   R   Q   Y   S   D   K   S   G   R   V   S   R   S   G   S   I

2485 GGT ACT CTA GCT AAC GCG AAT ATT GAT TCC TCA CCA GCG AAT AAC TCT AAT CCA ACT AAA
 829  G   T   L   A   N   A   N   I   D   S   S   P   A   N   N   S   N   P   T   K

2545 TTA GAG AAG ATA CGA TCA TCA AGT TCA TTG GAG TTA GAC TCT GAG AAA AAT GAA AAA ATT
 849  L   E   K   I   R   S   S   S   S   L   E   L   D   S   E   K   N   E   K   I

2605 GCA TAT ATA AAA AAT GTT TTG TTG GGA TTT TTG GAG CAC AAG GAA CAA CGG AAC CAA TTA
 869  A   Y   I   K   N   V   L   L   G   F   L   E   H   K   E   Q   R   N   Q   L

2665 CTT CCT GTA ATT TCT ATG TTG TTA CAA CTG GAC AGT ACT GAT GAA AAA AGA CTG GTT ATG
 889  L   P   V   I   S   M   L   L   Q   L   D   S   T   D   E   K   R   L   V   M

2725 TCT CTG AAG TAA AAA TGT AGC TGA AAG
 909  S   L   K  OCH
```

sequence or transmembrane domain. Therefore Imh1 is not likely to be a nuclear protein or an integral membrane protein. However, from the sequence features of Imh1 little can be learned about the function of this protein. My finding that *IMH1* is a multicopy suppressor of *gcs1* cold sensitivity suggests that Imh1 might also have a role in vesicular transport, and this notion is also supported by the recent finding that *IMH1* on a low-copy plasmid suppress the *ypt6-169*ts mutation, as Ypt6 is a Ypt/Rab protein that is involved intra-Golgi vesicular transport (Li and Warner, 1996).

20. Imh1 is structurally similar to Rabaptin-5

When I used the Imh1 protein sequence for a BLASTp search against Genbank, I found that Imh1 shows weak similarity in sequence with many proteins containing coiled-coil motifs, including myosin heavy chain (reviewed in Kiehart, 1990). The coiled-coil motif comprises two amphipathic right-handed α-helices which adopt a left-handed supercoil so that the non-polar faces of the two α-helices are continually adjacent (reviewed in Adamson *et al.*, 1993). Like those proteins with coiled-coil structure, the primary sequence of Imh1 contains many so-called heptad repeat sequences, in which hydrophobic amino acid residues are often found in positions *a* and *d* of the *abcdefg* heptad sequence (Figure 26). To learn whether Imh1 protein could have many α-helical structures I used the PeptideStructure program of the GCG software package (Devereux *et al.*, 1984) to predict the secondary structure of Imh1. As shown in Figure 27, using both Chou-Fasman and Garnier-Osguthorpe-Robson methods (Chou and Fasman, 1978; Garnier *et al.*, 1978), most of the protein is predicted to fold into α-helix structures. This secondary-structure feature and the primary heptad repeat sequences suggest that Imh1 probably contains coiled-coil motifs. Based on this structure analysis, most of the Imh1 protein could form a coiled-coil structure similar to that of myosin heavy chain, so the whole Imh1 protein could have a rod-like tertiary structure.

The predicted structure of Imh1 is also similar to that of a protein called Rabaptin-5

Figure 27. Analysis of the secondary structure of Imh1.

The secondary structure of Imh1 was analyzed using PeptideStructure and PlotStructure of the GCG software (Devereux *et al.*, 1984). "KD Hydrophilicity" displays the hydropathic character of Imh1; "CF Turns", "CF Alpha Helices", and "CF Beta Sheets" display the secondary structure of Imh1 predicted using the method of Chou and Fasman (1978); "GOR Turns", "GOR Alpha Helices", and "GOR Beta Sheets" display the secondary structure of Imh1 predicted using the method of Garnier *et al.* (1978). The predictions for surface probability (indicated by "Surface Prob."), flexibility (as indicated by "Flexibility"), antigenic index (as indicated by "Jameson-Wolf [Antigenic Index]"), and glycosylation sites (as indicated by "Glycosyl. Sites") of Imh1 are also shown.

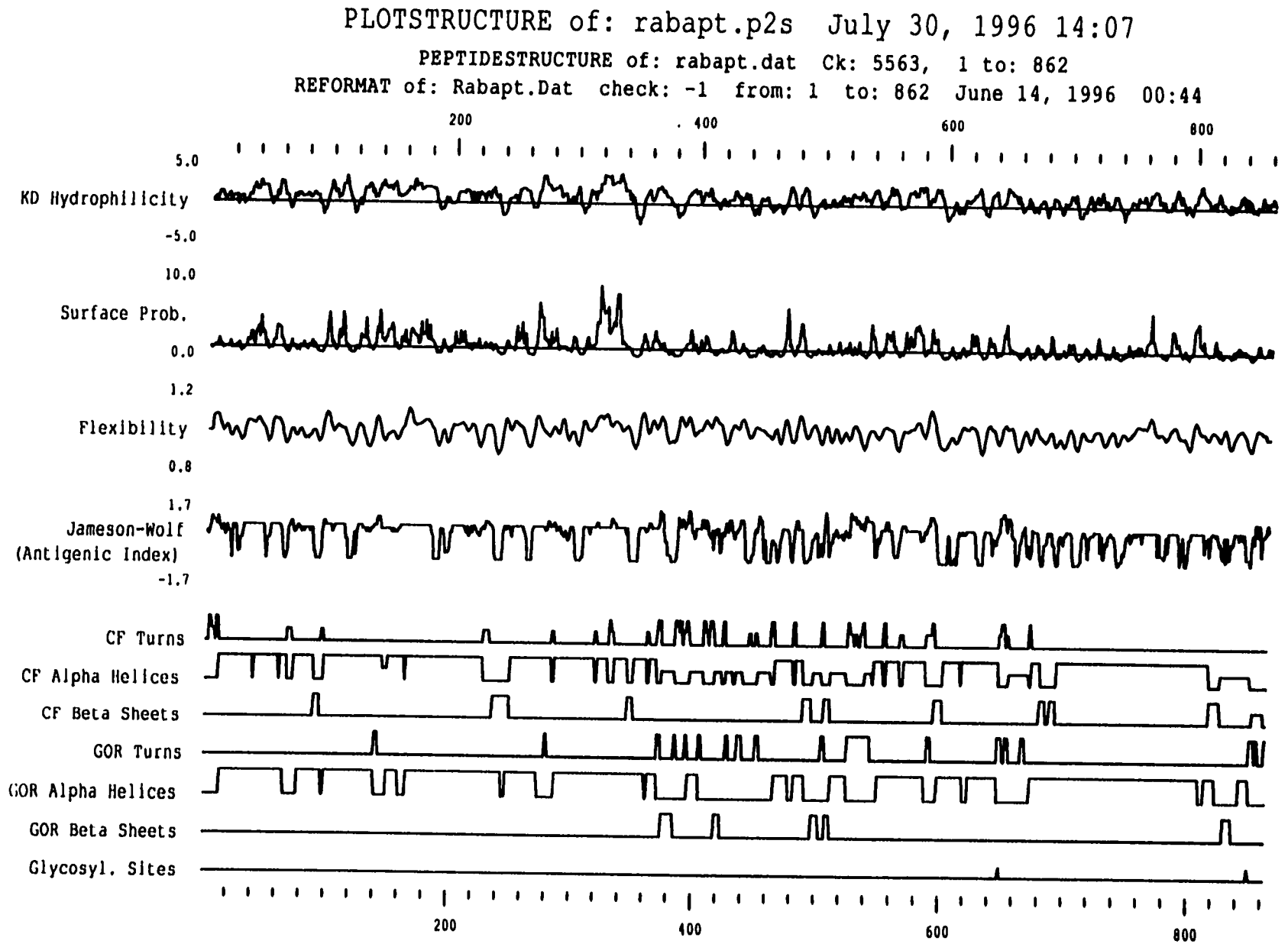
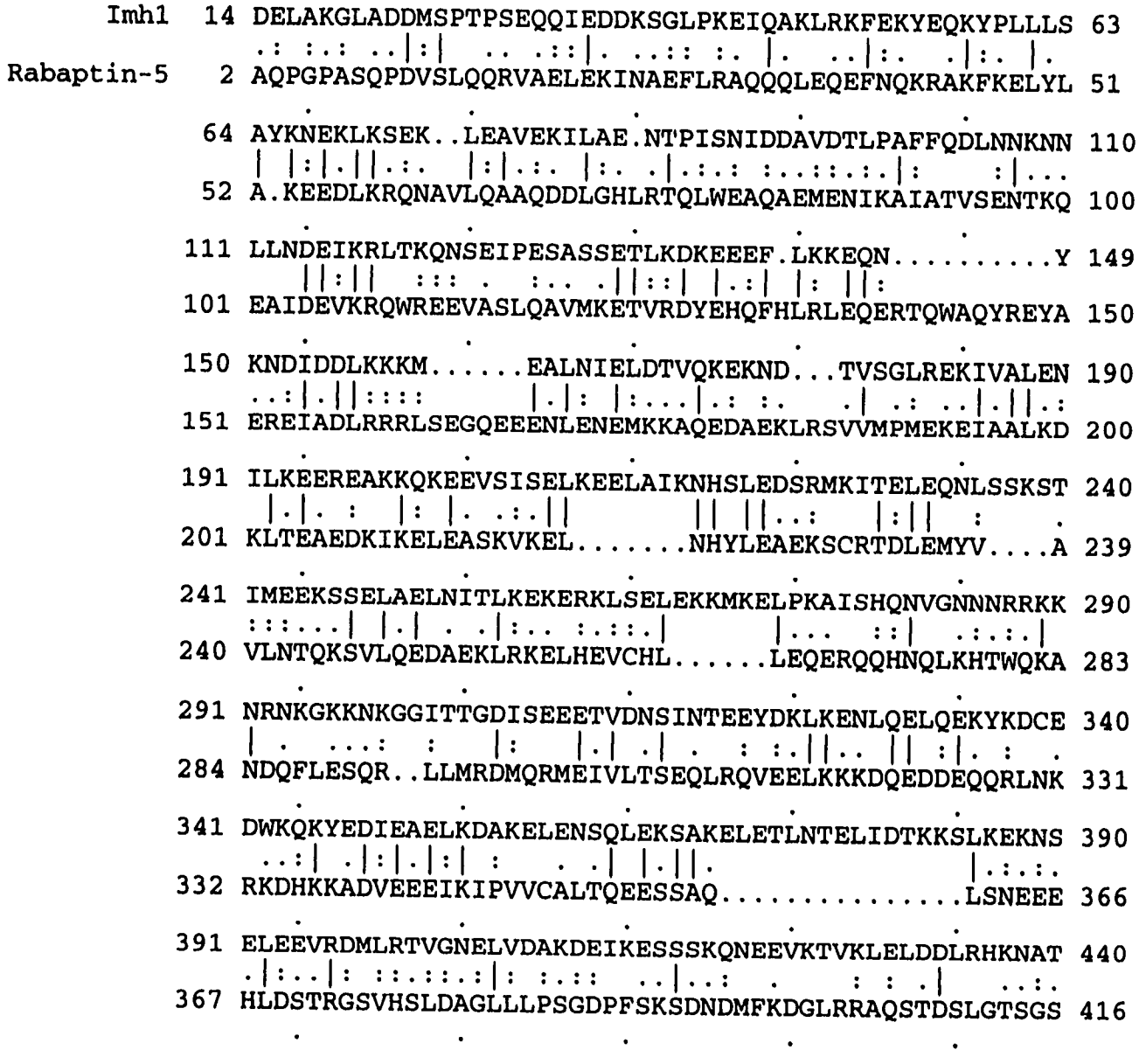
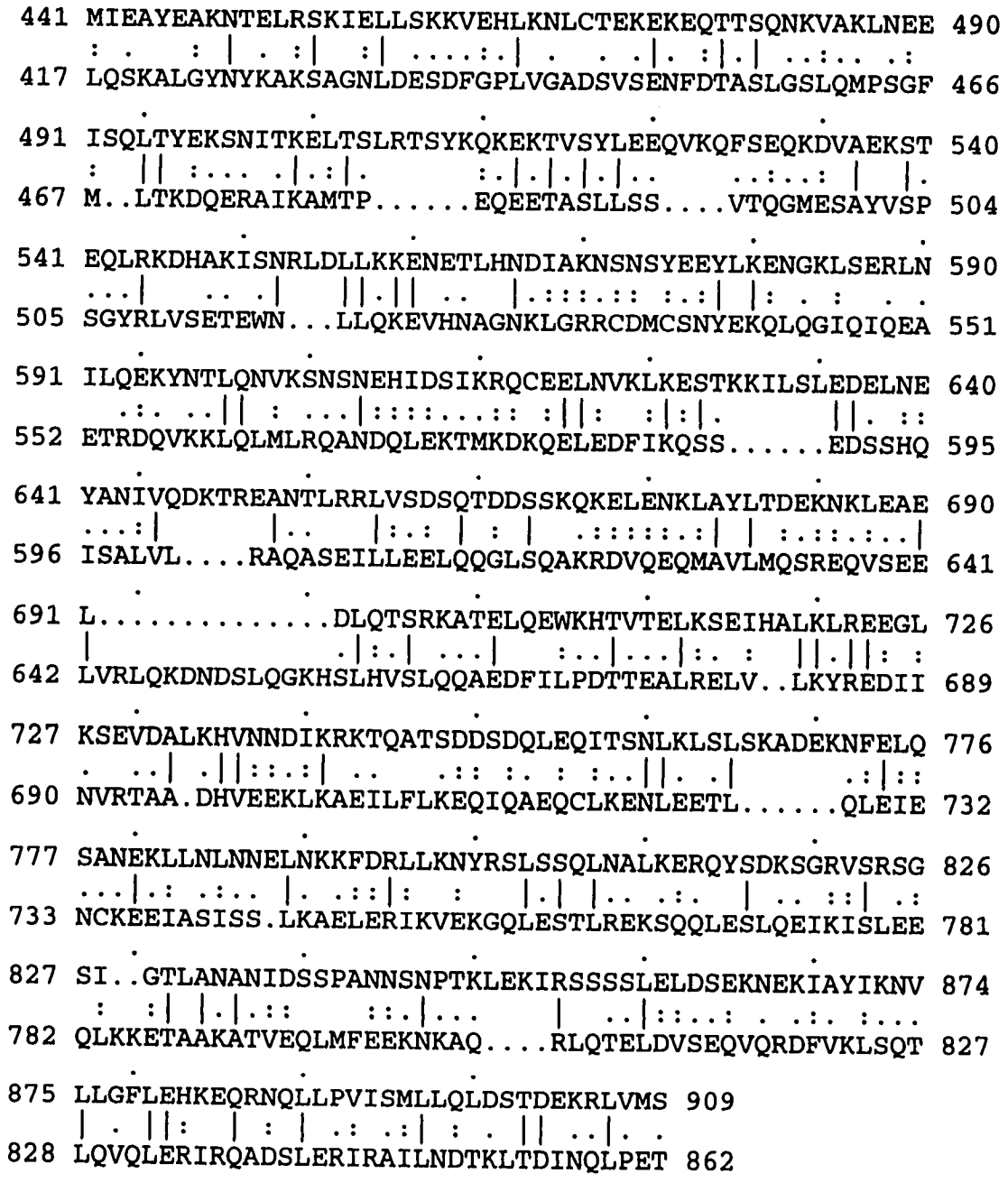

PLOTSTRUCTURE of: rabapt.p2s July 30, 1996 14:07
PEPTIDESTRUCTURE of: rabapt.dat Ck: 5563, 1 to: 862
REFORMAT of: Rabapt.Dat check: -1 from: 1 to: 862 June 14, 1996 00:44
200
400
600
800
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
200
400
600
800

Figure 28: Imh1 is similar in sequence to Rabaptin-5

Alignment of Imh1 and Rabaptin-5 predicted polypeptides. "|" indicates where Imh1 and Rabaptin-5 have the identical amino acid; ":" indicates where those two proteins have highly conserved amino acids; and "." indicates where those two proteins have less conserved amino acids. Positions of amino acids are indicated to the left of aligned sequences.


Imh1 14 DELAKGLADDMSPTPSEQQIEDDKSGLPKEIQAKLRKFEKYEQKYPLLLS 63
Rabaptin-5 2 AQPGPASQPDVSLQQRVAELEKINAEFLRAQQQLEQEFNQKRAKFKELYL 51
64 AYKNEKLKSEK..LEAVEKILAE.NTPISNIDDAVDTLPAFFQDLNNKNN 110
52 A.KEEDLKRQNAVLQAAQDDLGHLRTQLWEAQAEMENIKAIATVSENTKQ 100
111 LLNDEIKRLTKQNSEIPESASSETLKDKEEEF.LKKEQN.....Y 149
101 EAIDEVKRQWREEVASLQAVMKETVRDYEHQFHLRLEQERTQWAQYREYA 150
150 KNDIDDLKKKM.....EALNIELDTVQKEKND...TVSGLREKIVALEN 190
151 EREIADLRRRLSEGQEEENLENEMKKAQEDAEKLRSVVMPMEKEIAALKD 200
191 ILKEEREAKKQKEEVSISELKEELAIKNHSLEDSRMKITELEQNLSSKST 240
201 KLTEAEDKIKELEASKVKEL.....NHYLEAEKSCRTDLEMYV....A 239
241 IMEEKSSELAELNITLKEKERKLSELEKKMKELPKAISHQNVGNNNRRKK 290
240 VLNTQKSVLQEDAEKLRKELHEVCHL.....LEQERQQHNQLKHTWQKA 283
291 NRNKGKKNKGGITTGDISEEETVDNSINTEEYDKLKENLQELQEKYKDCE 340
284 NDQFLESQR..LLMRDMQRMEIVLTSEQLRQVEELKKKDQEDDEQQRLNK 331
341 DWKQKYEDIEAELKDAKELENSQLEKSAKELETLNTELIDTKKSLKEKNS 390
332 RKDHKKADVEEEIKIPVVCALTQEESSAQ.....LSNEEE 366
391 ELEEVRDMLRTVGNELVDAKDEIKESSSKQNEEVKTVKLELDDLRHKNAT 440
367 HLDSTRGSVHSLDAGLLLPSGDPFSKSDNDMFKDGLRRAQSTDSLGTSGS 416


441 MIEAYEAKNTELRSKIELLSKKVEHLKNLCTEKEKEQTTSQNKVAKLNEE 490
417 LQSKALGYNYKAKSAGNLDESDFGPLVGADSVSENFDTASLGSLQMPSGF 466
491 ISQLTYEKSNITKELTSLRTSYKQKEKTVSYLEEQVKQFSEQKDVAEKST 540
467 M..LTKDQERAIKAMTP.....EQEETASLLSS....VTQGMESAYVSP 504
541 EQLRKDHAKISNRLDLLKKENETLHNDIAKNSNSYEEYLKENGKLSERLN 590
505 SGYRLVSETEWN...LLQKEVHNAGNKLGRRCDMCSNYEKQLQGIQIQEA 551
591 ILQEKYNTLQNVKSNSNEHIDSIKRQCEELNVKLKESTKKILSLEDELNE 640
552 ETRDQVKKLQLMLRQANDQLEKTMKDKQELEDFIKQSS.....EDSSHQ 595
641 YANIVQDKTREANTLRRLVSDSQTDDSSKQKELENKLAYLTDEKNKLEAE 690
596 ISALVL....RAQASEILLEELQQGLSQAKRDVQEQMAVLMQSREQVSEE 641
691 L.............DLQTSRKATELQEWKHTVTELKSEIHALKLREEGL 726
642 LVRLQKDNDSLQGKHSLHVSLQQAEDFILPDTTEALRELV..LKYREDII 689
727 KSEVDALKHVNNDIKRKTQATSDDSDQLEQITSNLKLSLSKADEKNFELQ 776
690 NVRTAA.DHVEEKLKAEILFLKEQIQAEQCLKENLEETL.....QLEIE 732
777 SANEKLLNLNNELNKKFDRLLKNYRSLSSQLNALKERQYSDKSGRVSRSG 826
733 NCKEEIASISS.LKAELERIKVEKGQLESTLREKSQQLESLQEIKISLEE 781
827 SI..GTLANANIDSSPANNSNPTKLEKIRSSSSLELDSEKNEKIAYIKNV 874
782 QLKKETAAKATVEQLMFEEKNKAQ....RLQTELDVSEQVQRDFVKLSQT 827
875 LLGFLEHKEQRNQLLPVISMLLQLDSTDEKRLVMS 909
828 LQVQLERIRQADSLERIRAILNDTKLTDINQLPET 862

(Figure 28), which was recently isolated as a protein that binds specifically to Rab5 in its GTP-bound form (Stenmark *et al.*, 1995). Rab5, a member of the Rab/Ypt protein family discussed above, has been shown to function in the early endocytic pathway (Bucci *et al.*, 1992) and Rabaptin-5 is thought to be a direct effector of Rab5 (Stenmark *et al.*, 1995). Overexpression of Rabaptin-5 alone resulted in an expansion of early endosome compartment, and this effect is similar to that caused by overexpression of either Rab5 or Rab5Q79L. Furthermore, Rabaptin-5 is required for Rab5-stimulated fusion between early endosomes in a cell-free system, indicating a important role for Rabaptin-5 in endocytic membrane transport (Stenmark *et al.*, 1995). Rabaptin-5 has 865 amino acid residues and a calculated molecular weight of 101 kD, which is comparable to that of Imh1 (105 kD); Rabaptin-5 is also hydrophilic, and it has a calculated pI of 4.8, a little lower than the 5.42 pI of Imh1; most of Rabaptin-5 is predicted to adopt a coiled-coil structure, which is similar to that of Imh1. The similarities between Imh1 and Rabaptin-5 suggest that, like Rabaptin-5, Imh1 could also be a effector of certain Ypt/Rab GTP-binding proteins. Since increased Imh1 and increased Ypt31 have the same effect in suppressing the *gcs1* mutant phenotype, it will be interesting to see whether Imh1 is a direct effector of Ypt31.

21. *IMH1* is not an essential gene

To gain insight into the function of the *IMH1* gene, I decided to generate *imh1* mutations and study the phenotype of cells lacking Imh1. To generate a *imh1Δ* strain, linearized *IMH1* deletion plasmid, pCSG1::HIS3 (Figure 25), was transformed into wild-type diploid W303 cells so that one copy of the chromosomal wild-type *IMH1* gene was deleted, as confirmed by Southern analysis. The resultant diploid cells were sporulated and subjected to tetrad analysis. This analysis showed that all four spores from a tetrad could germinate and form colonies at 23°C, and no difference was detected between wild-type spores and *imh1Δ* mutant spores (identified by the *HIS3* nutritional marker at

the *imh1Δ* locus, data not shown). Therefore, the *IMH1* gene is not essential for spore germination or mitotic growth at 23°C. When I checked the growth of these *imh1Δ* haploid cells at a higher temperature of 37°C or a lower temperature of 15°C, no differences were observed between these mutant cells and wild-type haploid cells. Thus, Imh1 protein activity is not essential for vegetative cell growth over a wide range of temperatures. This finding that Imh1 protein is dispensable suggests that there might be other Imh1-like proteins in yeast that can provide Imh1 activity. However, a search for Imh1-like proteins in the completed yeast genome databank did not find any proteins that show significant sequence similarities with Imh1. Therefore it is possible that, under the normal growth conditions I have tested, cells can grow well without Imh1 activity.

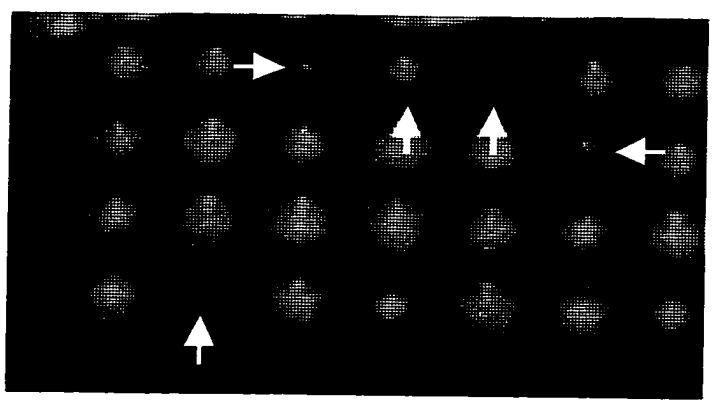
22. Genetic interaction between *arf1Δ* and the *ypt32Δ ypt31*cs mutant situation

It has been reported that the mammalian ARF1 protein is associated with *trans*-Golgi network (TGN) and with TGN-originated vesicles (Barr and Huttner, 1996). This ARF1 localization is similar to that reported for the mammalian Rab11 protein (Urbe *et al.*, 1993). Since two of my suppressor proteins, Ypt31 and Ypt32, are yeast homologues of Rab11, I was interested to see whether there is any genetic interaction between a yeast *arf* mutation and mutations in the *YPT31* and *YPT32* genes. As discussed above, yeast Arf1 and Arf2 form an essential family, and cells lacking both proteins are dead. The mammalian ARF1 is able to rescue the lethality of *arf1Δ arf2Δ* double mutations, indicating that mammalian ARF1 and yeast Arf1 + Arf2 have similar cellular functions (Stearns *et al.*, 1990a, b). Therefore I decided to test whether there is any genetic interaction between mutations in the *ARF1* + *ARF2* and *YPT31* + *YPT32* genes. It was found previously in our lab that cells containing both a *gcs1Δ* mutation and an *arf1Δ* mutation form smaller colonies than do cells containing only one of these mutations (Poon *et al.*, 1996). Since the *gcs1Δ* mutation is synthetically lethal with *ypt32Δ ypt31*cs mutant situation (see above), I decided to test if there is any synthetic enhancement when

Figure 29: Synthetic enhancement between *arf1Δ* and the *ypt32Δ ypt31*cs mutant situations.

Haploid *arf1Δ* mutant cells (strain 10b) were crossed with haploid *ypt32Δ ypt31*cs mutant cells (strain YTH12B), and the resultant diploid cells were sporulated and subjected to tetrad analysis. Four spores from each ascus, separated and arranged at different positions (from top to bottom) on the surface of YEPD medium, were incubated at the permissive temperature of 29°C for 6 days to allow germination and colony formation. Colonies formed by spores bearing the *arf1Δ ypt32Δ ypt31*cs genotype, as identified by nutritional markers, are indicated with white arrows.

arf1Δ crossed with *ypt32Δ ypt31*cs



an *arf1Δ* mutation is combined with the *ypt32Δ* and *ypt31*cs mutations. To do this, the *arf1Δ* mutant strain 10b was crossed with strain YTH12B, which contains the *ypt32Δ* and *ypt31*cs mutations, and resultant diploid cells were sporulated and subjected to tetrad analysis; spores were germinated at a permissive temperature of 29°C, and genotypes were determined by the nutritional markers integrated at the corresponding mutant loci and by cold-sensitivity phenotypes. As shown in Figure 29, spores containing the *arf1Δ*, *ypt32Δ* and *ypt31*cs mutations formed smaller colonies than did other spores within these tetrads. Thus there is a synthetic enhancement of impairment between an *arf1Δ* mutation and the *ypt32Δ ypt31*cs mutant situation, a genetic interaction similar to that observed between a *gcs1Δ* mutation and an *arf1Δ* mutation, suggesting that yeast Ypt31/32 and the yeast Arf1 activities might also affect a common aspect of membrane trafficking.

It was also shown that increased expression of Gcs1 impairs the growth of *arf1Δ* cells (Poon *et al.*, 1996), and since Arf2 forms an essential family with Arf1 and is also a substrate of Gcs1, this result indicates that a balance between the Arf2 GTP-bound form and the Arf2 GDP-bound form, in cells lacking Arf1, is important for cell growth. I transformed *arf1Δ* cells with a multicopy *YCK2* plasmid (pXW123), or a multicopy *IMH1* plasmid (pXW110), or a multicopy *YPT31* plasmid (pXW130) and found no effects on *arf1Δ* cell growth when these genes are overexpressed. Perhaps increased dosage of *YCK2*, *YPT31*, or *IMH1* does not change the balance between Arf2 GTP-bound form and Arf2 GDP-bound form and boosting Yck2, Ypt31, or Imh1 activity does not directly affect Arf1- and Arf2-GAP activity when Gcs1 is absent; instead, increased Yck2, Ypt31 and Imh1 activity suppress *gcs1* cold sensitivity by activating a parallel pathway that bypasses the need for Gcs1.

IV DISCUSSION

1. General remarks

Using a multicopy-suppressor approach, I have identified seven yeast proteins that may function in the same type of cellular process as that mediated by Gcs1. My analysis provides important clues about the biological functions of Gcs1. The finding that proteins associated with membranes (Yck1, Yck2, and Yck3; Vancura *et al.*, 1994; Wang *et al.*, 1996) and proteins regulating vesicle transport (Ypt31 and Ypt32; D. Gallwitz, personal communication) are among the multicopy suppressors of the *gcs1* cold sensitivity implicates a role for Gcs1 in intracellular vesicular transport. This idea has proven correct because of our recent finding that Gcs1 is a GTPase-Activating Protein (GAP) for yeast Arf1 and Arf2 proteins, which have been shown to be involved in vesicular transport. My study also identified some novel gene products (Yck3 and Imh1) that might function in parallel with Gcs1, providing an additional basis for studying the biological functions of Gcs1. All of the suppressors were isolated by their ability to suppress the cold sensitivity of cells lacking the Gcs1 protein. Since none of these suppressor proteins is structurally similar to Gcs1, it is unlikely that increased amount of any of those suppressor proteins actually replaces Gcs1 function; instead, each of these suppressor proteins activates a pathway that can bypass the need for the Gcs1 protein.

In this study, I found that increased activities of membrane-associated yeast casein kinase I isoforms, Yck1, Yck2 and Yck3, suppressed *gcs1* cold sensitivity, and that the membrane association of these kinases is essential for suppression. The need for membrane association indicates that suppression could take place by modifying some membrane-associated activity, and suggests that casein kinase I activity may be involved in regulating intracellular vesicular transport. I also found that increased activity of an essential pair of small GTP-binding proteins, Ypt31 and Ypt32, suppresses the *gcs1* cold sensitivity. Ypt31 and Ypt32 belong to the Ypt/Rab family of small-GTP binding

proteins, which have been shown to be involved in regulating vesicle transport at various stages (reviewed in Novick and Brennwald, 1993). My finding that an increase in Yck casein kinase I activity suppresses the *ypt32Δ ypt31*cs cold sensitivity further supports a role for Yck proteins in vesicular transport. I also found that increased activity of a novel protein (Imh1) suppresses *gcs1* cold sensitivity. Furthermore, Imh1 shows homology with a mammalian protein, Rabaptin-5, which is thought to be the effector of Rab5 (Stenmark *et al.*, 1995). Therefore, Imh1 protein probably also has a role in regulating membrane trafficking, although its role remains undefined.

2. Suppression of *gcs1Δ* is mediated through a second Arf1-GAP pathway that is inherently cold sensitive

Unlike other small-GTP binding proteins, purified mammalian Arf1 protein has no detectable intrinsic GTPase activity *in vitro* (Kahn and Gilman, 1986); thus a GAP is needed for the activation of Arf1-GTPase activity and the decoating of coated vesicles. A putative GTPase-defective mutant yeast Arf1 protein, Arf1Q71L (the glutamine at position 71 was changed to leucine; Kahn *et al.*, 1995), is lethal for cell growth, indicating that the ability of Arf1 to cycle between GTP-bound and GDP-bound forms is essential for cell growth. In contrast, a *gcs1Δ* mutant cell, with no Gcs1 protein, has no detectable growth defect at temperatures higher than 15°C. Therefore, Gcs1 could not be the sole Arf- GAP in yeast, and there is probably a second Arf1-GAP ("GAP2") in yeast. The copy suppressors isolated in this study probably allow vesicular transport to take place more efficiently using another Arf1-GAP pathway. As discussed in the Introduction, a major function of Arf proteins is to drive, in a GTP-dependent manner, the assembly of sets of cytosolic coat proteins onto the ER or Golgi membranes as well as to determine the timing of the release of those proteins back to the cytosol (reviewed in Donaldson and Klausner, 1994). Therefore, Arf proteins and those coat proteins are important for the formation and the subsequent decoating of coated vesicles. So far, two

type of coats, COPI and COPII, have been characterized in yeast (reviewed in Salama and Scheckman, 1995). It has been shown that Sec23, a component of the COPII protein complex, has GAP activity for the Arf-like protein Sar1 (Yoshihisa *et al.*, 1993). This result suggests that while purified Arf proteins have very low intrinsic GTPase activity, their GTPase activity could be activated when they bind to lipids and coat proteins during the process of vesicle formation. Therefore it is possible that COPI coat proteins also have GAP activity for Arf1, and it would be useful to test this possibility directly *in vitro*.

In *gcs1Δ* null mutant cells (strain Gwk9A), which are conditionally defective only for the resumption of cell proliferation from stationary phase, there is no Gcs1 protein present. Therefore, the cold sensitivity of these *gcs1Δ* mutant cells is not caused by a cold-sensitive mutant Gcs1 protein, but actually reflects an inherent cold sensitivity of the pathway mediated by GAP2 activity, which is only revealed when there is no Gcs1 protein present and a Gcs1 pathway is not functioning. From this point of view, a multicopy suppressor of *gcs1Δ* cold sensitivity does not suppress a defect in Gcs1 protein, but instead suppresses an inherently cold-sensitive process. There is some evidence which suggests that the vesicular transport machinery is inherently somewhat cold sensitive. For example, vesicle transport in mammalian cells does not function effectively at the low temperature of 15°C. Similarly, mutations in many yeast genes encoding proteins involved in vesicular transport, such as mutations in the *YPT1* (Segev *et al.*, 1988) and *YPT31* plus *YPT32* (D. Gallwitz, personal communication) genes, produce cold sensitivity. One possibility for this inherently cold sensitivity of vesicular transport is that a reduced lipid fluidity, caused by the low temperature, requires higher protein activities to organize membrane components. The same argument would apply to Arf1 + Arf2 activity and, in fact, decreased Arf1 + Arf2 activity, as in the *arf1Δ* mutant cells, produces a cold-sensitivity phenotype (Stearns *et al*, 1990a). Therefore, the cold sensitivity of *gcs1Δ* cells reflects the cold sensitivity of a pathway mediated by a second Arf1 GAP.

3. Yck casein kinase I activity and vesicular transport

Increased activity of any of the three prenylated casein kinase I isoforms, Yck1, Yck2 and Yck3, bypasses the need for the Gcs1 protein, while increased dosage of the non-prenylated yeast casein kinase I isoform, Hrr25, does not suppress *gcs1* cold sensitivity. This observation suggests that proper membrane association of casein kinase I activity is important for suppression. Yck1 and Yck2 have been shown to be predominantly associated with the plasma membrane, and the C-terminal prenylation signal of Yck2 is important for its plasma-membrane association (Vancura *et al.*, 1994). I found that the C-terminal prenylation motif is also important for the suppression of *gcs1* cold sensitivity, indicating that plasma-membrane association is important for these proteins to serve as copy suppressors of *gcs1*. Fusion of the prenylation motif of Yck1 to the C-terminal end of the Hrr25 protein allowed the resulting chimeric protein, Hrr25-CC, to suppress *gcs1* cold sensitivity. Therefore, the C-terminal portion of Yck1 may facilitate the association of Hrr25, which has a different intracellular localization to that of Yck1, to where Yck1 normally functions and suppress the *gcs1* cold sensitivity. Hrr25 is involved in DNA repair (Hoekstra *et al.*, 1991), and is predominantly associated with nuclei (Vancura *et al.*, 1994). A chimeric protein, Hrr25-Yck2, with the Hrr25 protein kinase domain associated with the prenylation signal of Yck2 while lacking a potential nuclear targeting sequence TKKQKY, suppressed *gcs1* cold sensitivity to a better extent than did Hrr25-CC. This observation indicates that it is the non-nuclear and membrane-associated casein kinase I activity that is important for suppressing the effects of *gcs1*. Consistent with this idea, a portion of Yck3 co-fractionates with the plasma membrane (Wang *et al.*, 1996).

The importance of membrane association of Yck casein kinase I activity for suppression of a defect caused by the absence of Gcs1, an Arf1-GAP that is important for vesicular transport, suggests that Yck casein kinase I might also have a role in vesicular

transport and may be localized to similar intracellular membrane compartments as Arf1 does. Mammalian Arf1 has been localized to the Golgi apparatus (Helms *et al.*, 1993; Tanigawa *et al.*, 1993), to secretory vesicles originated from the *trans*-Golgi network (Barr and Huttner, 1996), and to endosomes (Whitney *et al.*, 1995), and a mammalian Arf1 GAP has also been localized to the Golgi apparatus (Cukierman *et al.*, 1995). Interestingly, one mammalian casein kinase I isoform, casein kinase I alpha, has also been localized to vesicular structures such as Golgi apparatus and endoplasmic reticulum, and has been shown to be associated with purified synaptic vesicles (Gross *et al.*, 1995). Therefore, at least in mammalian systems, there is evidence that some casein kinase I isoforms are associated with intracellular membrane compartments and they may have a role in regulating vesicular transport. Due to the small size of the yeast Golgi apparatus and secretion vesicles, the localization of yeast Arf1 and Gcs1 has yet to be determined. Given the genetic interaction seen between *gcs1* and *yck* mutations, it is possible that yeast Yck casein kinase I isoforms are also associated intracellular membrane compartments.

The wide intracellular distribution of different casein kinase I isoforms indicates that casein kinase I activity is involved in various cellular processes. The suppression of a defect in the yeast Arf1-GAP activity by the increased activity of Yck casein kinase I indicates a role for casein kinase I in regulating vesicular transport. Results from our lab have also provided evidence that Yck activity is important for vesicular transport, in this case for a yeast endocytic process. When stationary-phase *yck2Δ yck1Δ yck2*-2ts mutant cells (strain YI228) were stimulated to resume cell proliferation at 15°C, a temperature that is permissive for these cells, endocytosis (monitored by the uptake of the fluorescent dye FM4-64) occurred at a much slower rate than in wild-type cells (Wang *et al.*, 1996). The *gcs1Δ* mutant cells (strain Gwk9A), under the same conditions, have a similar but more severe defect in endocytosis. Even though *gcs1Δ* mutant cells were able to take up FM4-64 dye into the cell, no dye was detected in the vacuoles. Therefore, insufficient

Yck activity causes a defect in endocytosis similar to that produced by a *gcs1Δ* mutation. Furthermore, overexpression of Yck2 casein kinase I activity suppresses the *gcs1Δ* endocytosis defect (Wang *et al.*, 1996). These observations strongly suggest that both the Gcs1 activity and the Yck casein kinase I activity are involved in endocytosis.

Interestingly, when YI228 cells (temperature-sensitive for growth due to the *yck2Δ yck1Δ yck2-2*ts mutations) were transferred to the restrictive temperature of 37°C, no obvious immediate endocytic defect was observed (G. C. Johnston, personal communication). One reason for this observation might be that Yck casein kinase I is important for several cellular activities and YI228 cells arrest at the restrictive temperature of 37°C for reasons which are not related to membrane trafficking. It is, however, also possible that this observation is due to the long time needed to manifest the temperature sensitivity of YI228 cells. It takes about 10-14 hours before YI228 cells become arrested for cell proliferation at the restrictive temperature of 37°C, which is much longer than the time for *sec* mutant cells (cells with defects in secretion; Novick and Schekman, 1979). Therefore, the endocytic activity assay conditions based on *sec* mutant cells may not be appropriate for assaying endocytosis in YI228 cells at 37°C, because even at the restrictive temperature the mutant cells go through several generations before they exhibit a growth defect. A long incubation of YI228 cells at the restrictive temperature before assay is also problematic, because after this long time arrested cells could exhibit an endocytic defect due to indirect reasons. Therefore, a faster arresting *yck* mutant strain might be needed for a better assessment of endocytosis under conditions of decreased of Yck activities.

How would increased casein kinase I suppress a decrease in Arf1-GAP activity? Being a protein kinase, Yck casein kinase I is likely to suppress the *gcs1* cold sensitivity by changing the phosphorylation status of a protein substrate. Identifying substrates of Yck casein kinase I that are involved in regulating vesicular transport will certainly help us to understand the function of Yck casein kinase I in this transport process. In

mammalian cells, a synaptic-vesicle-associated casein kinase I isoform has been shown to phosphorylate a subset of vesicle proteins, including SV2, a transmembrane synaptic-vesicle-specific protein (Gross *et al.*, 1995).

While Yck casein kinase I activity is probably involved in vesicular transport as discussed above, other studies suggest that Yck casein kinase I could have other functions in the cell. First, increased dosage of *YCK1* and *YCK2* allows wild-type yeast cells to grow in media of high salinity without prior adaptation (Robinson *et al.*, 1992) and the C-terminal prenylation motif is important for this dosage suppression, suggesting the suppression is mediated by phosphorylation of some membrane proteins, possibly ion channels. Second, increased expression of *YCK1* and *YCK2* weakly suppresses the growth defects of *snf1* and *snf4* mutant cells on media with sucrose or raffinose as sole carbon source (Robinson *et al.*, 1992). Snf1 protein kinase (Celenza and Carlson, 1986) and Snf4 protein (Celenza and Carlson, 1989; Celenza *et al.*, 1989) are necessary for the relief of glucose repression. However, the increased expression of Yck1 and Yck2 does not appear to relieve the glucose repression in *snf1* and *snf4* mutant cells, for the expression of invertase was not induced in those suppressed cells (Robinson *et al.*, 1992). Therefore, it is possible that by modifying the activities of some hexose transporters, increased Yck casein kinase I activity allows the *snf1* and *snf4* mutant cells to utilize low-level glucose more efficiently. Given the wide distribution of casein kinase I activity in the cell, it is possible that casein kinase I could have many different protein substrates and therefore have multiple functions (Vancura *et al.*, 1994).

In both of the above situations it is also likely that increased casein kinase I activity allows cells to change their plasma-membrane activity to adapt to new environments (high salinity and low glucose conditions, respectively). Newly synthesized membrane proteins such as ion channels and hexose transporters rely on the secretory pathway for translocation to the plasma membrane. On the other hand, endocytosis-mediated internalization and degradation of membrane proteins might also

be needed to complete the change of plasma-membrane activity. Similarly, when stationary-phase cells encounter fresh nutrients, changes in plasma-membrane as well as cell-wall activities are needed for cells to increase biosynthesis activities and prepare for activation of the cell-division cycle. Therefore, it is possible that through its activity in vesicular transport, increased casein kinase I activity promotes changes in plasma-membrane activity in all three situations.

4. Casein kinase I and yeast cell morphogenesis

While a function for Yck casein kinase I in vesicular transport has not been previously reported, Yck1 and Yck2 proteins have been shown to be important for yeast cell morphogenesis, which is related to intracellular vesicular transport. When *yck1Δ yck2-2ts* mutant cells are transferred to the restrictive temperature of 37°C, these cells exhibit hyperpolarized bud growth and are also impaired for cytokinesis (Robinson *et al.*, 1993). These morphogenic problems caused by insufficient Yck casein kinase I activity are related to vesicle transport. Yeast cells exhibit polarized membrane growth. Newly synthesized membrane is predominantly delivered to the bud in the form of vesicles, and the isotropic fusion of vesicles with the bud plasma membrane drives the enlargement of the bud (Schekman and Novick, 1982). However, in cells with insufficient Yck casein kinase I activity the normal transition from apical to isotropic bud growth (Lew and Reed, 1993) is impaired, and the buds become elongated, indicating that the normal re-targeting of vesicles is impaired. Interestingly, the punctuate localization pattern of Yck1 + Yck2 at the plasma membrane (Vancura *et al.*, 1994) is similar to that of the yeast cortical actin patches, which have been suggested to play key roles in directing cell membrane and cell wall growth (reviewed in Welch *et al.*, 1994). These observations suggest that the Yck1 and Yck2 activities might also be important for the re-targeting of membrane growth.

The morphogenic defects caused by insufficient Yck casein kinase I activity

resemble those produced by mutations in the *CDC55* gene, which encodes the B regulatory subunit of the protein phosphatase 2A (Healy *et al.*, 1991). Genetic studies suggest that a *cdc55* mutation counteracts the effects of Yck1 and Yck2, perhaps by increasing protein phosphatase 2A activity to dephosphorylate certain casein kinase I substrates (Robinson *et al.*, 1993). In keeping with this view, increasing the Pph22 protein phosphatase 2A catalytic subunit activity also produces hyperpolarized buds (Ronne *et al.*, 1991). One of my findings also supports the notion of balanced casein kinase I and PP2A activity: increased expression of Cdc55 suppresses the *gcs1* mutant phenotype, perhaps through decreasing protein phosphatase 2A activity towards certain casein kinase I substrates. Thus, membrane growth as mediated by casein kinase I may be involved in the suppression of *gcs1* by Cdc55.

5. Two subgroups of yeast casein kinase I enzymes

With the identification of the novel *YCK3* gene as described above and the determination of the entire yeast genome sequence, we now know that there are only four yeast casein kinase I isoforms in yeast. Casein kinase I activity has been found in many eukaryotic systems: four casein kinase I genes have been found in the fission yeast *S. pombe* (*hhp1*$^+$, *hhp2*$^+$, *cki1*$^+$, *cki2*$^+$) (Dhillon and Hoekstra, 1994; Wang *et al.*, 1994) and four casein kinase I isoforms (α, β, γ, δ) have also been isolated from bovine sources (Rowles *et al.*, 1991). However, we do not know whether there are more casein kinase I genes in those two genomes.

Genetic studies revealed that we can divide the four yeast casein kinase I genes into two subgroups. *YCK1* and *YCK2* form an essential gene family (Wang *et al.*, 1992; Robinson *et al.*, 1992), and *YCK3* and *HRR25* form another essential gene family (Wang *et al.*, 1996). However, there is some evidence that these two subgroups of casein kinase I enzymes have overlapping activities. First, increased dosage of *YCK3* or *HRR25* suppresses the *yck1Δ yck2*-2ts temperature sensitivity, indicating that increased Yck3 or

Hrr25 activity can at least to some extent perform the essential Yck1 and Yck2 functions. Second, intracellular-localization studies for these casein kinase I enzymes also suggests that these proteins share some overlapping localization. All four enzymes are mostly associated with particulate fractions: Yck1 and Yck2 are predominantly associated with the plasma membrane (Vancura *et al.*, 1994), while most of the Hrr25 protein is associated with intact nuclei (Vancura *et al.*, 1994), consistent with its role in DNA repair. However, a small portion of Hrr25 is also associated with plasma-membrane fractions. The intracellular localization of Yck3 is similar to that of Hrr25, with about half of the protein associated with intact nuclei, and a small portion associated with plasma membrane. The majority of Yck3 that is not associated with nucleus is also not associated with plasma membrane (Wang *et al.*, 1996), indicating that Yck3 only minimally overlaps with Yck1 and Yck2 in intracellular localization. The observation that increased dosage of *YCK3* or *HRR25* can suppress the *yck1Δ yck2-2*ts temperature sensitivity supports the notion that the small portion of Yck3 and Hrr25 associated with plasma membrane could supply Yck1 and Yck2 activity. Therefore the two subgroups of yeast casein kinase I isoenzymes indeed share some common function.

The ubiquitous existence of casein kinase I activity in various eukaryotic systems and the wide distribution of casein kinase I enzymes inside a cell indicate that the casein kinase I activity is important for many cellular processes. Because two subgroups of yeast casein kinase I enzymes are mainly located in different parts of the cell, it is likely that the two groups of casein kinase I enzymes normally recognize different protein substrates and regulate different cellular processes, with only limited overlapping functions.

6. Yck3 is a novel casein kinase I isoform

While Yck3 parallels Hrr25 in intracellular localization and in function, Yck3 has several distinctive characteristics that suggest Yck3 could have functions different than those of

Hrr25. First, when compared to the protein kinase domains of Yck1, Yck2, and Hrr25, the protein kinase domain of Yck3 contains three insertions, which might allow Yck3 to interact with some other regulatory or effector molecules (Wang *et al.*, 1996), indicating that its function might be regulated differently to that of other casein kinase I enzymes. Second, Yck3 has a C-terminal lipid-modification motif, which is absent in Hrr25 and more complex than those of Yck1 and Yck2. While Yck1 and Yck2 terminate with a CC motif, which is a consensus sequence for geranylgeranylation (Wang *et al.*, 1992), Yck3 terminates with a CCCCFCCC motif, which could contain many potential prenylation motifs. Therefore, the lipid modification of Yck3 might differ from that of Yck1 and Yck2, which might ultimately contribute to the different intracellular localization patterns of those casein kinase I enzymes. Third, while increased dosage of *YCK3* suppresses *gcs1* cold sensitivity, increased dosage of *HRR25* does not, which suggests that Yck3 does not totally overlap with Hrr25 in function. These novel characteristics suggest that Yck3 might have its own distinctive functions inside the cell besides having overlapping functions with Hrr25 or the Yck1 + Yck2 casein kinase I enzymes.

7. Ypt31 and Ypt32 as multicopy suppressors of *gcs1* cold sensitivity

While the function of Yck casein kinase I in vesicular transport has yet to be further characterized, two other copy suppressors of *gcs1*, Ypt31 and Ypt32, have clearly been implicated in regulating intracellular vesicular transport. Growing biochemical and genetic evidence indicates that different members of the Ypt/Rab protein family have unique cellular functions and cannot replace each other (Novick and Brennwald, 1993). For example, the yeast Sec4 protein is essential for the Golgi-to-plasma-membrane vesicular transport (Salminen and Novick, 1987), and the Ypt1 protein is essential for the ER-to-Golgi and intra-Golgi vesicular transport (Segev *et al.*, 1988). Increased Sec4 does not compensate for the loss of Ypt1 activity, and increased Ypt1 does not compensate for the loss of Sec4 activity (Brennwald and Novick, 1993). Consistent with

unique cellular functions, different members of the Ypt/Rab family are localized to different intracellular membrane compartments, and increased expression of Ypt/Rab proteins does not lead to their mislocalization (Gorvel *et al.*, 1991; Brennwald and Novick, 1993). Mutations in the *SEC4* gene show strong genetic interactions with a group of *sec* mutations affecting post-Golgi vesicular transport, and increased Sec4 activity suppresses the secretion and growth defects caused by the same group of *sec* mutations (Salminen and Novick, 1987). These observations suggest that increased activity of a Ypt/Rab protein would probably affect only the vesicular transport process in which it is normally involved. Therefore, the effects of increased Ypt31 and Ypt32 activity, in my case suppressing the *gcs1* cold sensitivity, could reflect their normal functions.

Gallwitz's group found that yeast cells lacking Ytp31 and Ypt32 activity accumulate Golgi stacks similar to those found in *sec7* mutant cells, and also accumulate ER- and Golgi-modified forms of carboxypeptidase Y (D. Gallwitz, personal communication), indicating that Ypt31 and Ypt32 are involved in intra-Golgi membrane transport (between *cis*- and *trans*-Golgi comparments). Yeast Arf1 and Arf2 have also been implicated in vesicular transport (Stearns *et al.*, 1990a, b), as *arf1Δ* mutants have defects in processing secreted invertase and cells accumulate partially Golgi-modified forms of this enzyme. The genetic interactions shown between *gcs1Δ* and *arf1Δ* mutations (Poon *et al.*, 1996), between *arf1Δ* and *ypt32Δ ypt31*cs mutant situations, and between *gcs1Δ* and *ypt32Δ ypt31*cs mutant situations (see Results) suggest that Gcs1, Arf1 and Ypt31 + Ypt32 activities probably affect a common stage in intracellular vesicular transport.

While both Ypt31 and Arf1 are small GTP-binding proteins involved in regulating vesicular transport, they play different role in this process. Arf1 has been shown to be important for the formation and subsequent decoating of coated vesicles (Donaldson and Klausner, 1994). On the other hand, genetic and biochemical evidence indicates that Ypt/

Rab proteins are important for the docking and/or fusion of vesicles with target membranes: for example, ER-derived vesicles cannot dock at *cis*-Golgi membranes in *ypt1* mutant cells because of a failure in forming the docking protein complexes (Sogaard *et al.*, 1994), or they are important for activating v-SNAREs (Lian *et al.*, 1994). However, the exact role for Ypt/Rab proteins in the vesicle docking is not yet known, and Ypt/Rab proteins have not been found in purified docking protein complexes. Regarding my observation that increased Ypt31 and Ypt32 activity suppresses a decrease in Arf1-GAP (Gcs1) activity, it is possible that the increased Ypt31 and Ypt32 activity is either directly involved in the decoating process or activates the docking of vesicles in the absence of the usual vesicle decoating.

Although Arf1 has been shown to have Golgi-related activities (Helms *et al.*, 1993; Tanigawa *et al.*, 1993), recent findings indicate it could be involved at more than one stage of vesicular transport. In yeast, Arf1- and COPI-coated vesicles bud directly from the endoplasmic reticulum (Bednarek *et al.*, 1995), suggesting Arf1 and COPI are involved in the ER-to-Golgi vesicular transport. COPI, which associates with Arf1 during the formation of coated vesicles, has also been shown to be important for a retrograde vesicular transport process that retrieves ER-resident proteins back from the Golgi apparatus (Letourneur *et al.*, 1994), suggesting that COPI and Arf1 are important for cycling of proteins involved in ER-to-Golgi vesicular transport. In mammalian cells, Arf1 but not COPI has been shown to be important for secretory-vesicle biogenesis from the *trans*-Golgi network (Barr and Huttner, 1996), indicating a possible role for Arf1 in post-Golgi vesicle transport. Interestingly, the mammalian Ypt31 homologue Rab11 has also been found to be associated with *trans*-Golgi network (TGN) and TGN-derived secretory vesicles (Huber *et al.*, 1993), indicating a role for Rab11 in post-Golgi vesicular transport as well. In yeast, it is not known whether Gcs1, Ypt31, or Arf1 functions in more than one stage of intracellular membrane trafficking, and a further characterization of their localizations as well as their functions is needed before we can understand the

genetic interactions of their activities.

8. Gcs1-like open reading frames (GLOs)

Essential gene families are common in yeast. Within an essential gene family, any one gene product is not essential for cell growth, while the loss of all members of a gene family is lethal. For example, *YCK1* and *YCK2* form an essential gene family, for cells that lack either one of the genes are normal for cell growth, but the deletion of both genes is lethal. Yck1 and Yck2 are about 75% identical in sequence, and have similar intracellular localization and functions (Wang *et al.*, 1992; Robinson *et al.*, 1992). Since *GCS1* is not an essential gene but provides an essential function, a second Arf1-GAP activity is needed for cell growth. It is therefore possible that the *GCS1* gene also belongs to an essential gene family, and that other gene products within the family could supply GAP activity.

I performed a BLASTp search against the yeast genome databank and found that there are 6 different ORFs that encode proteins containing the hallmark Gcs1 Zn-finger motif and are similar in size to Gcs1: they are *GCS1*, *SPS18* (Coe *et al.*, 1992), *GLO3* (Ireland *et al.*, 1994), *YIE4*, *GLO5* (U33057), and *GTS1* (Mitsui *et al.*, 1994). The sequences of Yie4 and Glo5 were revealed by the genome project and the functions of those two gene products have yet to be characterized. Gts1 has been suggested to have a role in the of timing bud formation, for *gts1* mutant cells have a delay in forming the bud (Mitsui *et al.*, 1994). Sps18 and Glo3 are more similar to Gcs1 than are the other proteins, and are about 30% identical in sequence to Gcs1. Sps18 is a meiosis-specific protein, and is not expressed in mitotic cells (Coe *et al.*, 1992). However, it is not known whether Sps18 is expressed in *gcs1Δ* mutant cells; therefore we can not exclude the possibility that Sps18 replaces Gcs1 activity in mitotic *gcs1Δ* mutant cells. Nothing else is known regarding the function of Sps18. In contrast, Glo3 (Ireland *et al.*, 1994) has been implicated in vesicular transport, for *glo3Δ* cells have a defect in processing

secreted invertase (P. Poon, personal communication), and multicopy suppressors of *glo3Δ* include v-SNAREs (Sec22, Bet1, and Bos1; V. Saunders, personal communication) that are involved in ER-to-Golgi vesicle transport (Newman *et al.*, 1990). In a W303 genetic background, a *glo3Δ* mutation causes cold sensitivity, and haploid spores bearing both the *glo3Δ* and *gcs1Δ* mutations are not viable (P. Poon, personal communication), while in a 21R genetic background, synthetic enhancement of impairment was observed between a *gcs1Δ* mutation and the *glo3Δ yck3Δ* mutations (Figure 7). These observations indicate that Glo3 and Gcs1 activity are involved in a common cellular process; therefore Glo3 may be a good candidate for GAP2. However, unpublished results from our lab have also shown that Glo3 and Gcs1 do not form a "conventional" essential family like Yck1 and Yck2, or Arf1 and Arf2. First, Glo3 and Gcs1 failed to complement each other in function. Increased expression of Gcs1, either from a multicopy plasmid or from the *GAL1-10* promoter, did not suppress *glo3Δ* cold sensitivity; similarly, increased expression of Glo3 did not suppress *gcs1Δ* cold sensitivity (P. Poon, personal communication). Second, multicopy suppressors of *gcs1Δ* did not suppress *glo3Δ* cold sensitivity and, *vice versa*, multicopy suppressors of *glo3Δ* did not suppress *gcs1Δ* cold sensitivity (D. Carruthers, personal communication). Third, multicopy suppressors of *gcs1Δ* are likely to be involved in intra-Golgi or post-Golgi membrane transport, while multicopy suppressors of *glo3Δ* are involved in ER-to-Golgi vesicle transport (V. Saunders, personal communication). These observations suggest that while Gcs1 and Glo3 do affect some common features of intracellular membrane trafficking, as indicated by the strong genetic interaction, they probably also function at different stages of vesicular transport as well.

It is important to determine whether Glo3 has Arf1-GAP activity *in vitro*, and if so then Glo3 would also be an Arf1 GAP. The genetic interactions observed between *gcs1Δ* and *glo3Δ* mutations would therefore suggest that while Glo3 and Gcs1 do belong to the essential Arf1-GAP family, they mainly function at different stages of vesicular

transport with some overlapping activity. As discussed above, Arf1 has been implicated in a retrograde Golgi-to-ER vesicular transport process, in intra-Golgi membrane trafficking, and in secretory vesicle biogenesis from the *trans*-Golgi network. It is therefore possible that at those different stages of vesicular transport, different Arf1 GAPs are used for Arf1-GTPase activity. On the other hand, if Glo3 does not have Arf1-GAP activity it is possible that Glo3 is a GAP for other Arf-like proteins that overlap with Arf1 + Arf2 in function.

It would also be interesting to determine whether other Gcs1-like proteins also have GAP activity towards Arf1 and Arf2 or whether they are GAPs for other Arf-like proteins, since all these Gcs1-like proteins contain the putative Zn-finger domain that is important for Gcs1 Arf1-GAP activity. The yeast genome contains seven Arf-like proteins (including Arf1, Arf2, Arf3, Arl1 and Sar1) and, given the complexity of the vesicular transport machinery, it is likely that different Arf proteins are involved in different stages of vesicular transport and that different Arf proteins have different GAPs. For example, Sar1 is important for the formation of COPII coated vesicles in the ER-to-Golgi vesicular transport and a subunit of the COPII protein complex, Sec23, has been shown to have GAP activity towards Sar1 but not Arf1. However, it is not yet known whether Sar1 is also a substrate for Gcs1-like GAP activity, or whether the COPI protein complex has GAP activity for Arf1. Studying the functions of these Gcs1-like proteins will help us to further understand the regulation of intracellular vesicular transport.

9. The relationships among Gcs1, Ypt31, Imh1 and Yck1

This study not only found that increased activity of each of seven different proteins suppresses a defect in Arf1-GAP, but also provided genetic evidence that the cellular functions of all these proteins are related. Ypt31 and Yck1 are multicopy suppressors of the *gcs1Δ* cold sensitivity, and this suppression, as discussed above, is mediated through another Arf1 GAP. Therefore, increased Ypt31 or Yck1 activates a parallel pathway, in

which another Arf1 GAP (GAP2) is a component, that bypasses the need for Gcs1 activity. It is likely that both Ypt31 and Yck1 function downstream of this GAP2 activity, unless increased Ypt31 or Yck1 abundance directly activates the hydrolysis of Arf1-GTP, which is not likely given what is known about the functions of the Ypt/Rab proteins and casein kinase I. The observations that increased Gcs1 did not suppress either the *yck1Δ yck2-2*ts temperature sensitivity or the *ypt32Δ ypt31*cs cold sensitivity showed that increasing overall Arf1-GAP activity does not compensate for the loss of Ypt31 and Yck2, suggesting also that the Arf1-GAP activity might function upstream of Ypt31 and Yck1, since increasing an upstream activity might not rescue defects in downstream activities.

The observation that increasing the activities of both Yck2 and Ypt31 suppressed *gcs1* cold sensitivity better than did increasing the activity of either protein alone suggests that Ypt31 and Yck2 + Yck1 probably activate a common pathway, for activating a common process at two different steps might allow the process to take place more efficiently than activating the process at just one step. Increased Ypt31 activity did not suppress the *yck1Δ yck2-2*ts temperature sensitivity, while increased Yck1 activity suppresses the *ypt32Δ ypt31*cs cold sensitivity. This observation indicates that Yck2 probably functions downstream of Ypt31.

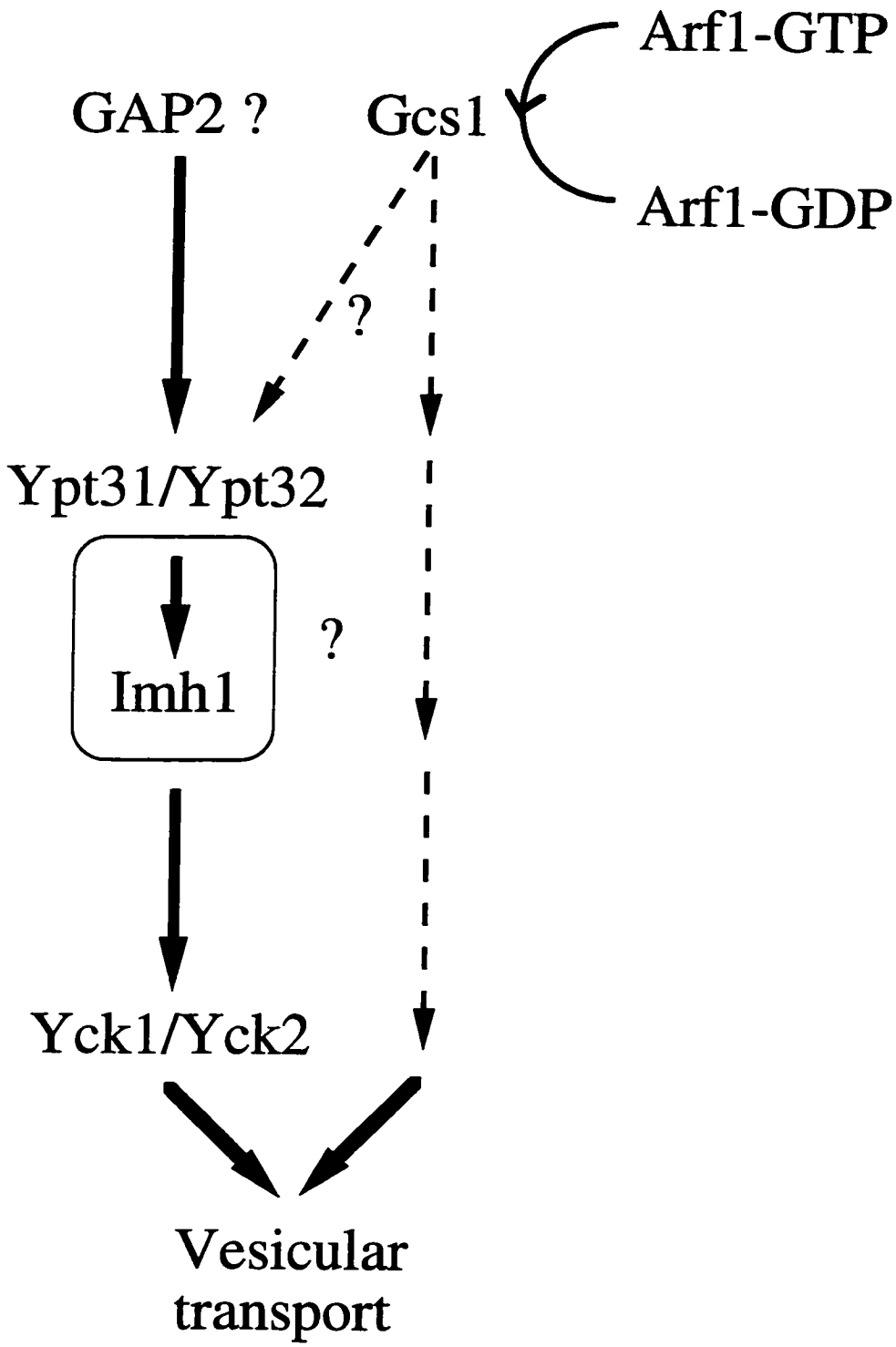
The structural similarities between Imh1 and Rabaptin-5 (Stenmark *et al.*, 1995), an effector of Rab5, suggest that Imh1 might also be an effector for a Ypt/Rab protein. Since increased Imh1 activity suppresses the *gcs1* cold sensitivity just like the increased Ypt31 does, it is certainly possible that Imh1 could be a direct effector of Ypt31. To test this hypothesis, I plan to apply a yeast two-hybrid approach (Chien *et al.*, 1991) to investigate whether Imh1 can interact physically with the GTP-bound form of Ypt31, analogously to the interaction of Rab5 and Rabaptin-5 (Stenmark *et al.*, 1995). Wild-type Rab/Ypt proteins have intrinsic GTPase activity, so the GTP-bound form of a Rab/Ypt protein can be naturally converted to its GDP-bound form. Therefore to test whether

Imh1 interact specifically with the GTP-bound form of Ypt31 it would be useful to see whether Imh1 interacts with a Ypt31 GTPase-deficient mutant protein, which is predominantly in the active GTP-bound form. As discussed above, the Ypt31-Q72L protein has a substitution analogous to that in Rab5Q79L, which is a GTPase-deficient mutant protein (Stenmark *et al.*, 1995); therefore Ypt31-Q72L could also be a persistently active Ypt31 protein suitable for testing interaction with Imh1. On the other hand, the Ypt31-S27A protein, which has a substitution similar to that in Rab5S34N that results in an inactive Rab5 protein (Stenmark *et al.*, 1995), could be an inactive Ypt31 protein and also a good control for testing whether Imh1 can interact with the GDP-bound form of Ypt31. To test the binding of the Imh1 protein to different forms of Ypt31, wild-type *YPT31*, *ypt31-S27A*, and *ypt31-Q72L* have been cloned into plasmid pGAD424 to generate fusion proteins that have the Gal4 transactivation domain fused to the corresponding Ypt31 protein, and DNA sequences encoding most of the Imh1 protein have been cloned into plasmid pGBT9 to generate a fusion protein that has the Gal4 DNA-binding domain fused to Imh1. The pGBT9-*CSG1* plasmid and one of the pGAD424-*YPT31* plasmids will then be co-transformed into strain YI53, that lacks endogenous Gal4 protein, so that Gal4 activity can only be restored if there is an interaction between Ypt31 and Imh1 that brings the Gal4 DNA-binding domain and activation domain together. The Gal4 activity, as indicated by expression from the *GAL1-10* promoter, can be monitored by the expression of the reporter protein β-galactosidase. These planned experiments might identify a possible interaction between Imh1 and one form of the Ypt31 protein. At the time of this writing this work is still in progress, so the relationship between Ypt31 and Imh1 remains undefined.

As summarized in Figure 30, regarding the activities that lead to the suppression of *gcs1* cold sensitivity, I propose that a second Arf1 GAP (GAP2) functions upstream of Ypt31 + Ypt32, with Imh1 as a possible direct downstream effector of Ypt31 + Ypt32 and the Yck1 + Yck2 casein kinase I enzyme functioning further downstream of Ypt31.

Figure 30: Relationships among Ypt31, Imh1, Yck1 and the Arf1-GAP activities.

The hydrolysis of GTP changes Arf1 from its GTP-bound form to its GDP-bound form (as indicated by a curve and an arrow), and this process is activated by GTPase-activating proteins Gcs1 and a possible (indicated by "?") GAP2. The GAP2 functions upstream of Ypt31 + Ypt32 (as indicated by the arrow); Imh1 is possibly (indicated by "?") a direct downstream effector of Ypt31 + Ypt32; and Yck1 + Yck2 functions further downstream of Ypt31 + Ypt32.


Arf1-GTP
GAP2 ?
Gcs1
Arf1-GDP
?
Ypt31/Ypt32
Imh1
?
Yck1/Yck2
Vesicular
transport

As discussed above, this GAP2-mediated pathway should be considered to be parallel with a Gcs1-mediated pathway. However, since GAP2 and Gcs1 perform the same function in activating Arf1 GTPase activity, they may share the same downstream partners. While many of the observations presented in this thesis could be explained by alternative models, the overall results fit better with this working model (Figure 30). To understand the mechanisms by which these multicopy suppressors alleviate *gcs1* cold sensitivity, further studies characterizing the functions of each component of this pathway are needed.

Regarding the relationships among Gcs1, Ypt31, Yck2 and Imh1, an important question yet to be addressed is at which stage(s) of intracellular vesicular transport do we see the genetic interactions of these proteins. The indications that Arf1 is involved in several stages of vesicular transport does not necessary mean that Gcs1, Ypt31, Imh1 and Yck2 function in all these several stages. Since each Rab/Ypt protein is found at a particular stage of a vesicular transport pathway (Novick and Brennwald, 1993), it is unlikely that Ypt31 and Ypt32 function at all these stages of vesicular transport like Arf1 does. As discussed above, the existence of other Gcs1-like proteins makes it also possible that there are several Arf1 GAPs, and each GAP is important for one particular stage of vesicular transport. On the other hand, the Gcs1 Arf1 GAP may well participate in several stages of vesicular transport just like Arf1 does, and if so the suppression of *gcs1Δ* cold sensitivity by Ypt31 would suggest that even though the absence of Gcs1 could potentially cause defects in several stages of vesicular transport, the associated growth defect can be alleviated by boosting Ypt31 activity associated with one certain stage of vesicular transport. Although this hypothesis might sound a little strange, recent findings of Arf1 functions suggest that it is a possibility. As discussed above, while both COPI and Arf1 have been shown to be important for the ER-to-Golgi and intra-Golgi vesicular transport (reviewed in Donaldson and Klausner, 1994), only Arf1 but not COPI is needed for the biogenesis of secretory vesicles in the TGN (Barr and Huttner, 1996),

suggesting that Arf1 may interact with different "coats" or does not interact with any coat at certain stages of membrane trafficking. Therefore, the absence of one Arf1 GAP (Gcs1) could certainly produce different effects at those different stages of vesicular transport, especially if COPI and those other coat proteins have different GAP activities towards Arf1 (as discussed above, the Sec23 component of COPII coat has GAP activity towards the Arf1-like protein Sar1). Therefore, future studies on the functions and localization of Gcs1 as well as Arf1 are needed before we can have a clearer understanding of Gcs1 function in vesicular transport, and the identification of *gcs1* multicopy suppressors will certainly help us to tackle the question from several different angles.

10. Some thoughts on Gcs1 and the resumption of cell proliferation from stationary phase

A) Gcs1 is active in proliferating cells

gcs1 mutant cells are impaired only for the resumption of cell proliferation from stationary phase, and then only when these *gcs1* mutant cells are stimulated at the restrictive temperature of 15°C. On the other hand, actively proliferating *gcs1* mutant cells can continue to proliferate indefinitely when transferred to 15°C as long as they are supplied with fresh nutrients (Drebot *et al.*, 1987; Ireland *et al.*, 1994). At first glance, this mutant phenotype could imply that the Gcs1 protein is only active when stationary-phase cells are stimulated to resume cell proliferation, while actively proliferating cells do not have Gcs1 activity at all. However, results from our lab suggest that this is not the case. First, Gcs1 is expressed in proliferating cells, while its expression level is low in stationary-phase cells (Ireland *et al.*, 1994). Second, genetic interactions shown between *gcs1Δ* and *arf1Δ* mutations (Poon *et al.*, 1996), and between *gcs1Δ* and the *yck2Δ yck1Δ yck2*-2ts mutant situations (Figure 2), indicate that Gcs1 activity is present in proliferating cells and at temperatures higher than 15°C; the loss of Gcs1 activity

enhanced the impairment caused by an *arf1Δ* or by the *yck2Δ yck1Δ yck2*-2ts mutations. Therefore, Gcs1 is not only important for stationary-phase cells to resume cell proliferation, but is also active for the maintenance of cell proliferation. As discussed above, the cold sensitivity during the resumption of cell proliferation by *gcs1Δ* cells is caused by an inherently cold-sensitive GAP2-mediated parallel pathway. When stationary-phase *gcs1Δ* cells are stimulated to resume cell proliferation at a permissive temperature of 29°C, the GAP2 pathway is able to mediate vesicle transport, which is needed for the cells to resume cell proliferation. On the other hand, when stationary-phase *gcs1Δ* cells are stimulated to resume cell proliferation at the restrictive temperature of 15°C, the GAP2 pathway, enfeebled by the cold, fails to supply adequate activity for vesicle transport. The difference between stationary-phase and actively proliferating cells in the requirement for Gcs1 at the low temperature of 15°C may also be due to an insufficient GAP2 activity in stationary-phase cells. Given the fact that the levels of most of the proteins present in proliferating cells decrease when cells enter stationary phase (Choder, 1991), GAP2 levels may well decline similarly.

B) Stationary-phase cells rely on membrane trafficking to adapt to fresh medium

Yeast cells respond to nutrient starvation by entering stationary phase, so that they can survive long periods of nutrient limitation until they encounter a favorable environment and resume cell proliferation. To do so, stationary-phase cells acquire physiological characteristics that are different to those of proliferating cells. Accordingly, stationary-phase cells exhibit patterns of gene expression different from those of proliferating cells. Upon the stimulus brought upon by the addition of fresh medium, stationary-phase cells change their gene-expression pattern, lose stationary-phase characteristics, and resume cell proliferation. This reorganization of cell activities relies on vesicular transport to translocate newly synthesized proteins to proper membrane locations, to degrade stationary-phase-specific proteins, and to modify plasma-membrane and cell-wall

activities.

While *gcs1* mutant cells fail to resume cell proliferation at the restrictive temperature of 15°C when fresh nutrients are resupplied, they are able to lose some of their stationary-phase characteristics and change their gene-expression pattern (Drebot *et al.*, 1987; Ireland *et al.*, 1994). This observation is consistent with the idea that a defect in vesicular transport causes *gcs1* mutant cells to fail to reorganize cellular activities to resume cell proliferation. As discussed above, stationary-phase cells have a different cell-wall structure to that of actively proliferating cells and this difference in cell-wall structure is mainly caused by the difference in mannoprotein structure. Newly synthesized mannoprotein relies on the vesicular transport machinery to be correctly modified and transported to the cell surface. Also as discussed above, changes in extracellular environment would cause cells to change their plasma-membrane properties as well. Different hexose transporters and ion channels might be utilized by cells to adapt to a new environment, and all those plasma-membrane proteins rely on the vesicular transport machinery for translocation to the cell surface. Based on the close relationship between vesicular transport and the yeast cell growth, it is conceivable that intracellular vesicular transport is necessary for nutrient-starved stationary-phase cells to adapt to fresh growth medium and resume cell proliferation.

11. Conclusions

The *gcs1* mutant phenotype provides genetic evidence that stationary phase is a unique developmental state. The identification of multicopy suppressors of the *gcs1* mutant phenotype helped us to further understand the biological activities needed for quiescent cells to resume cell proliferation. The finding that Gcs1, as a yeast Arf1-GAP, functions in the intracellular vesicular transport, highlights the importance of the vesicular transport machinery in reorganizing cellular activities for stationary-phase cells to resume cell proliferation. This study isolated new components that are involved in the vesicular transport process, and provide the basis for future studies of their functions.

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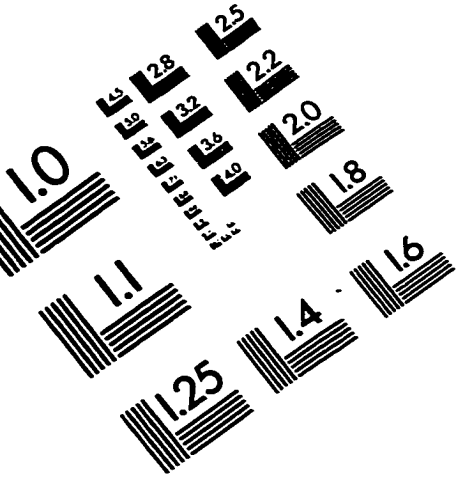
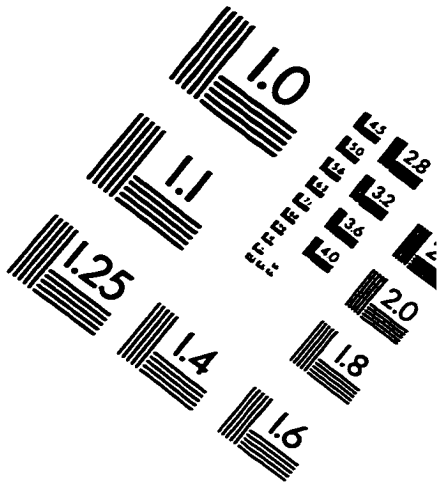
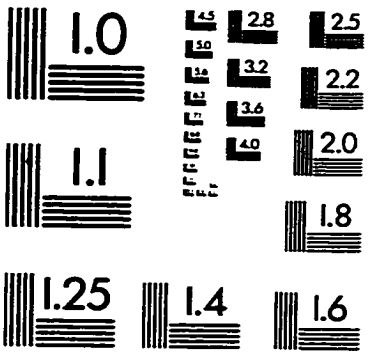
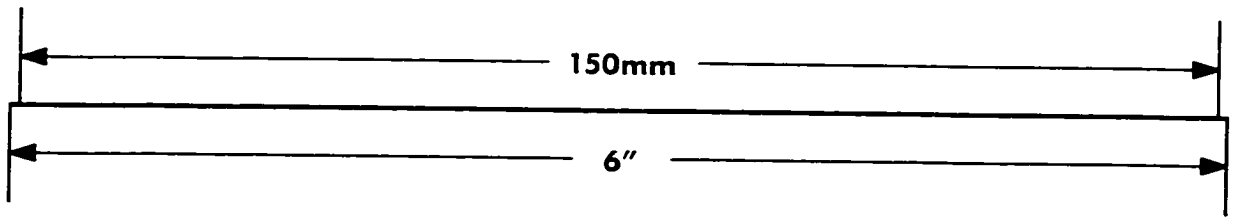
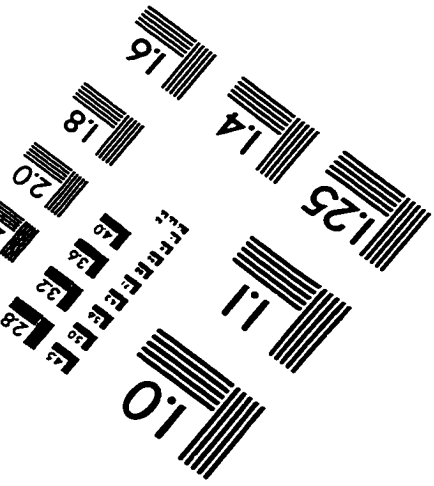
IMAGE EVALUATION TEST TARGET (QA-3)

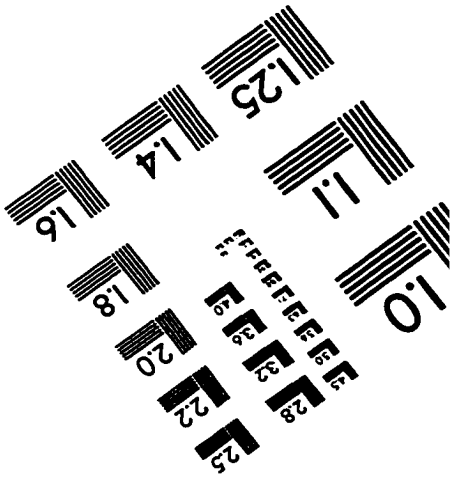












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