# $SCF^{Grr1}$ -Mediated Ubiquitination of Gis4 Modulates Glucose Response in Yeast

By

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CERTIFICATE OF APPROVAL

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***Abstract***

The cell cycle depends upon proper nutrient sensing and metabolism. Grr1 is a yeast protein that regulates the stability of the G1 phase cyclins and whose mutant phenotype is strongly implicated in nutrient uptake. My work is centered around the discovery that Gis4, a previously uncharacterized protein, is a novel substrate of Grr1 and provides insight as to how Grr1 affects pathways responsible for carbon source metabolism.

I show that Gis4 interacts with, and is ubiquitinated by $SCF^{Grr1}$, but that ubiquitinated Gis4 is a stable protein. Gis4 protein levels correspond directly to Grr1 protein levels and the Gis4 protein exists as three discrete bands, none of which appear to be ubiquitinated. The ubiquitinated species only appear above the highest molecular weight form of these three bands.

Gis4 is particularly interesting compared to other substrates of Grr1 in that it does not appear to undergo the classic ubiquitination-degradation pathway. All other known substrates of Grr1 are rapidly degraded upon ubiquitination by $SCF^{Grr1}$. The stability of ubiquitinated Gis4 suggests that an F box protein does not confer a specific fate to each of its substrates; rather that fate can depend on the substrate itself.

This work also sheds light on the role Grr1 plays in glucose metabolism and Snf1-dependent transcripts. Specifically, Gis4 interacts with Snf1 and is able to alter the activity of Snf1 kinase and Snf1-

dependent transcripts. Gis4 interacts with Snf1, but not in the absence of Grr1, or in the absence of a functional SCF complex. This indicates that ubiquitination is a key event for the Snf1-Gis4 interaction to occur. Furthermore, over-expression of Gis4 activates the Snf1 kinase complex and deletion of Gis4 prevents de-repression of Snf1-dependent transcripts under de-repressing conditions.

These results implicate Gis4 in linking the $SCF^{Grr1}$ glucose induction pathways and the Snf1-complex glucose repression pathways and suggest the following model. Gis4 is ubiquitinated by the $SCF^{Grr1}$ ubiquitin ligase and ubiquitinated Gis4 is then able to associate with the Snf1 kinase complex and increase its activity. Active Snf1 kinase causes the de-repression of the glucose-repressed transcripts SUC2 and CYC1, and may also affect other genes.

Identification of Gis4 furthers our understanding of how Grr1 appears to affect the glucose induction pathways and ultimately gives further insight into the role of E3 ubiquitin ligases in modulating protein activity.

# Chapter 1

# Introduction

## Ubiquitination, an overview

Understanding protein degradation, particularly ubiquitination-dependent degradation, is important to understanding cellular physiology. Nearly all mammalian proteins are degraded at some point, though their rates of degradation vary. Eighty percent of protein degradation occurs through the ubiquitin-proteasome system; additionally, a portion of the remaining 20% degraded via the lysosome is also ubiquitinated [1]. Effective degradation is critical to a variety of pathways. Its role in the cell cycle is well documented because miscues in ubiquitination contribute to proliferative diseases such as cancer.

Ubiquitination is the process by which the small, 76-amino-acid-protein ubiquitin is post-translationally attached to another protein. Ubiquitin is highly conserved among species; it is expressed from yeast to humans. Ubiquitin is unique in that its C-terminal residue, G76, attaches to a lysine residue either on a substrate or on other ubiquitin molecules. The addition of one or more ubiquitins alters the activity or stability of the protein.

A ubiquitin-activating enzyme (E1) activates ubiquitin by forming an ATP-dependent thio-ester linkage between a cysteine on the E1 and the ubiquitin molecule. The E1 enzyme interacts with a ubiquitin-conjugating enzyme (E2), and the ubiquitin molecule is passed from the E1 to a cysteine on the E2. The activated ubiquitin molecule is now

primed for attachment to a substrate. The E2 enzyme associates with an E3 or ubiquitin ligase, which selects the substrate to be ubiquitinated. Ubiquitin ligases can then aid in the conjugation of the ubiquitin molecule to a lysine residue on the substrate. (Reviewed in [2]) (Figure 1.)

Most eukaryotes have a single E1 enzyme, several E2's and a large number of E3's. This allows for the selection of a vast number of targets for ubiquitination through combinatorial control. [3] Additionally, this dictates that ubiquitination affects a wide variety of physiological reactions within a cell and serves as a potential area for therapeutics for a variety of diseases, particularly cancer. [1] However, to date a variety of substrates have been identified for many E3-ligases, but many more ligases and substrates have yet to be discovered. Additionally, while there are some clues as to how substrates are selected, the mode of how substrates are targeted for ubiquitination is unknown. Further investigation of binding will contribute to the effectiveness of therapeutics with regard to E3-ligases.

*Ubiquitin Ligases*

There are four defined types of ubiquitin ligases: the RING finger family, the HECT family, the U box family, and the PHD family (Figure 2). Each contains its own functional region for ubiquitin transfer to its substrate. The HECT and RING families are well described, but the U

box and PHD families are recent additions and comparatively little is known about these families.

A ubiquitin ligase with a RING (*R*eally *I*nteresting *N*ew *G*ene) finger domain does not accept the ubiquitin molecule onto itself; rather, it coordinates the passing of the ubiquitin molecule from the E2 enzyme directly to the substrate. The RING finger is defined by its zinc-binding region, which contains conserved coordinating cysteine and histidine residues. RING domain ligases can exist as a single subunit (e.g. Mdm2 and BRCA1) or as a multi-subunit complex (e.g. the SCF and Anaphase Promoting (APC/C) complexes). All known proteins with cullin homology domains (found in the SCF and APC/C E3 complexes) can assemble with the RING domain, suggesting a conserved core for these ubiquitin ligases.

By contrast to the RING domain family, the HECT (*H*omologous to *E*6-AP *C*-*T*erminus) domain-containing ligase can accept the ubiquitin molecule from the E2 onto its own cysteine, which it can then pass on to its substrate (Reviewed in [2]). HECT E3s appear to bind the substrate through the N-terminus, whereas the C-terminus contains the conserved cysteine for ubiquitination transfer. This means that the mechanism of ubiquitination is different in different families of ligases even though the end result remains similar. The first of these E3 ligases to be described was E6-AP; it ubiquitinates and targets p53 for rapid degradation [1].

The U box family of ligases, which is less well described than the others, contain the 75-amino acid U box domain. The domain is similar to the RING-finger domain, except that it lacks the zinc-coordinating cysteine residues [4]. This family is predicted to be particularly small; there are 468 predicted RING proteins, but only 19 predicted U box proteins in the human genome [4]. The canonical U box ligase, Ufd2, has been described as having E4 activity *in vitro*, but no *in vivo* substrates have been described to investigate this theory.

Another U box ligase, CHIP, has chaperone-associated E3 activity and is responsible for ubiquitinating substrates that require Hsc70 or Hsp90 for maturation [4]. This makes CHIP an interesting cancer target, because it is shown to mediate a "degradative pathway for abnormal proteins" that can impact the "development and/or aggressiveness of several types of adenocarcinomas, including breast and ovarian cancers via the proto-oncogene HER2/Neu. The CHIP ubiquitin ligase controls both the association of Hsp90 chaperones with HER2 and its down-regulation induced by Hsp90 inhibitors, supporting the hypothesis that modulation of substrate-specific E3 ligases can lead to targeted tumor inhibition." [1].

The PHD (Plant Homeodomain) family of proteins, like the U box family, resembles the RING finger domain; it is a specialized zinc-finger motif. It is best described for MEKK1 and its ubiquitination of ERK1/2. The PHD domain has also been described in the Kaposi's sarcoma-

associated MIR (modulator of immune recognition) proteins that ubiquitinate MHC (major histo-compatibility) class I proteins.

**Function of ubiquitination**

Ubiquitination causes the status of a substrate to change. In some cases, the protein is degraded by the 26S proteasome. Alternatively, the activity or localization of the protein may change. This depends on the type of ubiquitination that occurs—either mono- or poly-ubiquitination (also known as chain elongation). Mono ubiquitination, the attachment of a single ubiquitin molecule to one or more lysines, can be a signal for receptor internalization, vesicle sorting, DNA repair, and gene silencing. For example, under mitomycin C treatment (a DNA damaging agent that causes double strand breaks by cross-linking DNA), FANCD2 becomes mono-ubiquitinated. Mono-ubiquitinated FANCD2 is then targeted to the chromatin-associated nuclear foci where it interacts with a variety of DNA repair enzymes. (Reviewed in [5])

In contrast, poly-ubiquitination adds multiple ubiquitin molecules to lysines to form elongated chains. Specifically, there are seven lysines in ubiquitin upon which ubiquitination can occur: position (K)6, 11, 27, 29, 33, 48, and 63 [5, 6]. However, substrates degraded by the proteasome that have been discovered to date are generally thought to be ubiquitinated using chains formed through K48, or possibly K63 [6]. Substrates ubiquitinated by chains formed with K63 are implicated in

DNA repair, translational control, endocytosis, and protein kinase activation. [5, 6]. Ubiquitin chains formed with K29 are thought to direct substrates for proteasomal degradation, but perhaps by recruiting a novel factor [7]. The chains formed using the alternative lysines have as yet undefined consequences.

Classically, chains formed using K48 target the protein for destruction by the 26S proteasome. This chain form is the most abundant in proteins studied so far [5, 6]. The proteasome is a large, multi-subunit complex of proteases that exists in the nucleus and the cytoplasm to digest the ubiquitinated substrate. The proteasome requires a chain of at least four to five ubiquitin molecules linked in tandem by K48 to accept the substrate for degradation [8, 9].

As the chains using non-K48 linkages become more defined, our understanding of the classically defined function of K48 chains becomes less clear. For example, recently, Flick and colleagues [10] suggested that proteins ubiquitinated with K48 linkages may not always lead to degradation. They demonstrate that Met4 ubiquitination by $SCF^{Met30}$ regulates Met4 activity in a proteolysis-independent manner. Mass spectrometry of mutant ubiquitin molecules reveals that the ubiquitination of Met4 takes the form of ubiquitin chains with K48 linkages. This is surprising because it is the first substrate to have a ubiquitin chain through K48 and yet is not subjected to degradation.

Flick *et al.* suggest that the K48-linked chain on Met4 may be too short to induce proteolysis (two or three ubiquitin molecules), or that the chain is obscured by inter-molecular interactions. This new identification of a stable protein ubiquitinated via K48 linkages demonstrates yet another way in which ubiquitin chains can regulate protein activity, but it also underscores how little is known about the poly-ubiquitination process.

The mechanism of poly-ubiquitination is also unclear. One possibility is that a chain of ubiquitin molecules is passed from the E1 to the E2 and is attached as a chain to the substrate. Alternatively, chain elongation may be guided by an E4 after the E3 has attached a single ubiquitin molecule (i.e. the yeast UfD2 E4 enzyme or the E4-like protein CHIP). [11, 12] Finally, a chain of four ubiquitin molecules may be provided by the induced tetramerization of Cdc34, since purified Cdc34 has been shown to form dimers and higher-order oligomers *in vitro* [13]. None of these possibilities has yet been established as the preferred method of chain formation.

**The SCF complex**

While much remains to be elucidated about the ubiquitination process, a number of the proteins known to be involved have been identified. One of these is the SCF (Skp1-cullin-*F* box) complex, a well-studied RING family E3 enzyme. (Figure 3). The SCF complex is highly conserved; each subunit has a homologue in yeast, plants, and humans,

and these subunits are able to complement functionally in other species [14-17].

SCF is composed of several proteins: Skp1, cullin, and F-box. Skp1 is a scaffolding protein and interacts with the cullin protein [18, 19]. The cullin binds Hrt1/Roc1/Rbx1, the protein that contains the RING domain [15, 17]. The cullin and Hrt1/Roc1/Rbx1 proteins work together to recruit the E2 enzymes that can directly ubiquitinate the substrate selected by the F box protein [16, 17]. The variable F box protein associates with Skp1 through its F box motif [14].

F box proteins are named for their first family member, cyclin F [20]. They are typically dissimilar outside of the F box motif, and it is thought that the F box protein confers substrate selection upon the SCF complex through a secondary protein-protein interaction motif [2, 18, 19] that is generally C-terminal to the F box. Interestingly, no F box protein studied so far has more than one F box motif [20].

F box proteins can be classified based on their secondary protein-protein interaction domain. F box proteins that contain a WD-40 domain (Fbw's) recognize phospho-Serine or Threonine consensus sequences (e.g. Cdc4 and hβ-Trcp-1). WD-40 domains are protein: protein interaction domains and consist of short (roughly 40 amino acids) repeats that often end in Trp-Asp (W-D) [21]. They form a beta-propeller structure using 4-16 of the short repeated sequences [21].

Another class, the FBL's, has a variable number of leucine rich repeats (LRR) that form a horseshoe structure with a parallel beta sheet on the concave side and mostly helical elements on the convex side [22]. LRR's are also protein: protein interaction domains and are formed by 20-28 amino acid repeats rich in leucine [22]. These F box proteins do not necessarily require a phosphorylated substrate, and may only select substrates that are part of a particular complex (e.g. yGrr1 and hSkp2).

Finally, there are the Fbxs, which contain one of several miscellaneous domains, including CASH (carbohydrate-interacting), cyclin box, CH (calponin homology), TDL (Traf-domain like), zinc-finger, or proline-rich domains. [20]

Each subunit of the SCF complex has been crystallized, though the complex itself has not. The proposed structure, which is based on the combined subunit crystals, indicates that the cullin forms a slightly curved, rigid, and elongated structure that interacts with Hrt1/Roc1/Rbx1 and the E2 on one end and Skp1 and the F box protein on the other end. The curve to the cullin is such that the F box protein is brought within 50 Å of the E2, suggesting that a bound substrate would bridge the gap. This would explain why the SCF complex does not directly bind the ubiquitin molecule; it just has to position the E2 and the substrate closely together to yield a ubiquitinated substrate (Figure 3).

The SCF complex has been studied in budding yeast because of the simplicity of this system and more specifically because the cell cycle in this organism is closely related to that of humans. In *Saccharomyces cerevisiae* there are at least 17 potential F box proteins, 11 E2 enzymes, and only 1 E1 enzyme [3, 23]. To date, however, only 3 yeast F box proteins have been studied in detail: Met30, Cdc4, and Grr1. Each of these uses the E2 enzyme Cdc34 exclusively *in vivo* [23]. $SCF^{Met30}$ is important for methionine metabolism, and its substrates include Met4, and the protein kinase important for the G2/M transition, Swe1. [10, 24, 25]. $SCF^{Cdc4}$ is important for cell cycle regulation and ubiquitinates, among others, the cell-cycle inhibitors Sic1 and Far1, as well as the pre-replication protein Cdc6 [18, 19, 26]. $SCF^{Grr1}$ is important for cell cycle progression and glucose metabolism and ubiquitinates the G1 cyclins, Cln1 and Cln2, as well as Gic1, which is required for initiation of budding and cell polarization [2, 17, 27-29]. As described here, $SCF^{Grr1}$ also ubiquitinates Gis4.

**$SCF^{Grr1}$**

As mentioned above, $SCF^{Grr1}$ is responsible for the ubiquitination of several different substrates. Grr1 interacts with these known substrates through a binding domain containing 12 Leucine Rich Repeats (LRR's) [30-33]. Grr1 is a protein of 135 kDa and was originally identified in 1984 as a glucose repression mutant (Glucose Repression

Resistant) in *Saccharomyces cerevisiae* [34]. Flick and Johnston began to characterize Grr1 in detail in 1991, describing a protein that is constitutively expressed at low levels and is a primary response element in the glucose repression pathway (described below) [30]. Grr1 was associated with protein regulation when it was found as part of a screen of mutants that stabilized the yeast G1 cyclins, Cln1 and Cln2 [35]. In 1997, Li and colleagues established that Grr1 was is part of the ubiquitin-proteolysis machinery via its association with Skp1 [33] and ubiquitinated Cln1-2, preparing them for degradation.

Grr1 is an unstable protein that is itself ubiquitinated and degraded. The stability of Grr1 (as well as yCdc4) is dependent upon an intact Skp1, Cdc53 (the yeast cullin), and Cdc34, as well as an intact F box domain [26, 36, 37]. However, it is unclear whether the F box protein is ubiquitinated in the absence of another substrate or if F box protein ubiquitination is a result of the substrate ubiquitination. As such, two theories have been proposed: 1) F box proteins are degraded alongside their substrates; thus the level of F box proteins should be proportional to that of the substrates [26]. 2) The F box proteins are shielded from degradation by the substrates and are thus ubiquitinated and degraded in the absence of substrates. In this case, the F box protein level should be inversely proportional to the level of its substrate [2]. These concepts have yet to be tested fully.

*Substrate interaction*

Grr1 interacts with Cln2 through its LRR domain. [38]. Kishi *et al.* compared the LRR domain to the defined ribonuclease I LRR domain, enabling them to generate a model of Grr1's LRR domain. The model demonstrated an abnormally high number of basic residues along the concave surface of the domain, and several of these amino acids were shown to be critical for the binding and degradation of Cln2 [31]. Additionally, like other SCF substrates, Cln2 must be phosphorylated at a minimum of 4 sites for efficient degradation [39]. One theory for substrate selection by Grr1 is that the basic residues along its concave surface serve to recruit the acidic regions of the substrate or its phosphate groups [31].

While this theory may serve for Cln1, Cln2, and Gic2, a recently discovered substrate of Grr1—Mth1 (and possibly its paralogue, Std1)—may not bind Grr1 in the same fashion as the other substrates [40]. Mth1 and Std1 are involved with Grr1 in the glucose induction pathway [41]. Speilewoy and colleagues have recently shown that the type 1 casein kinase Yck1 phosphorylates Mth1, and this phosphorylation event is required for the Grr1-dependent degradation of Mth1 [42]. The authors found that while the degradation of Mth1 requires the LRR domain of Grr1, mutations of the basic residues along the LRR that abrogate binding of Grr1 to Cln2 and Gic2 have relatively little effect on Mth1 degradation. The authors propose that Mth1 is a member of

another class of Grr1 substrates, one that does not require the same amino acid residues on Grr1 that are critical for Cln2 and Gic2 binding [42]. This model suggests that binding to Grr1 may be important to determining the fate of the substrate.

*Physiological role of Grr1 in the cell*

Grr1 has been studied with respect to its interaction with the G1 cyclins and, to some degree, with Gic2. While the role of Grr1 in the cell cycle is well documented, Grr1 also plays a role in other processes, as indicated by its deletion from yeast strains. Cells lacking Grr1 are viable [30], but have a variety of defects, including elongated cell morphology, a defect in aromatic amino acid transport [30], and a defect in taking up cobalt [43]. Its mutants also have enhanced filamentous growth [44] and increased sensitivity to osmotic stress and nitrogen starvation [30]. These defects may reflect a broad role for Grr1 in sensing environmental changes.

Grr1 also plays a role in yeast glucose metabolism. Cells lacking Grr1 grow slowly on glucose, and inactivating Grr1 alters the transcription of genes involved in non-glucose aerobic metabolism [30, 34, 45]. This thesis describes in greater detail how Grr1 is able to alter the transcription of these genes.

**Glucose repression, an overview**

Glucose induction and repression are crucial processes for energy efficient metabolism of carbon sources. Glucose repression is the process by which genes that are unnecessary for glucose metabolism are prevented from being transcribed (Reviewed in [46, 47]). Simultaneously, genes that are required for glucose metabolism are up-regulated (see Figure 4A). As levels of glucose drop, the cell begins to transcribe the glucose-repressed genes to enable the cell to metabolize other carbon sources in the environment (de-repression) (Figure 4B).

Glucose repression occurs because glucose is the preferred carbon source and is the most efficient source for energy [46]. Other carbon sources must be converted into usable forms before being used for energy and thus increase the cost to the cell of using these forms. For example, the disaccharides raffinose and sucrose require the transcription, translation, and secretion of an invertase to convert them into monosaccharides that can be taken up by hexose transporters. Otherwise, the raffinose and sucrose would sit unused outside the cell. The cell must spend energy to metabolize these non-glucose carbon sources, and thus it does not invest in these pathways unless necessary. [46]

## Glucose Induction and Grr1

Grr1 is a critical component in communicating the nutrient-sensing signal and ultimately crucial for the glucose induction pathway. To metabolize glucose, a signal that glucose is available in the environment is transmitted through the glucose sensors Snf3 and Rgt2 to Grr1. Specifically, the carboxy terminal tails of the membrane proteins Snf3 and Rgt2 interact with the proteins Std1, Mth1, and Yck1/2 [40, 41]. In response to glucose, the casein kinases Yck1/2 are stimulated to phosphorylate Std1 and Mth1 [40, 48]. However, upon phosphorylation, $SCF^{Grr1}$ degrades the Mth1 and possibly Std1. Std1 and Mth1 are responsible for maintaining the transcriptional repressor, Rgt1, in a hypo-phosphorylated, promoter-bound state. [40, 42, 49]. Rgt1 represses glucose transporters (HXT's), among others, and requires the general co-repressors Ssn6 and Tup1 [50]. When Std1 and Mth1 are degraded, Rgt1 becomes phosphorylated and unable to repress the glucose transporters [49]. (Reviewed in [46]; see Figure 4A). The glucose transporters are induced by glucose and require Grr1 for this induction.

## Glucose de-repression and Snf1

Concordant with the glucose induction pathway described above, a variety of transcriptional repressors act on glucose-repressed genes, such as the MAL, SUC, and GAL families of genes that are required for

metabolism of maltose, raffinose and sucrose, and galactose, respectively. As glucose is depleted, these genes are no longer repressed and the cell can now metabolize other carbon sources. This occurs through Snf1, a protein kinase. Briefly, Snf1 is stimulated in low-glucose conditions and phosphorylates transcriptional repressors like Mig1. This phosphorylation event prevents Mig1 from repressing genes such as those in the MAL, SUC and GAL families. This allows the cell to use other available carbon sources.

These families of genes have many members and not all members of the family are found in all strains of yeast. SUC2, GAL1, and GAL4 are well studied because they are required components of their respective pathways. SUC2 codes for an invertase that is secreted out of the cell to allow uptake of the disaccharides raffinose and sucrose. GAL1 codes for galactokinase, which is important for the first step of galactose catabolism and whose expression regulated by Gal4p. Gal4 is a DNA-binding transcription factor required for the activation of the GAL genes in response to galactose.

De-repression of genes in low glucose conditions is controlled primarily through the protein kinase Snf1. Snf1 is activated by one of three upstream kinases, Elm1, Tor1, or Pak1, through its phosphorylation at residue T210 [51-55]. Glc7, and its subunit Reg1,

form a protein phosphatase that is critical to the down-regulation of Snf1 [56].

Snf1 is a homologue of AMPK, the AMP-activated protein kinase in mammals [57]. AMPK is activated by AMP:ATP ratios as well as by upstream kinases. Residue T210 in Snf1 is also conserved in AMPK, and this residue is phosphorylated by upstream kinases. Additionally, the subunits of the AMPK kinase complex are similar to the subunits of the Snf1 kinase complex (see below). While the manner of induction may differ for Snf1 and AMPK, they both react to stressful conditions and work in a similar fashion, suggesting that the Snf1 pathway in yeast may be highly conserved.

Snf1 has two domains, its kinase domain and its regulatory domain. Phosphorylation of Snf1 dissociates the Snf1 kinase domain from its regulatory domain and allows the Snf1 binding partner, Snf4, to interact with its regulatory domain (preventing re-association with the kinase domain). The mechanistic details of this conformational change are currently unknown.

Snf1 and Snf4 interact with one of several beta subunits (Sip1, Sip2, Gal83) and together form an active kinase complex [47, 58-60] (See Figure 5). The beta and gamma subunits of the Snf1 complex share similarity to the subunits of AMPK, and these beta subunits have been shown to regulate how Snf1 selects substrates, as well as the localization of Snf1 [60-62].

When Snf1 is activated in low-glucose conditions, this kinase phosphorylates Mig1 (among other substrates) and prevents it from entering the nucleus and repressing transcription [46, 58, 63, 64]. (Figure 4B.) In contrast, when glucose levels are high, Mig1 represses GAL and SUC families of genes (among others), and like Rgt1, requires the general repressors Ssn6 and Tup1. De-repression allows transcription of genes required to metabolize non-glucose carbon sources that may be present in the environment. Accordingly, *snf1Δ* cells are unable to grow using any carbon source other than glucose.

While this section has described briefly the concept of glucose induction and repression and their main players, the mechanism is nevertheless much more complex. Studies have shown that Std1, generally considered part of the glucose induction pathway, is able to activate Snf1 when over-expressed [65-67]. Conversely, Snf1 is able to inhibit HXT expression when activated, either through physiological conditions or when its negative regulators are removed [66]. This means that the pathways of induction and repression affect each other at many different points using many different proteins. Grr1, Std1, Snf1, Mig1/2/3, all contribute to cross-talk within this network. The redundancy of these regulatory methods underline the importance of carbons source metabolism; there are a variety of ways that metabolism

can occur because carbon sources are critical to the cell. As such, the glucose induction and repression pathways are particularly complex.

**Gis4-previous work**

The complexity of this network makes sense in light of the importance of carbon-source metabolism. The protein described here, Gis4, adds another layer to this network. I have identified Gis4 as a protein that interacts with both Grr1 and Snf1 and has an effect on the glucose repression pathway. Gis4 had been previously identified in a study that screened for suppressors of a triple mutant cell (*snf1 mig1 srb8*), which was unable to be grown on galactose [68]. Over-expressing Gis4 allowed growth on galactose for these triple mutant cells and also permitted *snf1Δ* cells to grow on raffinose [68].

Sequence analysis of Gis4 indicates no homologous proteins in other organisms and no functional domains. However, Gis4 appears to contain a C-terminal signal for farnesylation, a signal generally thought to cause a protein to interact with the plasma membrane [69], though the presence of a farnesyl group at the C-terminus has yet to be documented. All farnesylated proteins discovered to date associate with the plasma membrane at some point, but Gis4 appears to be localized to the cytoplasm in glucose—though its localization has also not been documented. However, its presence at the cytoplasm would not

preclude Gis4 from associating with the plasma membrane under the proper conditions.

Finally, cells deleted for Gis4 are viable on glucose, raffinose and galactose, and the cells show no change in morphology [70, 71]. Thus, Gis4 is not an essential protein to the cell, even under specific carbon-source conditions. Other conditions may exist that require Gis4, but these have not yet been identified.

Overall, details about Gis4 as a protein are relatively few. There are no obvious clues that would aid in directing study about it (i.e. homologues with defined functions in other organisms). Therefore, its interaction with Grr1 is of interest but is of little consequence, given the knowledge listed above. This thesis contributes to the body of work about both Gis4 and Grr1 and characterizes the interaction between these proteins.

**Significance**

Grr1 is known to have an effect on glucose-repressed genes. Flick and Johnston showed in 1991 [30] that when Grr1 is inactivated, the invertase coded for by SUC2 and required for sucrose and raffinose utilization is slightly repressed in low- or no-glucose conditions and is activated when glucose levels are high (see Figure 6). This is the opposite of how SUC2 is regulated in wild-type cells. Additionally, when Grr1 is inactivated, GAL1 is unable to be repressed in glucose, but showed no signs of altered activity in low-glucose conditions.

How Grr1 is able to change the regulation of these Snf1-dependent transcripts has been a mystery. Logically, the nutrient system and the cell cycle are coupled. Sufficient levels of metabolites must be maintained for the cell to commit to another round of cell division. The thesis I present here provides insight into how the two systems, glucose repression and the cell cycle, can be connected through $SCF^{Grr1}$. My hypothesis is that Grr1 is able to affect the glucose repression pathways through the protein Gis4 and to ultimately affect the expression of Snf1-dependent carbon source transcripts. This hypothesis is based on the observations that Grr1 is able to influence the expression of genes like SUC2 although there is no evidence that Grr1 directly affects Snf1 activity (the major regulator of SUC2 expression).

The goal of this work is to achieve a better understanding of $SCF^{Grr1}$. At the beginning of this work, there were relatively few known Grr1 interactors. To understand the nature of ubiquitination by $SCF^{Grr1}$ requires an understanding of its substrates. To that end, I employed a yeast two-hybrid screen to identify other proteins that interact with Grr1 and to characterize them with relation to Grr1. As substrates are identified, insights into ubiquitination and into the pleiotropic effects of a *grr1*Δ mutant may be better understood.

My work centers around Gis4, a previously uncharacterized protein—which, I demonstrate, interacts with Grr1. Gis4 is of particular interest compared to other substrates of Grr1 because it is a stable protein, while all other known $SCF^{Grr1}$ substrates are unstable. Gis4 does not appear to undergo the classic ubiquitination-degradation pathway, suggesting that one F box protein does not confer the same fate to all of its substrates. The substrates must "direct" the ligase in different ways to yield different outcomes using the variety of ubiquitination chains.

This work also illuminates the role Grr1 plays in glucose metabolism and Snf1-regulated transcripts. Specifically, it appears that Grr1 is necessary for Gis4 to interact with Snf1 and that Gis4 is able to change the activity of Snf1-dependent transcripts. Together, these

findings indicate that the glucose induction and repression pathways are coordinated and influence the activity of one another.

# Chapter 2

# Materials and Methods

**Table 1: Strains Used in this study**

| Strain | Genotype | Reference or Source |
|---|---|---|
| SLY 241 | W303 | Mike Tyers |
| SLY 476 | Y187-<br>MATa, ura3- 52, his3- 200, ade2- 101, trp1- 901, leu2- 3, 112, gal4Δ, met–, gal80Δ, URA3 : : $GAL1_{UAS\text{-}GAL1TATA}$ -lacZ | Clontech |
| SLY 477 | AH109-<br>MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : $GAL1_{UAS\text{-}GAL1TATA}$-HIS3, $GAL2_{UAS\text{-}GAL2TATA}$-ADE2, URA3 : : $MEL1_{UAS\text{-}MEL1\ TATA}$-lacZ | Clontech |
| SLY 488 | SLY 241+ GRR1:: GRR1-6His Leu2 | [Berset, 2002 #129] |
| SLY 610 | SLY 241+ GRR1::grr1 LEU2 | This study |
| SLY 648 | SLY 241 + GIS4::GIS4-TAP URA3 | This study |
| SLY 651 | SLY 610 + GIS4::GIS4-TAP URA3 | This study |
| SLY 653 | S288C + GIS4::$kan^R$ | Invitrogen |
| SLY 673 | SLY 648 + GRR1:: GRR1-6His Leu2 | This study |
| SLY 674 | S288C + TAP-Grr1 | This study |

Table 2: Plasmids used in this study

| Plasmid | Relevant Characteristics | Reference or Source |
|---|---|---|
| pSL 126 | CUP1:UBI4 | [72] |
| pSL 127 | CUP1:UBI4-myc | [72] |
| pSL 134 | CUP1:UBI4$^{K48RG76A}$ | [73, 74] |
| pSL 135 | CUP1:UBI-myc-6His | [73, 74] |
| pSL 136 | CUP1:UBI4 $^{K48RG76A}$ -myc-6His | [73, 74] |
| pSL 310 | pGADT7 | Clontech |
| pSL 311 | pGADT7-SV40 Large T Fusion | Clontech |
| pSL 312 | pCL (full length GAL4 protein) | Clontech |
| pSL 313 | pGBKT7 | Clontech |
| pSL 314 | pGBKT7- p53 | Clontech |
| pSL 315 | pGBKT7- Lamin | Clontech |
| pSL 325 | ADH:Grr1-HA | Y Hsiung/ C. Wittenberg |
| pSL 353 | pGBKT7-Grr1 | This Study |
| pSL 356 | pGADT7-Cln2 | This Study |
| pSL 405 | pGBKT7-Cdc28 | This Study |
| pSL 410 | pGBKT7-Grr1$^{P321A}$ (f box mutant) | This Study |
| pSL 441 | Spo13 URS, GAL1:URA3 | [75] |
| pSL 507 | pENTR/D-TOPO-Grr1 | This Study |
| pSL 510 | pENTR/D-TOPO-Gis4 | This Study |
| pSL 511 | pYES-DEST52-Gis4 | This Study |
| pSL 512 | pYES-DEST52 | Invitrogen |

| | | |
|---|---|---|
| pSL 525 | pDEST32-Grr1 | This Study |
| pSL 527 | pDES22-Grr1 | This Study |
| pSL 551 | pGADT7-ySkp1 | This Study |
| pSL 595 | GAL1:lacZ | J. Hopper |
| pSL 597 | SUC2:lacZ | S. Turkel |
| pSL 598 | HIS4:lacZ | [76] |
| pSL 599 | CYC1:lacZ | S. Turkel |
| pSL 601 | pDEST22-Gis4 | This Study |
| pSL 602 | PDEST32-Gis4 | This Study |
| pSL 605 | $LexAD_{87}$-Snf1 | M. Carlson |
| pSL611 | TEF promoter (MCS) | [77] |
| pSL 619 | HXT1:lacZ | M. Johnston |
| pSL 623 | pGAD-Snf4 | [78] |
| pSL 624 | pGBT9-Snf1 | [78] |
| pSL 632 | pGADT7-Gis4 (ML) | This Study |
| pSL 633 | pGADT7-Gis4 (FL) | This Study |
| pSL 634 | pGADT7-Gis4 (ΔN) | This Study |
| pSL 635 | pGADT7-Gis4 (MS) | This Study |
| pSL 641 | pGADT7-Gis4 (ΔC) | This Study |
| pSL 699 | TEF:Gis4-V5 6His | This Study |
| pSL 700 | TEF:FLAG-Gis4 | This Study |

**Yeast Two Hybrid Screen:** The yeast two hybrid screen was conducted using the Matchmaker (Clontech) system. The SLY477 strain was sequentially transformed with pGBKT7-Grr1$^{P321A}$ and then with the yeast genomic library in the vector, pGAD424 and selected for by growing on plates lacking tryptophan or leucine and tryptophan and histidine containing 2.5 mM 3-Amino-Trizole, respectively. $2.4*10^4$ clones were selected and tested by growth on plates lacking leucine, tryptophan, and adenine. Clones that still appeared positive were re-transformed and the selection was conducted again, according the Clontech's Matchmaker Yeast Two Hybrid Protocol. Clones were classified by their levels of interaction (weak to strong) and sequenced. Clone #5 encoded amino acids –84-363 of Gis4. The reading frame was verified by sequence analysis to insure that Gis4 was the protein expressed.

**Deletion mutant analysis:** Regions of Gis4 were amplified by PCR according to figure 8b. PCR products were ligated into pGADT7 of the Matchmaker System by Clontech. Constructs were tested for their interaction in SLY 477 and SLY476 as mentioned above. Additionally they were tested for LacZ activity according to Clontech's Matchmaker protocols.

**PEST Analysis:** The Gis4 sequence was examined for the presence of PEST domains using the program, PESTFIND, developed by Martin C.

Rechsteiner and Scott W. Rogers. PESTfind produces a score ranging form about -50 to +50. By definition, a score above zero denotes a possible PEST region, but a value greater than +5 sparks real interest.

(http://www.at.embnet.org/embnet/tools/bio/PESTfind/)

**Cultures and Conditions:** When preparing protein, yeast cells were harvested at log phase and lysed using glass beads in lysis buffer (50 mM Tris-HCl [58], 0.1% NP-40, 250 mM NaCl) containing protease inhibitors and phosphatase inhibitors (5 mM EDTA, 5 mM EGTA, 0.1 mM orthovanadate). Debris was removed and protein concentrations were measured using the BioRad Bradford Assay.

For the *skp1-12*$^{ts}$ cells, cultures were grown at 25° C to log phase, then transferred to 37° C (as appropriate) for 2 hours. Cells were examined for morphology changes to insure the effectiveness of the temperature shift.

For expressing tagged and untagged ubiquitin from the CUP1 (copper sensitive) promoter, one hour after temperature shift (if necessary), the ubiquitin constructs were induced for 1 hour with 50 mM $CuSO_4$ to relieve repression of the promoter and the cells were harvested in 8 M Urea.

**Co-Precipitations and Immunoblotting:** Gis4-TAP (see figure 8 for schematic) was purified using Calmodulin Binding Resin (Stratagene) according to manufacturer's protocols. Snf1 was purified using $Ni^{2+}$-NTA Resin for 2 hours at 4°C and eluted using 250-500 mM Imidazole- see experiment for specific elution conditions. Flag pull downs were conducted using FLAG-M2 beads (Sigma Chemical Co.) according to manufacturer's protocols.

Proteins prepared as above were run on SDS-PAGE gels and samples were probed with the appropriate antibodies: anti-PAP and anti-FLAG-M2 (Sigma Chemical Co), anti-RGS-His (Qiagen), anti-myc (Oncogene Biochemical Co.), anti-Snf1 and anti-Cdc28 (Santa Cruz Antibodies). Secondary antibodies used goat anti-mouse-HRP (Invitrogen) and donkey anti-goat-HRP (Santa Cruz Antibodies).

**Ubiquitination Assays:** Strains containing CUP1-driven ubiquitin constructs were induced for 1 hour with 50 mM $CuSO_4$ and harvested. Cells were lysed using glass beads in 8 M Urea and NEM (Sigma Chemical Co.) on ice. Debris was removed and protein concentrations were measured using the BioRad Bradford Assay. Ubiquitinated proteins were purified using $Ni^{2+}$ NTA resin and washed twice with 8M Urea. Proteins were eluted with 500mM Imidazole and SDS sample buffer and boiled. Samples were subjected to SDS-PAGE and probed with the appropriate antibodies.

**Stability Assays:** Yeast cells were grown to log phase and treated with cycloheximide (Sigma Chemical Co., 50 mg/mL). Samples were harvested at indicated time points over ice and frozen until lysis and examined by immunoblotting as described above.

**β-Galactosidase Activity-Snf1 Activity and Reporter Construct analysis:** Yeast strains with the appropriate plasmid containing the reporter construct were grown to log phase in the presence of 2% carbon source and harvested. 10 μg of whole cell extract was added to 80 μl of 4 mg/mL o-nitrophenyl galactopyranoside (ONPG, Sigma Chemical Co.) and volume was brought up to 350 μL using Z buffer (16.1g/L $Na_2HPO_4$-$7H_2O$, 5.5 g/L $Na_2H_2PO_4$, 0.75 g/L KCl, 0.246 g/L $MgSO_4$-$7H_2O$, adjusted to pH 7.0 and autoclaved. Clontech Matchmaker System Protocol). The reaction was stopped by the addition of 200 μL 1.0 M $NaCO_3$ (Sigma Chemical Co.) once the solution turned yellow and it was quantified by absorbance at 420 nm wavelength. Unit Activity represents color development in minutes per μg of protein. The assay was done using 3 different transformants, each in triplicate, for each carbon source and plasmid.

# Chapter 3

# Results

**Grr1 interacts with Gis4**

Genetic evidence suggests that Grr1 plays a role in a variety of functions that are distinct from its interaction with the SCF complex or with Grr1's known substrates, Cln1-2 and Gic2 [27, 28, 38]. This conclusion is based upon the fact that *grr1Δ* mutants have severely reduced growth on glucose and raffinose, suggesting that Grr1 plays a role in metabolism or transport of these carbon sources. However, concomitant deletion of Cln1-2, or Gic2 does not suppress this phenotype, indicating that these proteins are not involved in the reduction in growth seen in the *grr1Δ* mutants.

Further investigation into the role of Grr1 in carbon source uptake and metabolism points to specific genes that are affected by the deletion of Grr1. Indeed, when Grr1 is inactivated, transcription of SUC2, an invertase required for metabolism of sucrose and raffinose, is altered. Grr1 also affects other genes that are glucose-regulated, such as CYC1 (an isoform 1 of cytochrome C) and the MAL family of genes, which is required for maltose metabolism [30]. Finally, *grr1Δ* mutants are reduced in their transport of aromatic amino acids [30] and in cobalt transport, compared with wild-type cells [43].

To identify proteins that Grr1 interacted with that could explain these pleiotropic effects, I conducted a yeast two-hybrid screen. Our rationale was to prevent substrate ubiquitination upon interaction with $SCF^{Grr1}$, which would augment our ability to select and identify

substrates that might otherwise not be identified because of a transient interaction and subsequent degradation by $SCF^{Grr1}$. Therefore, to improve the probability of finding substrates, I used a mutant form of Grr1 that has a conserved residue of the F box motif [14] mutated to abrogate its binding to Skp1 and the SCF complex. Using this mutant, $Grr1^{P321A}$ (for details, see Appendix II) as bait, I screened $2.4X10^4$ clones and isolated 39 positives. Here, I describe one clone, Gis4, a protein of previously unknown function (Figure 7A). For further information on the yeast two-hybrid screen and other positive interactors, see Appendix I.

Gis4 is a protein of 774 amino acids and has no homology to known proteins. Sequence analysis did not indicate any catalytic domains of known function or any common protein: protein interaction domains. However, its sequence suggested four strong PEST motifs (See Figure 8A). PEST motifs were described in 1986 as sequences rich in proline (P), aspartate (D), or glutamate (E), serine (S) and threonine (T) residues. Rechsteiner and Rogers developed an algorithm to detect PEST motifs in protein sequences and called it PESTFind [79]. Their algorithm defines PEST sequences as hydrophilic stretches of amino acids greater than or equal to 12 residues in length. Such regions contain at least one P, one E or D and one S or T. They are flanked by lysine (K), arginine (R) or histidine (H) residues, but positively charged residues are disallowed within the PEST sequence. PESTfind produces a score ranging form

about -50 to +50. By definition, a score above zero denotes a possible PEST region, but a value greater than +5 sparks real interest" [79].

Using the PESTFind algorithm [79], Gis4 is predicted to have four potential PEST motifs in its amino acid sequence. Scores above 5 units indicate a strong potential for a PEST motif; Gis4's motifs score 7.72, 8.09, 7.51, and 5.12. (See Figure 8 for details.) The importance of these PEST domains will be discussed below.

To further examine binding, and to confirm our yeast two-hybrid results, I needed to detect Gis4 in experiments. An antibody against Gis4 was not available, so an epitope tag was used. To this end, a TAP tag was fused to the C-terminus of chromosomal Gis4, as described previously [80]. The TAP (Tandem Affinity Purification) tag contains a calmodulin binding peptide and the IgG -protein A binding motif, separated by a Tobacco Etch Virus (TEV) protease cleavage site (Figure 8B). This allows dual purification by calmodulin binding resins and IgG sepharose (or agarose) matrices. The TAP tag is also detectable by an anti-PAP antibody by virtue of its protein A tag and can be used in immunoblotting (Figure 8B).

Next, I examined the ability of tagged Gis4 to interact with endogenous Grr1 *in vivo*. Lysates from strains that chromosomally expressed Gis4-TAP, or Grr1-6His, or both, were incubated with a calmodulin binding resin to adsorb Gis4-TAP. Gis4 appears as three discrete bands, as described below. I confirmed that Gis4 interacts with

Grr1 (Figure 7B), as observed in the two-hybrid screen. Grr1 appears in the bound fraction only when tagged Gis4 is present (lane 6).

To determine which regions of Gis4 are important for binding to Grr1, deletion constructs of Gis4 were analyzed for their interaction with Grr1 using the yeast two-hybrid methodology. The Gis4 protein pulled out from the initial screen spanned nucleotides –84 to 393. The presence of the 5' UTR was of concern, because it could mean that the reading frame was incorrect or that the 5' UTR was responsible for the interaction with Grr1. However, sequencing of the fragment indicated that the reading frame would allow the translation of the fragment to be identical to the N-terminal region of Gis4. Furthermore, I confirmed that full length Gis4 was able to interact with Grr1 *in vivo*. Therefore, I continued to examine and characterize Gis4.

To identify regions important for binding, I used full-length Gis4, the central fragments ML (nt 196-661) and MS (nt 393-661), as well as the N- or C- termini of the protein, and determined if they interacted with full-length Grr1 using the two-hybrid methodology (Figure 8C). Using several two hybrid reporter constructs, I found that full-length Gis4 bound to Grr1 most effectively; followed by the ML fragment and then the MS fragment. The N- terminal and C- terminal fragments appeared to bind weakly to Grr1 with similar reporter activity. These data suggested that the region between amino acids 196-661 is important for binding. Because the MS fragment has less activity than the ML fragment, this

implicates the region 196-393 as an important factor in the interaction. But it also suggests that it is not the only important region or that the region must be longer, because the DC-terminal fragment contains this section yet has a weak binding ability.

PEST motifs generally indicate unstable proteins [79]; however, there are exceptions [81], including Gis4, as I show in Figure 3. Interestingly, the larger region of Gis4, which is important for Grr1 binding, contains several of these PEST motifs; similarly, the domain in Cln2 that is important for Cln2's interaction with Grr1 contains a PEST motif [39]. This could indicate that the PEST domains are important to signal for an interaction with Grr1 in these proteins. Furthermore, PEST motifs are found within each of the N- and C- terminal fragments that flank this Grr1-binding region, perhaps accounting for their similar affinities for Grr1. Additionally, the region of Grr1 pulled out from the two-hybrid screen contained two of these PEST domains. Other substrates of Grr1 have at least putative, if not strong, potentials for PEST domains (Figure 8), indicating that this region is an important aspect of the Grr1-substrate interaction.

**Gis4 protein levels are directly proportional to Grr1 protein levels**

Because Grr1 is a subunit of the SCF ubiquitin ligase, I explored the possibility that Gis4 is substrate of the $SCF^{Grr1}$ complex. Initially, I examined the levels of Gis4 protein in relation to Grr1 and found that

Gis4 is present at moderate levels in cells that express endogenous Grr1 (Figure 9, lanes 1-4). When Grr1 is over-expressed from an ADH promoter, Gis4 levels are increased (Figure 9, lanes 9-12). In contrast, when Gis4 is expressed in a *grr1Δ* strain, Gis4 protein levels are significantly decreased (Figure 9, lane 5-8). This result was particularly intriguing: for Gis4 to be a substrate of $SCF^{Grr1}$ for ubiquitination and degradation, I would have expected the levels to be inversely related. Instead, I found that Gis4 levels were directly proportional to the levels of Grr1.

**Gis4 is a stable protein**

While I found that Gis4 protein levels are directly proportional to Grr1 protein levels, this did not necessarily exclude the possibility that Gis4 is a substrate of Grr1, since ubiquitinated proteins are not always immediately degraded. Because Grr1's role as part of an E3-ubiquitin ligase is well documented, I investigated the stability of Gis4. To measure the stability of the Gis4 protein I used a *GAL1*-driven Gis4 construct that can be expressed in galactose and then quickly repressed when cells are filtered into glucose-containing medium. These results indicated that Gis4 was stable up to 3.5 hours after glucose repression, a time course that is very stable for a yeast protein (data not shown).

Since Grr1 and Gis4 might participate in carbon-source utilization, I could not rule out that these results were misleading, because stability

is measured by employing a carbon source shift. Therefore, to examine the stability of Gis4 without affecting the carbon source, I also analyzed Gis4 protein stability after adding cycloheximide (CHX), which inhibits translation and allows the protein to be followed over time because no new proteins are being translated. I found that, again, Gis4 appeared to be stable for at least 90 minutes (Figure 10A), which is still stable given that the half-life of yeast is about 120 minutes. After 90 minutes the cells appeared to be unhealthy when viewed microscopically, and Gis4 was markedly reduced at 120 minutes, presumably due to toxic effects of CHX on the yeast.

To confirm the effectiveness of our CHX experiments, I also assayed the unstable protein Grr1. This protein appeared to have a half-life of 15-30 minutes (Figure 10B), a time period that is corroborated by other publications [36], and thus indicated that Gis4 was indeed a stable protein. In the event that Grr1 may affect Gis4 stability, I examined the levels of Gis4 when Grr1 was absent (Figure 10C). Gis4 appeared to be stable under these conditions as well.

These results indicate that Gis4 is not degraded upon ubiquitination by Grr1. While this is possible, it has not been shown for any known substrate of Grr1. However, if Gis4 were degraded, it might have a shorter half-life, but Gis4 would also be stabilized when Grr1 was inactivated—precisely the opposite of what I found.

**Gis4 is ubiquitinated in a Grr1-dependent manner**

When Gis4 was expressed from its own promoter, it appeared as three bands, the fastest running at the predicted size of 89 kDa (or 109 kDa with the 20 kDa TAP tag fusion). The cause of variation in the expression levels of these different bands has yet to be determined. There is a large shift between each of the bands, of approximately 15-20 kDa for each band. All three bands can be seen in a *grr1Δ* strain, albeit at a reduced level.

The observed variations among these bands could have several explanations. For example, the sequence prediction suggested that Gis4 is a candidate for C-terminal farnesylation. Gis4 ends in –Cys-Ala-Iso-Met, and it fits the required -CAAX (cysteine, alanine, an aliphatic amino acid, followed by any amino acid) motif indicative of C-terminal farnesylation. However, in these experiments, the C-terminal TAP tag made farnesylation unlikely. The presence of the TAP tag eliminated the ability of the –CAAX motif to be recognized and become farnesylated. Thus, the three bands were not likely caused by farnesylation.

Another possibility that could cause these different bands is through transcriptional regulation. First, yeast do not generally make use of transcriptional splice variants, and the Gis4 sequence does not indicate that it is unusual in this respect. Additionally, when Gis4 is expressed from the Gal1 promoter, which allows transcription of the gene in the absence of glucose, Gis4 still bears this pattern of three bands

(data not shown). Therefore, these bands were not due to a transcriptional alteration because the three bands are seen when Gis4 is expressed from an endogenous or exogenous promoter.

Gis4 is therefore most likely being post-translationally modified. To test this possibility, and given that Grr1 is part of a ubiquitin ligase, I examined whether Gis4 is ubiquitinated in a Grr1-dependent manner. While Gis4 is a stable protein, there are examples of proteins that are ubiquitinated and not degraded, including Met4 and Histone 2B [10, 82]. Thus, I asked whether the three bands were ubiquitinated forms, and I looked for the higher molecular-weight species "smear" indicative of ubiquitination.

To determine if any of the three bands of Gis4 were ubiquitinated, I conducted an affinity purification of tagged Gis4 and probed the extracts for both the presence of Gis4 and for tagged ubiquitin (Figure 11A). The first lane was probed for FLAG-Gis4 and shows the typical three bands of Gis4, as well as the presence of the IgG heavy chain from the purification process. The next two panels were probed for myc-ubiquitin. Lane 2 was from the same extract as lane 1, yet only one band appeared. However, purified extracts lacking Gis4 probed for myc-ubiquitin (lane 3) also had this band, indicating that the band was not specific to Gis4. Therefore, none of the three bands of Gis4 appeared to be ubiquitinated since no particular band was specific to both Gis4 and tagged ubiquitin.

To enhance the ability to detect Gis4-ubiquitin conjugates *in vitro*, I used cells expressing a polyhistidine and myc -tagged mutant ubiquitin (6His-Myc-Ubi$^{K48R, G76A}$). Expressing this mutant resulted in accumulation of short-chain ubiquitinated target proteins, since K48 is required for ubiquitin chain elongation and G76 is essential for cleavage by ubiquitin isopeptidases [83]. Lysate prepared from cells expressing the 6His-Myc-Ubi$^{K48R, G76A}$ was passed over $Ni^{2+}$-NTA matrix, and bound proteins were eluted using SDS sample buffer and immunoblotted for Gis4-TAP with anti-PAP antibody. Ubiquitin-conjugated Gis4 accumulated predominantly in a mono-ubiquitinated form, in contrast to Cln2, which accumulated several higher molecular-weight species, indicative of multi-ubiquitination (Figure 12A, lane 7 only).

I repeated the experiment with both tagged and untagged wild-type ubiquitin. Both were expressed in the Gis4-TAP strain in the presence (Figure 12A) or absence of Grr1 (Figure 12B). In this experiment—as well as in others throughout this paper—I used 5 times the amount of protein when working with *grr1Δ* cells, to compensate for the lower levels of Gis4 seen in these mutants. I precipitated ubiquitinated species with the polyhistidine epitope tag, as before, and probed for Gis4. A series of bands appeared above the highest molecular-weight band of Gis4, and these bands appeared to have the characteristic laddering typical of ubiquitination (Figure 12A, lanes 4 and 6). Significantly, the appearance of these bands was dependent upon the presence of Grr1 (Figure 12B,

lanes 4 and 5). However, to characterize the type of ubiquitination and to differentiate between multiple mono-ubiquitination and multi-ubiquitination requires further study. There are 61 lysines available for possible ubiquitination in the full-length protein sequence of Gis4, making mono-ubiquitination possible even in light of the large-molecular-weight species seen in the figure.

The retention of non-ubiquitinated Gis4 on the $Ni^{2+}$-NTA resin was not specific, as demonstrated by the equivalent levels retained from cell extracts expressing ubiquitin in either its tagged or untagged forms. Gis4 also does not appear to have any internal sequence that would substitute for a poly-His epitope tag. Longer exposures of these non-specific bands in untagged ubiquitin-containing strains did not show higher molecular-weight species (compare lane 5 against lanes 4 or 6). Additionally, it was unlikely that I lost ubiquitin molecules that are on Gis4 during extract preparation because I lysed yeast cells quickly under denaturing conditions on ice and added NEM, an isopeptidase inhibitor. Thus, I conclude that the appearance of these bands indicates that Gis4 was ubiquitinated in a Grr1-dependent manner.

**Functional Analysis of Gis4**

We know that Gis4 is ubiquitinated in a Grr1-dependent fashion, but what is the function of Gis4 or of ubiquitinated Gis4? To investigate this question, I tested the ability of *gis4Δ* cells to grow on a variety of carbon

sources (data not shown); these mutants were viable in all cases and had normal morphology (not shown). Cells that over-expressed Gis4 were viable and had a normal morphology as well. However, I found that over-expression of Gis4 in a *grr1Δ* strain was lethal on galactose and raffinose (Figure 13), suggesting a functional interaction between Grr1 and Gis4. Because Gis4 seemed to be involved in carbon-source utilization, I tested whether the deletion mutants grew at different rates on different carbon sources (Figure 14). This did not appear to be the case. I also tested the levels of Gis4 in the various carbon sources. It appeared that Gis4 levels were relatively equal in glucose and in galactose, but increased in raffinose and glycerol (Figure 15).

These results demonstrated that Gis4 was not an essential protein and was not absolutely required for metabolism of different carbon sources. However, the over-expression of Gis4 in *grr1Δ* mutants was lethal on specific carbon sources, indicating a functional interaction, if not an essential one.

**Gis4 interacts with the Snf1 kinase in a Grr1 dependent manner**

Data from the genome-wide affinity purification by Ho *et al.* [84] suggested that Gis4 interacts with Snf1, a major regulator of genes in response to glucose levels. Additionally, the *snf1Δ* phenotype was partially suppressed on raffinose by over-expression of Gis4; and *snf1Δ gis4Δ* was synthetically lethal on galactose [68], suggesting a functional

connection between Gis4 and the Snf1 kinase pathway. Therefore, I wanted to determine the functional relationship between Gis4 and Snf1.

First, I purified Gis4-TAP protein using calmodulin-binding resin and probed for Snf1 binding (Figure 16a). While there was significant binding in the wild-type cells (lane 4), there was a very low level of Snf1 binding when Grr1 or Gis4 was absent (lanes 5- 6). This Grr1 dependency on Gis4-Snf1 binding suggested that Grr1 is critical to the Gis4-Snf1 interaction. Grr1 could be required to bridge this interaction, it or could be important to ubiquitinate Gis4 so that it can bind Snf1. Previous data suggested that Snf1 was in a complex that included Gis4 and ubiquitin [84], making it likely that Gis4 interacts with Snf1 in an ubiquitinated form.

To address these possibilities, I examined the ability of Grr1 to interact with Snf1 in a two-hybrid assay (Figure 16B). I found that Grr1 and Snf1 were unable to interact under these conditions, a finding that was especially striking in comparison to the strong Snf1-Snf4 interaction. While this result is preliminary, it suggests that Grr1 is not acting as a bridge between Snf1 and Gis4.

If Grr1 is not acting as a molecular bridge, then it might promote the Gis4-Snf1 interaction by ubiquitinating Gis4. Snf1 has 13 internal Histidine residues, making it easy to precipitate under native conditions using $Ni^{2+}$-NTA resin. I was able to precipitate Snf1 in the presence of tagged Gis4 and myc-ubiquitin (Figure 17A). The eluate from this

purification was subjected to a second purification, using IgG agarose to precipitate the forms of Gis4 that came down with Snf1. I found that Snf1 co-precipitated with the ubiquitinated form of Gis4, thus explaining the dependence upon Grr1 for the interaction (lane 5). Snf1 did not precipitate with the IgG beads in the absence of tagged Gis4, suggesting that the Snf1 seen in the lane 5 was specific to the Gis4 present at this purification.

Additionally, I tested the importance of ubiquitination in the Gis4-Snf1 interaction in a strain in which the SCF complex can be inactivated. I used a *skp1-12$^{ts}$* mutant that has a known defect in cyclin proteolysis, thus making it a relevant mutant for Grr1 studies [14, 33]. I assayed whether Gis4 could bind Snf1 in the absence of the SCF complex. I expressed FLAG-Gis4 in the *skp1-12$^{ts}$* mutant strain and precipitated Gis4 by virtue of its FLAG epitope from strains grown at 25°C and 37 °C. These strains were examined for the associated abnormal morphology at 37 °C, but not at 25 °C, to ensure that the temperature shift was effective. I found that at low temperatures, when the SCF complex is intact, Gis4 is readily able to bind Snf1, as I had seen before (See Figure 17B, lane 3). However, at the higher temperature, when the SCF complex is impaired, Gis4 is still precipitated (lane 4), but the Snf1 was no longer bound (lane 4); instead, it was all in the flow-through (lane 3). The newly unbound Snf1 indicated that ubiquitination is critical for the Gis4-Snf1 association. Together, these findings demonstrate that Grr1 is

required for the Gis4-Snf1 interaction and that this requirement depends on the ubiquitination of Gis4, allowing Gis4 to interact with Snf1.

**Gis4 over-expression positively affects Snf1 activity**

It appears that Gis4 interacts with Snf1, but this does not necessarily mean that Gis4 affects the Snf1 kinase activity. To determine if Gis4 binding to Snf1 does affect the activity of the Snf1 kinase, I employed a three-hybrid assay commonly used to measure a protein's effect on Snf1 activity [65]. Under low- and no-glucose conditions, the Snf1 kinase complex is active, and Snf1 interacts directly with its gamma subunit, Snf4. When glucose is high, the complex is inactive and Snf1 does not associate with Snf4. To determine if Gis4 can affect this complex and ultimately Snf1 activity, I tested for any Snf1-Snf4 interaction when Gis4 was over-expressed, by the strongly expressing TEF (*T*ranslation *E*longation *F*actor) promoter (Figure 18A) using the β-galactosidase assay.

Cells with and without the Gis4 over-expression vector were grown in glucose and galactose, and then assayed for β-galactosidase activity, which would indicate an interaction between Snf1 and Snf4. In glucose, the interaction was as low as the empty vector controls, but in galactose, the Snf1-Snf4 interaction was increased well beyond that of the negative controls (Figure 18A). When Gis4 was expressed, the Snf1-Snf4 interaction could be observed in both glucose and galactose conditions.

Thus, I found that Gis4 does activate Snf1 in repressing and de-repressing conditions at levels above wild-type cells without Gis4 over-expression.

Interestingly, when I precipitated FLAG-Gis4 that was over-expressed from the TEF promoter, I noticed that the form of Snf1 with which it interacted was shifted to a form that was less mobile. Figure 18B; compare lane 3 to lanes 1 and 2.) Given that I was over-expressing Gis4, it followed that Gis4 was interacting with the active, phosphorylated form of Snf1 (indicated by the asterisk); however, this possibility has not been tested directly. Such a test could be accomplished by employing phosphatases against this form of Snf1 and examining a shift in the molecular weight of the band compared with that of the bands in the other lanes.

**Deletion of Gis4 inhibits de-repression of Snf1-dependent transcripts**

We know from the above experiment that Gis4 can stimulate Snf1 activity when over-expressed, but can it alter Snf1-dependent transcripts? To answer this question, I tested whether Gis4 could affect transcription of Snf1-responsive genes. I obtained β-Galactosidase reporter constructs whereby GAL1, SUC2, CYC1, and HIS4 promoters were fused to LacZ. These genes are important for the metabolism of galactose, raffinose and sucrose, for the expression of cytochrome C in

the mitochondria, and for Histidine biosynthesis, respectively. I observed their expression by β-Galactosidase activity in wild-type cells and in *gis4*Δ cells under glucose, galactose, raffinose (Figure 19), and glycerol (not shown) conditions. HIS4: LacZ is not Snf1-dependent and is thus a negative control.

I found that in wild-type cells (Figure 19A) in galactose, GAL1 transcription was induced (compare lane 5 to lane 1). In galactose and raffinose, SUC2 transcripts were de-repressed (compare lanes 6 and 10 to lane 2). In all carbon sources, CYC1 was greatly induced (lanes 3,7, and 11), and HIS4 transcripts remained low (lanes 4,8, and 12).

These results were expected. In the presence of glucose, expression of GAL1 and SUC2 transcripts should be repressed, since the enzymes they encode are not required to metabolize glucose. CYC1, as our positive control, should be expressed in all conditions, but at a greater level under de-repressing conditions. However, the development of CYC1: LacZ was very strong and our assay may not be sensitive enough to detect this change at such high levels of activity. Ultimately, the wild-type cells expressed the reporter constructs as expected for each condition.

In sharp contrast, *gis4*Δ cells (Figure 19B) were unable to de-repress any construct except GAL1 in galactose (compare lane 5 to all other lanes). Surprisingly, CYC1 transcripts were repressed as well (see lanes 3,7, and 11). What is most interesting was the difference between the GAL1 and

the SUC2 de-repression. CYC1, SUC2 (and GAL1) are all Snf1-regulated [47, 85], yet GAL1 was de-repressed in *gis4*Δ cells, and SUC2 and CYC1 were not. This finding suggested a mode of de-repression that is specific to SUC2 and CYC1, but not to GAL1—a mode that uses Gis4 (described further in the Discussion). Given that SUC2 and CYC1 are both affected by the Gis4 deletion, it remains possible that Gis4 affects other Snf1-transcripts as well, and possibly to a greater degree than it affects SUC2 and CYC1.

# Chapter 4

# Discussion

## Gis4 is a novel substrate of the $SCF^{Grr1}$ Ubiquitin Ligase

The results of this dissertation demonstrate that Gis4 is a novel ubiquitinated substrate of SCFGrr1 that may facilitate communication between the glucose-repression and glucose-induction pathways. Grr1 has several reported substrates, but these substrate interactions do not explain how Grr1 affects carbon-source utilization. *grr1Δ* mutant strains have severely reduced growth on glucose and raffinose. When Grr1 is deleted, the transcription of SUC2, GAL, CYC, and MAL families of genes is changed [30]. I have identified a novel substrate, Gis4, that may facilitate communication between Grr1 and these transcripts.

## Gis4 protein levels are proportional to Grr1 protein levels

When working with classic ubiquitin ligases, it is easy to assume that substrates are degraded upon ubiquitination. This leads to the prediction that the substrate is also unstable and its levels are inversely proportional to that of the ligase. Gis4 as a substrate of $SCF^{Grr1}$ shows that this is not always a correct assumption. When Grr1 is over-expressed, the levels of Gis4 increase. Conversely, when Grr1 is deleted, the levels of Gis4 are almost undetectable. Interestingly, all three forms of Gis4 seen in wild-type cells are still expressed in *grr1Δ* cells, albeit at much lower levels, indicating that these three forms are not dependent upon the expression of Grr1 as would be the case if they were ubiquitinated forms of Gis4. Additionally, Gis4 over-expression in *grr1Δ*

cells grown on non-glucose carbon sources is lethal. Such lethality may be an artifact of over-expression, yet it is interesting to note that Gis4 levels are controlled with respect to Grr1, suggesting that ubiquitination alters the status of Gis4, but not its rate of stability. While these data are consistent with a protein that is not degraded in response to Grr1, the regulation of Gis4 by Grr1 is still unclear.

**Gis4 is ubiquitinated by $SCF^{Grr1}$, but remains a stable protein**

I have found that Gis4 ubiquitination is Grr1-dependent. To define what type of ubiquitination is occurring requires further analysis. Both multiple mono-ubiquitination as well as multi-ubiquitination, remain possibilities. However, ubiquitin mutants or mass spectrometry must be employed to identify how Gis4 is ubiquitinated.

The ubiquitination in and of itself is an interesting finding and implies that Grr1 may participate in ubiquitination that results in an outcome other than degradation for some substrates. The ubiquitination bands appear above the largest form of Gis4; other post-translational modifications may therefore exist or be required for Gis4 to become ubiquitinated, because the ubiquitination only occurs after the Gis4 has been modified to form the highest molecular-weight form. This is particularly true in light of the data that indicate that none of the three primary bands of Gis4 are ubiquitinated species. Since the ubiquitinated

product runs above the highest form of Gis4, it is possible that these modifications are required for Gis4 to become ubiquitinated by $SCF^{Grr1}$. The precise form of the three primary bands of Gis4 remains an open question. The fastest-running form runs at the expected molecular weight for unmodified Gis4. However, the other bands are not ubiquitinated and are presumably not farnesylated. Phosphorylation of Gis4 therefore remains a possibility. Substrates of the SCF complex are commonly phosphorylated before becoming targets for ubiquitination. This is true for Cln2; there are four phosphorylation sites that are critical to its interaction with Grr1 [39]. Cln1 and Cln2 have both been shown to interact with Grr1 in their phosphorylated forms. [17, 19]. Gic2 is phosphorylated at its C-terminus, and its active form is specifically ubiquitinated and degraded by the $SCF^{Grr1}$ [28]. (See Figure 8 for phosphorylation sites for these proteins.)

Gis4 has several potential sites for phosphorylation by CDK and other kinases. Gis4 has been shown to interact with several kinases, including Srb8/10, Kin2, and Snf1, but phosphorylation of Gis4 by these kinases has not been demonstrated. Preliminary experiments examining phosphorylation of affinity-purified Gis4 were conducted, but the data were inconclusive.

**Gis4 compared to other $SCF^{Grr1}$ substrates**

The partial mapping of Gis4 suggests that it binds Grr1 using at least one PEST motif. While PEST motifs are traditionally regarded as indicating instability, these motifs are not necessary for degradation. For example, ubiquitin-mediated degradation of the yeast Matα repressor is not dependent upon its PEST motif [86]. Similarly, we have previously reported that the single PEST motif in Cln2 is not sufficient for rapid degradation when fused to heterologous proteins [39]. However, this PEST motif is involved in binding to Grr1, since the inefficient binding of the critical phosphorylated domain in Cln2 to Grr1 is strongly enhanced when fused to the PEST motif. Gis4 has 4 PEST motifs at 8.09, 7.51, 7.72, and 5.12 (Figure 8). Cln2 has a single PEST motif scored at 6.99. Similarly, Cln1 has a single motif, scoring quite high at 14.12 (Using [79]). While Gic2 does not have any PEST motifs, Gic2 has at least one motif that could constitute "poor, but possible" PEST motifs. (See Figure 8.) We postulate that the PEST motifs in Gis4 are involved in binding to Grr1, similar to the PEST motif in Cln2, which is a demonstrated Grr1 substrate.

The importance of the PEST domain in binding can be determined by examining the substrates for binding to Grr1 with and without their respective PEST domains. Gis4 is a stable protein, irrespective of relative Grr1 protein levels. Again, the stability of Gis4 is compatible with multiple mono-ubiquitination, but also with multi-ubiquitination. Gis4

is the first substrate of $SCF^{Grr1}$ that is a stable protein. The substrates of Grr1 are so different that no immediate conclusion can be drawn. Further analysis of the substrates and their differences may illuminate details such as the mechanism of ubiquitination and how a E3 ligase differentiates between substrates.

Most substrates are assumed to bind through the LRR domain, but this has not been verified for Gis4. It would be interesting to determine whether Gis4 requires the same critical amino acids on Grr1 for Cln2 binding, or whether its binding more closely resembles that of the glucose signaling protein Mth1. Cln1-2 and Gic2 are known to interact with basic amino acids along the concave surface of Grr1's LRR's [31]. However, Speilewoy *et al.* demonstrated that Mth1 does not require these same amino acids for its interaction with Grr1 and suggest that substrates may be classified according to their preferred binding to Grr1 [42]. Further definition of these proposed classes and knowing which class Gis4 belongs to may provide more information about Gis4 and its interaction with Grr1.

**Gis4 interacts with and activates Snf1 kinase**

Given that Grr1 affects glucose metabolism, and that Gis4 interacts with Snf1, we investigated whether Gis4 explained the effects of Grr1 on glucose-repressed, Snf1-dependent transcripts. Initially, we verified that Gis4 was able to interact with Snf1, as had been shown

before. I also found that Grr1 was required for this interaction. Data from Ho *et al.* suggested that the Snf1 complex in which Gis4 was precipitated contained ubiquitin. This implies that the requirement for Grr1 was due to an ubiquitinated form of Gis4 interacting with Snf1. However, it remained possible that Grr1 was required to bridge Gis4 and Snf1, even though there was no suggestion that Grr1 interacted with Snf1 directly. A simple yeast two-hybrid assay with Grr1 and Snf1 suggested that Grr1 and Snf1 did not interact. However, we examined this further. When the SCF complex was inactivated, but Grr1 remained present, Gis4 was no longer able to interact with Snf1. This strengthens the hypothesis that ubiquitinated Gis4 interacts with Snf1 and accounts for the requirement for Grr1 in this interaction.

Ubiquitination may be the signal for Gis4 to interact with Snf1. Ubiquitination may change the status of Gis4 in terms of its binding partners, rather than acting as a signal for degradation. Given that Gis4 appears to be ubiquitinated in glucose and that Snf1 is inactive in glucose, another modification may be required after it is ubiquitinated, thus allowing Gis4 to interact with Snf1 under the appropriate conditions. Alternatively, there may be other factors or proteins involved in the ultimate regulation of this interaction. It remains clear, however, that ubiquitination is an important step in this process.

While Gis4 may interact with Snf1, such an interaction by itself does not indicate whether Gis4 can affect the activity of the Snf1 kinase

complex. Direct methods of assessing this would require a known substrate. Snf1 has many substrates, and it is not immediately clear which of these substrates Gis4 may affect. Other authors have used a more indirect method of assaying this by examining Snf1's activity through its interaction with Snf4 and examining reporter expression due to the interaction. Using this method, when Gis4 was over-expressed and Snf1 activity was assessed, I found that Gis4 was able to activate Snf1 in both repressing and de-repressing conditions.

Upon purification of Gis4 that had been over-expressed, I found that Gis4 could co-precipitate a higher-molecular-weight form of Snf1. Snf1 is active when it is phosphorylated at residue T210. It would follow that this is the form with which Gis4 interacts, because over-expression of Gis4 is able to activate the Snf1 complex even in glucose, making the presence of active Snf1 possible under these experimental conditions.

Other laboratories have recently created an antibody against phosphorylated Snf1. This antibody could be used to test whether the higher-molecular-weight form of Snf1 is truly phosphorylated Snf1. Alternatively, the incorporation of $\gamma$-$^{32}$P into the yeast could be used; the purification of the Gis4 should bring down a radio-labeled species that we could detect as phosphorylated Snf1.

Given that Gis4 is able to activate the Snf1 kinase complex, I considered the possibility that Gis4 may be an as yet unidentified beta subunit of the Snf1 complex. However, the 3 known beta subunits (Sip1,

Sip2, and Gal83) share a similar Snf1 binding domain, and Gis4 does not appear to share any amount of homology with these proteins. Additionally, the complex described by Ho *et al.* containing Snf1, Gis4, and ubiquitin, also contains Gal83 (but not the other beta subunits), suggesting that Gal83 already provides for the beta subunit required for Snf1 activity and that Gis4 has another role in the Snf1 kinase complex.

**Deletion of Gis4 inhibits de-repression of Snf1-dependent transcripts**

Interaction with Grr1 and activation of Snf1 could indicate that Gis4 communicates a signal from Grr1 to Snf1 to de-repress the appropriate transcript. Accordingly, when Gis4 is deleted, SUC2 and CYC1, the Snf1-dependent transcripts, are unable to become de-repressed, yet GAL1 is unaffected. Flick and Johnston found similar phenotypes in *grr1Δ* mutants [30]. GAL1 was defective in repression, yet SUC2 and CYC1 were defective in de-repression. Given that Gis4 protein levels and its association with Snf1 are Grr1-dependent, it follows that the *gis4Δ* and *grr1Δ* mutants have similar, but not identical phenotypes.

These data pose the question of why GAL1 and SUC2 are regulated differently in these mutants when both transcripts are glucose-repressed. Given that Mig1 is the primary, and possibly sole, transcriptional repressor of GAL1, it is unlikely that Mig1 is the target for Snf1

activation via Gis4. I propose that Gis4 aids in complete de-repression of SUC2 and CYC1 by relieving another, secondary, repressor.

SUC2 is repressed by a variety of proteins, including Mig2, Mig3, Ngr1/2, Med8, Gcr1, Hxk2, and possibly Rgt1 [63, 87-95]. This could explain why SUC2 remains repressed under de-repressing conditions, while GAL1 becomes activated. Under de-repressing conditions, Mig1 is phosphorylated by Snf1 and removed from various promoters and/or from its co-repressors Ssn6 and Tup1 [96]. However, under these conditions Gis4 is required to remove the additional repressors specific to the SUC2 and CYC1 promoters. Mig2 is an unlikely candidate since it appears that the effects of Gis4 are moderated through Snf1, and Mig2 is not a Snf1-dependent repressor [63]. But possible candidates include Mig3, Ngr1/2, Hxt2, and Rgt1, which all associate with Snf1.

The expression of Gis4 increases in raffinose (and glycerol). This increase could represent the requirement for de-repression of SUC2, an enzyme required for metabolism of raffinose. Gis4 levels do not change in different concentrations of glucose or galactose. Again, this finding corresponds with the fact that Gis4 has no effect on repression in glucose or galactose metabolism (i.e. GAL1 expression), but instead affects SUC2 expression.

Again, if Gis4 is in fact is important to relieve a second repressor from Snf1-dependent transcripts, it is possible that Gis4 affects other genes regulated by this "second repressor", perhaps even to a greater

degree. Identification of this repressor could be identified by creating double mutants of *gis4Δ* and of the deletion of potential genes for the repressors. The correct repressor mutant should be able to suppress the lack of de-repression seen in *gis4Δ* cells. This next step would help to refine the model I propose below.

**Grr1 communicates with the Snf1 pathway via Gis4**

I propose that Gis4 is ubiquitinated by Grr1 and is then able to interact with, and activate, Snf1. This allows for complete de-repression of the SUC2 and CYC1 transcripts. This model suggests that ubiquitination is a signal to allow Gis4 to communicate with Snf1. (See Figure 20.)

Flick and Johnston [30] have previously demonstrated that in *grr1Δ* mutants, repression of GAL and SUC2 was defective. However, only de-repression of SUC2 was defective, while de-repression of GAL was apparently normal, suggesting a more universal method of repression for the genes but a more selective mode of de-repression. Gis4 appears to be part of this selective de-repression for SUC2. Since Gis4 protein levels are low when Grr1 is deleted and it is not ubiquitinated, SUC2 (and potentially others) may not be successfully de-repressed in *grr1Δ* mutants due to the inactivity of Gis4. This would further explain Flick and Johnston's findings that *grr1Δ* mutant strains grow slowly on raffinose and glucose, but not galactose and glycerol. In

raffinose, SUC2 is not fully expressed in these mutants and similarly, Rgt1 is not inactivated in glucose. However, the de-repression of GAL is not affected, allowing these cells to grow well in galactose media.

Overall, it appears that in wild-type cells in glucose, Gis4 is ubiquitinated, but does not work with Snf1 to relieve transcriptional repressors, possibly because Snf1 is not activated (Figure 20a, panel A). In low- or no-glucose conditions (Figure 20a, panel B) Gis4 is required to remove additional repressors from certain Snf1-dependent transcripts, including SUC2 and CYC1. In the absence of Gis4, GAL1 is still completely de-repressed because Snf1 is able to phosphorylate and inactivate Mig1, its sole repressor. However, SUC2 and CYC1 are unable to be active at high levels because while Mig1 is removed, other repressors may still be in effect due to the absence of Gis4. Finally, in *grr1Δ* cells under low-glucose conditions (Figure 20b, panel C) these same genes would be expressed at low rates because Gis4 is also at low levels in *grr1Δ* cells. There may not be sufficient Gis4 to completely alleviate repression in low-glucose conditions, so these cells will have difficulty growing on raffinose etc. as seen previously. Additionally, Grr1 may be required for alleviation of secondary repressors; otherwise over-expression of *gis4Δ* would not be lethal on non-glucose carbon sources. However, I have not yet determined the identity of the secondary repressor.

It remains possible that Gis4 is able to affect other Snf1-dependent transcripts not tested here. Gis4 did not have a dramatic effect on the growth of cells in different media. This could be because it is simply part of a functionally redundant pathway. Candidates include SUC2, as well as GAL1 and CYC1, which are all commonly studied genes and are affected by many proteins and conditions.

Gis4 may have a role in other pathways that would yield a more dramatic result under appropriate conditions. For example, Grr1 and Snf1 have both been implicated in meiosis. In fact, Snf1 is responsible for regulation of IME1, the major initiator of meiosis. Additionally, meiosis only occurs under proper environmental conditions. Specifically, meiosis occurs in diploid cells in the absence of nitrogen and glucose, and it requires the presence of a non-fermentable carbon source [97]. While *gis4Δ* mutants do not appear to have meiotic defects, since Gis4 interacts with both proteins, it could potentially be important in this pathway. However, with *grr1Δ* cells having so many phenotypes and Snf1 having so many different substrates, there are other possibilities as well, including salt tolerance and adapting to stressful conditions.

The finer points of carbon-source sensing and metabolism have yet to be elucidated, including understanding how *grr1Δ* cells are able to express glucose repressed transcripts at high levels in glucose and how Gis4 relieves transcriptional repression at the molecular level.

Additionally, while ubiquitination of Gis4 is critical for its interaction with Snf1, the signal that differentiates high versus low glucose to Gis4 is unknown. Grr1 is thought to receive a signal from the glucose sensors Snf3 and Rgt2, but how this signal is transduced remains unclear as well. Perhaps understanding of the signaling mechanism that indicates levels of glucose will lead to further understanding of how Grr1, Gis4, and Snf1 cooperate to regulate glucose-repressed transcripts. Regardless, Gis4 is an example of a protein that is important to the cross-talk of glucose metabolism and signaling pathways.

**Ubiquitin Ligases and Cancer Therapeutics**

Ultimately, the study of ubiquitin ligases and their substrates is important both for the pathways that the substrates operate in, but also in understanding the general mechanism of ubiquitination. For example, ubiquitination is critical for proper progression of the cell cycle. Aberrant cell cycles may result in cancer. To prevent this, proteasome inhibitors have been used as possible therapeutics for cancer. However, the effects of these drugs are broad and effect pathways that may not be contributing to the aberrant growth.

More recently studies have focused on more specific targets such as ubiquitin ligases. Consequently, a variety of inhibitors of various parts of the ubiquitination pathway have been developed. For example,

Lactacystin and Bortezomib inhibit the 20S proteasome, and E3-specific inhibitors such as the Nutlins and Protac-1 inhibit Mdm2 and $SCF^{Skp2}$, respectively. Inhibition of the proteasome by Lactacystin and Bortezomib has been shown to promote apoptosis to a greater extent in transformed cells than normal cells, but is no longer studied as a therapeutic due to the broad and non-specific activities of these drugs. Nutlin-3 is a small molecule inhibitor of p53's Mdm3 binding pocket. This stabilizes the tumor-suppressor protein p53 leading to "cell cycle arrest, apoptosis, and growth inhibition of human tumor xenografts in nude mice" [98].

The successful application of E3-specific drugs underlines the importance in understanding the mechanism of ubiquitination. More importantly, it highlights the importance of grasping how substrates bind the ubiquitin ligases and become ubiquitinated. A more complete understanding of this binding may lead to a more focused effort to design drugs that affect the E3-ligases and ultimately may aid in the development of potential therapeutics for cancer treatment.

# Chapter 5

# Summary and Concluding Remarks

I have described the identification of Gis4 as a protein involved in glucose repression. It interacts with both Grr1 and Snf1, and data suggest it may coordinate the glucose repression/induction pathways. Gis4 is not an essential protein, nor is it critical for the Snf1 kinase complex or the $SCF^{Grr1}$ complex. However, it is the first clue as to how Grr1 can affect Snf1-dependent transcripts, and as such, this finding is important for understanding how the glucose repression and induction pathways communicate for efficient metabolism of carbon sources.

Given that the regulation of genes involved in the glucose repression and induction pathways is redundant and exceptionally complicated, Gis4 is most likely not the only protein involved in coordinating the pathways. Indeed, Std1 is also able to activate the Snf1 kinase, and Std1 may be degraded in a Grr1-dependent manner, like its paralogue Mth1. However, Std1's effects may be more specific to the glucose induction pathway; it appears to work at the level of Snf3/Rgt2 and at Rgt1/HXT expression but its interaction with Snf1 indicates another level of coordination between the pathways.

Crosstalk between the pathways has already been demonstrated to be complicated and functionally redundant. A variety of repressors and activators coordinate to properly repress or induce the correct genes under the appropriate conditions. The role demonstrated here for Gis4 in glucose repression and induction may be more complicated. For

example, the coordination of Gis4 protein expression with Grr1 protein levels may indicate another level of control for Gis4 in relation to Grr1 and the glucose-dependent pathways.

Another important feature of Gis4 is that it is a new substrate of $SCF^{Grr1}$. Understanding how these F box proteins select substrates for ubiquitination has yet to be made clear. Identifying additional substrates allows a better comparison to other substrates, adding to the pool of knowledge that is essential to further the understanding of substrate selection. However, identifying them is also technically challenging. Initial comparisons of Grr1 substrates, including Gis4, do not yield any similarities at the sequence level. The substrates vary greatly in size and function in a cell and are difficult to evaluate.

While the data presented here do not shed light specifically on substrate selection, it does describe a unique substrate that may aid in the understanding of substrate selection. Because Gis4 is the first stable substrate of Grr1 to be described, it may be evidence that the outcome of a substrate can be deduced based upon its interaction with its F box protein. For example, if Gis4 interacts with Grr1 in a novel way (not in the same region as the cyclins or Mth1), it provides evidence for a class of substrates with a different outcome than degradation.

The process of ubiquitin chain formation is also not well understood. Are ubiquitin chains or single ubiquitin molecules passed

from E1 to E2 to E3? Or does an E4 expand a ubiquitin chain after the E3 has mono-ubiquitinated a substrate? In either case, how does the E3 (or E4) ligase define the type and length of ubiquitin chain that is to be attached to the substrate? The identification of Gis4 as a stable substrate of $SCF^{Grr1}$ indicates that F box proteins do not confer a single fate upon a substrate. It instead suggests that the substrate dictates its own fate upon interaction with the SCF complex. Indeed, Gis4 is able to interact with Grr1 so it is able to interact with Snf1; however, Cln2 interacts with Grr1 to commence its degradation.

Flick *et al.* suggest that Met4 is not degraded, but instead has either a ubiquitin chain too small to be recognized by the proteasome or that the chain is obscured. A discrete laddering pattern is observed for Met4, consistent with a small chain. When ubiquitinated Gis4 is probed for, experiments indicate that the laddering is longer; the pattern is not as short as Flick *et al.* see with Met4. Gis4 may have longer, non-K48 linked chains, or they may be ubiquitinated at multiple lysines. That Gis4 has non-K48 chains is speculation, but it would be interesting to compare the type of ubiquitination on Gis4 compared to that on an unstable protein such as Cln2. This would also shed light onto the process of ubiquitination and the fate of ubiquitinated substrates.

To determine the type of ubiquitin chains that Gis4 is modified with requires further analysis. Mass spectrometry is a common method to do this and it may be used to identify the exact type of modification

occurring. Ubiquitin mutants exist that have one of ubiqutin's several lysines altered to another amino acid, so that chains cannot form using this lysine. Employing these mutants and probing for ubiquitinated Gis4 allows us to determine the type of chain employed by various substrates.

This work presents the first description of a protein that is ubiquitinated in a manner that is different from other substrates of $SCF^{Grr1}$. Moreover, it is the first description as to how Grr1 acts on the Snf1 pathway to regulate glucose repression. The substrates of Grr1 will be a useful tool for understanding the processes of ubiquitination and glucose repression further.

# Chapter 6

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# Chapter 7

# Appendices

## Appendix I

*"Searching for Grr1 interacting proteins-*

*the Yeast Two Hybrid Screen in detail"*

To identify other proteins that Grr1 interacts with, we conducted a yeast two hybrid screen with Grr1. It was our expectation that by preventing ubiquitination upon substrate interaction with $SCF^{Grr1}$, we would augment our ability to identify substrates that might otherwise not be identified because of a transient interaction and subsequent degradation by $SCF^{Grr1}$. To improve the probability of finding substrates, we used a mutant form of Grr1 that has a conserved residue of the F box motif [Bai, 1996 #18] mutated to abrogate its binding to Skp1 and the SCF complex. The mutant, $Grr1^{P321A}$, was synthesized by means of PCR. It replaced the F box motif in the wild type version of Grr1, already cloned into the bait plasmid.

Using this mutant, $Grr1^{P321A}$, and a yeast genomic library provided as a courtesy from Robb Moses, we screened $2.4X10^4$ clones and isolated 39 positives. Positives were selected first on media lacking adenine, and then on media lacking both adenine and histidine. (These plates also lacked leucine and tryptophan to maintain the bait and prey plasmids.) Positive clones were streaked out to obtain single clones and the plasmid DNA from these clones was harvested. To eliminate false-

positives, the DNA was retransformed into the strain already containing the Grr1$^{P321A}$ plasmid, and the interaction was re-tested.

Upon eliminating the false-positives, the clones were tested using the full range of reporter constructs in the PJ69 strain; growth on media lacking adenine and histidine, as well as assessed for β-galactosidase activity. Figure 21. We sequenced the strongest interactors initially learned that we had isolated Gis4 (YML006c), a transposable element (YDRW Ty2-2), as well as the open reading frames of YOL063c and YIL105c. The transposable element was dropped from the screen, but we examined what was known about the other ORF's to determine how they might relate to Grr1.

YIL105c was lacking part of the its N-terminus in the yeast genomic library. It was identified with Mck1 as part of an immuno-precipitation, a kinase involved in IME1 transcription and meiosis [Weng, 2003 #373]. We postulated that YIL105C could be involved with the mitotic CDK complex. Grr1 is required to fully repress initiation of meiosis and Grr1 prevents glucose repression of later stages of meiosis via Cln2 and Rgt1.

Alternatively, YIL105c may be involved in the actin cytoskeleton. A mutation in YIL105C is synthetically lethal in combination with a mutation in Mss4, a gene known to be involved in actin cytoskeleton organization [Weng, 2003 #373]. The mutation in YIL105c indicates a genetic association between YIL105c and actin cytoskeleton organization.

This is interesting because $SCF^{Grr1}$ is known to target Gic2 for degradation and Gic2 is involved in actin polarization in mother/daughter cells. It was possible that its association with Grr1 is along the lines of the actin cytoskeleton, somewhat similar to that of Gic2.

YOL063 is not a well characterized ORF. It lacked its N-terminus as part of the yeast genomic library. Database queries suggest that it interacts with Mga1 [Weng, 2003 #373]. Mga1 has been tentatively identified as a gene involved in filamentous growth [Christie, 2004 #371]. Grr1 mutants have an increase in filamentous and pseudohyphal growth. It could be possible that the effects seen in Grr1 mutants are through its interaction with YOL063c, and indirectly, with Mga1.

We also identified Gis4 in the screen. At the time of this work, I also found that Gis4 interacted with Snf1 according to a genome wide immuno-precipitation screen, and that ubiquitin may be part of this complex. Additionally, there was more information about Gis4; Balciunas *et al.* had published one paper identifying it as part of a suppression screen for proteins that could permit the *snf1Δmig1Δsrb8Δ* mutant cells to grow on galactose. Given the information we had at the time, I pursued Gis4 and its interaction with Grr1 further.

## *Appendix II*

"Characterizing the Grr1-Cln2 interaction"

### Background

*Cell Division and $SCF^{Grr1}$*

Ubiquitin is essential in eukaryotes and plays a critical role in a variety of pathways. Its role in the cell cycle is well documented. Cell division is a precarious event that needs to occur in a stepwise fashion. Ubiquitination and degradation prevents the cell cycle from being able to go in reverse; proteins are degraded and no longer available.

The cell cycle is tightly regulated and is advanced by cyclins and cyclin dependent kinases (CDK's). There are cyclins specific to each phase of the cell cycle and they interact with the CDK's, forming an active kinase complex. To prevent early entrance into each phase, there are also cyclin dependent kinase inhibitors (CKI's). These inhibitors are vital to ensure that the cell does not undergo the next phase if the cell is damaged in some way. (Reviewed in [Deshaies, 1999 #39])

In budding yeast, there are three major cyclin families; the G1 cyclins (Cln1-3), the S-phase cyclins (Clb5-6), and the mitotic cyclins (Clb1-4). There is only one common cyclin dependent kinase in yeast, Cdc28, to which each of the above cyclins bind. At G1, the cyclins Cln1-3 are required to pass "Start," the point of commitment to another round

of cell division that occurs just before DNA replication. Far1 is a CKI that inhibits the Cln-Cdc28 complexes to arrest cells at Start in response to mating pheromones. Similarly, Sic1 is a CKI that inhibits the Clb-Cdc28 complex that prevents premature DNA replication in G1 and later, aids in exit from mitosis (Reviewed in [Willems, 1999 #366]).

To undergo a round of cell division, phase-specific cyclins and CKI's like Far1 and Sic1 need to be removed at appropriate times. As such, they are targets of ubiquitination and degradation. For example, the Cln2-Cdc28 complex, phosphorylates Sic1. Phosphorylation of Sic1 signals it to be targeted by $SCF^{Cdc4}$ for ubiquitination and degradation [Feldman, 1997 #27]. Upon degradation of Sic1, the G1 cyclins become phosphorylated by auto-phosphorylation from Cdc28 and are targeted for ubiquitination and degradation by $SCF^{Grr1}$ [Barral Y, 1995 #15;Ceccarelli, 2001 #96;Lanker, 1996 #133]. This process allows the cell to enter S phase because the S-phase inhibitor has been degraded. Ubiquitination prevents cycling backwards into G1 because there are now no G1 cyclins to signal and indicate G1 phase.

Ubiquitination is a critical event for the cell cycle and is maintained by two major ubiquitin ligase complexes: the SCF complex and the Anaphase Promoting Complex (APC). The SCF complex is important for the G1-S transition (at "Start") and at the entry into mitosis. The APC complex is required for the cell to enter Anaphase and to exit mitosis. Each complex selects the proper substrate to be

degraded at these critical intervals. The group of substrates specific to each complex is not necessarily related in sequence or function, which makes understanding these ubiquitin ligases difficult.

As mentioned earlier, the G1 cyclins are ubiquitinated by the SCF complex. While the G1 cyclins are similar in sequence and in function, not all substrates of a particular F box protein are similar. Sequence analysis of several Grr1 substrates (Cln1, Cln2, Gic2) does not indicate any obvious similarities that would explain why they bind Grr1. One similarity between substrates is that they are generally phosphorylated before being targeted for ubiquitination. This is a potential signal that the substrate should be degraded. As mentioned above, Cln2 is phosphorylated by Cdc28/Cln complex as G1 phase progresses forward [Barral Y, 1995 #15;Ceccarelli, 2001 #96]. Additionally, analysis of the domain in Grr1 that interacts with Cln2, the Leucine Rich Repeat (LRR), by molecular modeling suggested that the concave side of the LRR domain has a large number of positively charged residues. The negative charge of the phosphate groups may aid the interaction between Grr1 and Cln2. Binding experiments by co-immuno-precipitation indicates that these positively charged residues in Grr1 are important for the interaction with Cln2 [Hsiung, 2001 #87].

In addition to phosphorylation, Cln2 contains a PEST motif ([Salama SR, 1994 #11]. PEST motifs are acidic domains that have been thought to indicate an unstable protein [Rechsteiner, 1996 #262]. Our

studies have shown that this PEST motif is important in the interaction between Grr1 and Cln2 [Berset, 2002 #129]. These acidic residues may contribute additionally to the ionic interaction that may be occurring through the phosphate groups and the PEST domains on Cln2 and the basic residues along the inside of Grr1's LRR domain [Hsiung, 2001 #87].

*Experimental Design/Hypothesis*

Grr1 and Cln2 have been shown to interact by means of the LRR domain of Grr1 and a region containing a PEST motif in Cln2 [Berset, 2002 #129]. We believe that this interaction is contributed to by a variety of ionic interactions and I set out to examine this in detail. To identify critical residues important for the Grr1-Cln2 interaction, I wanted to use the reverse yeast two hybrid system and identify mutations in the two proteins that contribute to the interaction. The mutations would be generated by error-prone PCR. I expect that residues important for the LRR and PEST motifs will be identified as well as charged residues along these regions.

The reverse two hybrid system is similar to the classic two hybrid system in that protein-protein interactions are assessed by virtue of their ability to drive expression of reporter constructs using proteins fused to either a DNA binding domain (BD) or an Activation Domain (AD). Traditionally, these reporter constructs encode an enzyme required for

growth- without expression of the enzyme, the yeast will not grow on selective media. Genes frequently used include ADE2, HIS3, and URA3, allowing growth on media lacking adenine, histidine, and uracil, respectively.

However, when cells expressing URA3 are grown in the presence of 5-fluoro-orotic acid (5FOA), the enzyme orotidine-5'-phosphate decarboxylase coded for by URA3 converts the 5FOA into 5-fluorouracil, a toxic substance. This can be used then to screen for proteins that do not interact. In our case, the reverse two hybrid screen would allow us to select for Grr1 or Cln2 constructs that have been mutated in such a way that the two proteins no longer interact. This would give us a set of mutations to examine to characterize the Grr1-Cln2 interaction in detail.

## Materials and Methods

***Yeast Two Hybrid Assay:*** The yeast two hybrid screen was conducted using the MatchMaker (Clontech) system and the ProQuest (Invitrogen) system. The SLY477 strain was transformed with indicated plasmids and selected for by growing on plates lacking tryptophan and leucine. Clones were selected and tested by examining growth on plates lacking leucine, tryptophan, and either adenine, histidine, uracil, or containing 5FOA, as indicated. Clones were classified by their levels of interaction (weak to strong).

***LacZ Activity Assay:*** Yeast strains with the appropriate plasmid containing the reporter construct were grown to log phase in the presence of 2% glucose and harvested. 10 µg of whole cell extract was added to 80 µl of 4 mg/mL o-nitrophenyl galactopyranoside (ONPG, Sigma Chemical Co.) and volume was brought up to 350 µL using Z buffer (Clontech Matchmaker System Protocol [Zhu, 1997 #369]). The reaction was stopped by the addition of 200 µl 1M $NaCO_3$ (Sigma Chemical Co.), once the solution turned yellow and it was quantified by absorbance at the 420 nm wavelength. "Unit Activity" represents color development in minutes per µg of protein. The assay was done using 3 different transformants, each in triplicate, for each carbon source and plasmid.

***Strains Used in This Study***

| Strain | Genotype | Source |
|---|---|---|
| AH109 | MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : $GAL1_{UAS\text{-}GAL1TATA}$-HIS3, $GAL2_{UAS\text{-}GAL2TATA}$-ADE2, URA3 : : $MEL1_{UAS\text{-}MEL1\ TATA}$-LacZ | Clontech |
| PJ69 | MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS\text{-}GAL1TATA}$-HIS3; $GAL2_{UAS\text{-}GAL2TATA}$-ADE2, URA3::$GAL7_{UAS\text{-}GAL7TATA}$-lacZ | |
| MaV201 | MATa, leu2-3,112, trp1-901, his3Δ200, ade2-101, gal4Δ, gal80Δ, SPAL10:: URA3, GAL1:: lacZ, $HIS3_{UAS\ GAL1}$ ::HIS3@ LYS2, $can1^R$, $cyh2^R$ | Invitrogen |
| MaV203 "A" | pPC97 empty vector, pPC86 empty vector | Invitrogen |
| MaV203 "B" | pPC97-human RB aa 302-928, pPC86 human E2F1 aa 342-437 | Invitrogen |
| MaV203 "C" | pPC97-*CYH2* –*Drosophila* DP aa 1-337, pPC86 –*Drosophila* E2F aa 225-433 | Invitrogen |
| MaV203 "D" | pPC97 rat c-Fos aa 132-211, | Invitrogen |

| | | |
|---|---|---|
| | pPC86 mouse c-Jun aa 250-325 | |
| MaV203 "E" | pCL -full length GAL4 aa 1-881,<br>pPC86 empty vector | Invitrogen |

**Plasmids Used in This Study**

| **Plasmid** | **Genotype** | **Source** |
|---|---|---|
| pSL 310 | pGADT7 | Clontech |
| pSL 311 | pGADT7-SV40 Large T Fusion | Clontech |
| pSL 312 | pCL (full length GAL4 protein) | Clontech |
| pSL 313 | pGBKT7 | Clontech |
| pSL 314 | pGBKT7- p53 | Clontech |
| pSL 315 | pGBKT7- Lamin | Clontech |
| | pGBKT7-hSkp2 | S. Tokarz |
| pSL 353 | pGBKT7-Grr1 | This Study |
| pSL 356 | pGADT7-Cln2 | This Study |
| pSL 405 | pGBKT7-Cdc28 | This Study |
| pSL 410 | pGBKT7-Grr1$^{P321A}$ (f box mutant) | This Study |
| pSL 441 | Spo13 URS, GAL1:URA3 | [Vidal, 1996 #76] |
| pSL 551 | pGADT7-ySkp1 | This Study |

## Results

*Grr1 interacts with Cln2 in the Classic Two Hybrid System*

Initially it was important to ensure that Grr1 and Cln2 had strong, reproducible interactions in this type of system. Grr1 was cloned into a vector that fused its N-terminus to the GAL4 DNA binding domain. Similarly, Cln2 was cloned into a vector fusing its N-terminus to the GAL4 activation domain. I was able to confirm via Western blotting that my proteins were being expressed from the two hybrid vectors and that they were expressed at a size consistent to a fusion to their respective GAL4 domains (data not shown).

I assayed the two hybrid interaction of Grr1 and Cln2 as well as appropriate controls in the AH109 yeast strain. I tested the interaction using the AH109 reporters. The AH109 strain expresses these reporters at varying levels due to differing promoters and promoter strength. The strongest expression is from the ADE2 ($GAL2_{UAS}$-$GAL2_{TATA}$-ADE2) reporter, followed by the HIS3 ($GAL1_{UAS}$-$GAL1_{TATA}$-HIS3) reporter. The LacZ expression is particularly weak due to its MEL1 promoter ($MEL1_{UAS}$-$MEL1_{TATA}$-lacZ).

The Grr1-Cln2 interaction was clear on the growth media as well as on LacZ. It was strong enough to be identifiable, but not as strong as moderate positive controls. When a quantitative analysis was done on the interaction using beta-galactosidase activity of three clones, one had activity above that of the positive control and the other 2 were essentially

weaker interactions. It was proposed that the variation among the clones might represent a varied number of plasmids in each clone, causing differing amounts of activity (Figure 22).

To see if the strain background could enhance the Grr1-Cln2 interaction, we repeated the growth assays in a related two hybrid strain, PJ69. The PJ69 strain is a parent strain of Clontech's two hybrid strains AH109 and Y187. It has the same ADE2 and HIS3 reporters as AH109, but has a stronger promoter fused to LacZ, as seen in the Y187 strain ($GAL7_{UAS}$-$GAL7_{TATA}$-lacZ). Our results in this strain did not significantly differ from those we saw in AH109. (Figure 23, fourth line).

*Grr1 and Cln2 do not interact in the Reverse Two Hybrid System*

To test if the interaction we saw in AH109 and PJ69 was strong enough to work in the reverse two hybrid system required a URA3 reporter construct. However, these strains have only the adenine, histidine, and LacZ reporter constructs, so I went about creating a GAL4p:URA3 reporter construct in the PJ69 background, chosen because it has universally stronger promoters than AH109.

The construct was created according to the technique that Mark Vidal had already established- by using a Spo13 promoter with Gal4 binding domains and fusing it to URA3 [Vidal, 1999 #125]. I used PCR to make this Spo13p:URA3 fusion in preparation for chromosomal recombination. Because PJ69 contains a URA3 mutation, I included

enough of the URA3 gene in the fusion to eliminate the current mutation (URA3-52) where a transposable element was inserted into the gene, effectively eliminating protein function [Rose M, 1984 #2].

During the creation of this construct, we found that the Rosalie Sears lab (OHSU) had obtained a new two hybrid system from Invitrogen that employed the MaV201/3 strains. These strains contained the following reporter constructs: $GAL1_{UAS}$-HIS3, $GAL1_{UAS}$-LacZ, and GAL4 binding sites in a Spo13 promoter (with an intact repressor binding sequence) fused to the URA3 gene. The URA3 construct was identical to that designed by Mark Vidal. I investigated the Grr1-Cln2 interaction in this strain to determine if it was a viable alternative to creating a reverse two hybrid strain from the AH109 yeast.

I found that the interaction on histidine drop out media was as selective as seen in the previous strains. However, the Grr1-Cln2 interaction was apparently not strong enough to overcome the repression of the Spo13 promoter; the interaction was negative using selection media lacking uracil. Conversely, when grown on 5-FOA media (to select against interacting protein partners), the Grr1-Cln2 interaction was positive. This again indicates that they did not interact strongly enough to drive transcription of the URA3 gene. (Figure 24)

*Generation of an F box Mutant*

Because the interaction was not robust among our clones, we investigated a way to resolve this. One potential reason for the lack of activity is that Cln2 is a substrate that is ubiquitinated upon interacting with Grr1 and degraded. We postulated that if we disabled Grr1 from interacting with Skp1, thus preventing ubiquitination of substrates, we could strengthen the interaction between Grr1 and Cln2 in the two hybrid system. Bai *et al.* reported a sequence alignment of the F box motif in F box proteins and identified conserved sequences. The F box motif is responsible for the interaction of the F box protein with Skp1. I proceeded to mutate a conserved residue in the F box motif in Grr1 that Bai *et al.* showed was sufficient to abrogate binding to Skp1. Using PCR, I created $Grr1^{P321A}$.

This mutant was employed in the two hybrid assay with Cln2. As before, I verified that it was being expressed from its two hybrid vector. When examining the interaction of $Grr1^{P321A}$ with Cln2 using the reporters listed above, we found that the mutant appeared to have a stronger interaction with Cln2. However, upon further analysis we discovered that $Grr1^{P321A}$ and the empty vector drove some activity by itself, depending upon the strain used. This might indicate interesting possibilities for the role of Grr1 in transcriptional activation, but ultimately was not a useful tool to examine the Grr1-Cln2 interaction.

## Conclusions

Interactions between a ubiquitin ligase and its substrates are traditionally difficult to identify. This may be due to the transient interaction between the binding partners, but degradation of the substrate may add additional problems. In genome wide studies to define protein-protein interactions, identification of F box protein and substrate pairs have been rare. To date, no genome wide screen has pulled out Grr1 and Cln2. Grr1 has been identified with members of the SCF complex, and Cln2 has been detected in Cdc28 kinase complexes, but there is no published association of Grr1 and Cln2 in large yeast two hybrid screens [Peng, 2003 #246; Hazbun, 2002 #336; Uetz, 2000 #46]. Bacterial systems have not been used because substrates generally require specific modifications (i.e. phosphorylation) to prime them for ubiquitination.

In addition to the difficulty of identifying F box proteins and their substrates in protein interaction screens, it is also difficult to identify short-lived proteins that are ubiquitinated in a screen specifically looking for ubiquitinated proteins. Peng *et al.* purified ubiquitinated proteins via 6His-ubiquitin and identified the proteins by multidimensional liquid chromatography coupled with tandem mass spectrometry. They note, "We failed to detect a number of known, short-lived regulators of the cell cycle (e.g. Sic1, Cln1, Cln2, Clb1, Clb2, Clb3, and Clb4) [Peng, 2003 #246]. Many of these proteins are degraded so quickly after

ubiquitination that they can be measured only after stabilization either by chemical inhibition of the proteasome or after genetic deletion of required E2 or E3 ligases." While they did not look for protein interactions, this is important to note when trying to assay with Cln2.

Given the difficulties of working with a short lived protein (Cln2) that is degraded immediately upon interaction with Grr1, it is not surprising that the interaction is difficult to detect. One feasible option to study this further would be to inhibit the proteasome. However, yeast do not absorb proteasome inhibitors through their cell wall. Yeast with mutations in the proteasome are very sick and difficult to work with effectively.

My data ultimately suggests that the transient interaction between Grr1 and Cln2 is not strong enough, even when the proteins are over-expressed, to use as a measure in this type of large-scale screen. Unfortunately, when Grr1 is fused to the DNA binding domain and contains the F box mutation (P321A), it appears to have some background activity by itself. This makes it useless as way to stabilize the Grr1-Cln2 interaction. Even if the interaction is enhanced, it is not apparent over the background levels of $Grr1^{P321A}$ alone.

**Summarizing statement**

We conclude from this research that the reverse two hybrid system may not be an effective tool for our analysis of the Grr1-Cln2 interaction. While it would allow us to examine a large number of mutations quickly, it requires clear, reproducible interactions to screen the mutants effectively. Grr1 and Cln2 do not appear to provide a consistently strong enough interaction in the two hybrid system to use this tool.

# Appendix III:Genes/Proteins and their Mutant Phenotypes

| gene | aka | protein (if different) | function (if known) | mutant phenotype |
|---|---|---|---|---|
| ADE2 | sc | phosphoribosylamino-imidazole-carboxylase | catalyzes a step in the 'de novo' purine nucleotide biosynthetic pathway; | Null mutant is viable and requires adenine. ade2 mutants are blocked at a stage in the adenine biosynthetic pathway that causes an intermediate to accumulate in the vacuole; the intermediate gives the cell a red color. |
| AMPK | hs | AMP-activated protein kinase | Appears to act as a metabolic stress-sensing protein kinase switching off biosynthetic pathways when cellular ATP levels are depleted and when 5'-AMP rises in response to fuel limitation and/or hypoxia | |
| Cdc28 | CDK1, HSL5, SRM5 | cyclin-dependent protein kinase | Catalytic subunit of the main cell cycle cyclin-dependent kinase (CDK); alternately associates with | null mutant is inviable; arrests at G1/S transition arrests at G2/M transition |
| Cdc4 | sc | | F-box protein which associates with and directs SCF complexes (ubiquitin-protein ligases) to substrates | Null mutant is inviable. cdc4 mutants arrest in meiosis at the mononucleate stage with duplicated spindle pole bodies. |
| Cdc53 | sc | cullin | structural protein of SCF complexes (which also contain Skp1p, Cdc34p, and an F-box protein) involved in ubiquitination; SCF promotes the G1-S transition by targeting G1 cyclins and the Cln-CDK inhibitor Sic1p for degradation | null mutant is inviable; Cells arrest in G1 with active Cln kinases but no Clb-associated Cdc28p kinase activity |
| Clb | sc | G2 and M cyclins | cell cycle transitions | null mutant is viable |
| Cln1/2/3 | sc | G1 cyclins | promotes the G1-S transition | null mutant is viable, but triple deletion of Cln's is inviable |
| Cul1 | hs | | Essential component of the SCF ubiquitin ligase complex, Serves as a rigid scaffold that organizes the SKP1-F-box protein and RBX1 subunits. Aids in positioning of the substrate and the ubiquitin-conjugating enzyme. | |

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|---|---|---|---|---|
| Elm1 | sc LDB9 | Serine/threonine protein kinase | regulates cellular morphogenesis, septin behavior, and cytokinesis; required for the regulation of other kinases; forms part of the bud neck ring | null mutant is viable. Exhibits growth defect on a fermentable carbon source. Overexpression causes elongated bud formation |
| Far1 | sc | Cyclin-dependent kinase inhibitor | mediates cell cycle arrest in response to pheromone; also forms a complex with Cdc24p, Ste4p, and Ste18p that may specify the direction of polarized growth during mating; potential Cdc28p substrate | null mutant is viable |
| GAL1 | sc | Galactokinase | phosphorylates alpha-D-galactose to alpha-D-galactose-1-phosphate in the first step of galactose catabolism; expression regulated by Gal4p | Null mutant is viable and cannot utilize galactose. |
| GAL4 | sc GAL81 | | DNA-binding transcription factor required for the activation of the GAL genes in response to galactose; repressed by Gal80p and activated by Gal3p | Null mutant is viable, cannot utilize galactose as sole carbon source |
| GAL83 | sc SPM1 | | One of three possible beta-subunits of the Snf1 kinase complex, allows nuclear localization of the Snf1 kinase complex in the presence of a nonfermentable carbon source; contains glycogen-binding domain | null mutant is viable |
| Gic1/2 | sc | | Protein of unknown function involved in initiation of budding and cellular polarization, interacts with Cdc42p via the Cdc42/Rac-interactive binding (CRIB) domain | Null mutant is viable; gic1 gic2 double null is temperature sensitive at 33 degrees C |
| Gis4 | sc | | CAAX box containing protein of unknown function, proposed to be involved in the RAS/cAMP signaling pathway | null mutant is viable |
| Glc7 | sc CID1, DIS2, PP1, DIS2S1 | protein phosphatase type I | involved in many processes including glycogen metabolism, sporulation, and mitosis; interacts with multiple regulatory subunits; predominantly isolated with Sds22p | null mutant is inviable |

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|---|---|---|---|---|
| Grr1 | sc | CAT80, COT2, SSU2 | F-box protein component of the SCF ubiquitin-ligase complex, required for Cln1p and Cln2p degradation; involved in carbon catabolite repression, glucose-dependent divalent cation transport, high-affinity glucose transport, and morphogenesis | Null mutant is viable, resistant to high levels of divalent cations, sensitive to sulfite, and defective in high affinity glucose transport and glucose repression; null mutant also exibits an elongated cell morphology |
| HIS3 | sc | HIS10 | imidazoleglycerol-phosphate dehydratase | catalyzes the sixth step in histidine biosynthesis | mutations cause histidine auxotrophy and sensitivity to Cu, Co, and Ni salts |
| Hrt1 | sc | RBX1, ROC1, HRT2 | RING finger containing subunit of Skp1-Cullin-F-box ubiquitin protein ligases (SCF) | null mutant is inviable |
| Mig1 | sc | CAT4, SSN1, TDS22 | Transcription factor involved in glucose repression; C2H2 zinc finger protein which resembles the mammalian Egr and Wilms tumour proteins | Null mutant is viable, exhibits partial derepression of numerous glucose regulated transcripts; MIG1 overexpression and deletion studies suggest that other repressors such as MIG2 may act in a redundant fashion with MIG1 |
| Mig2 | sc | MLZ1 | Involved in repression, along with Mig1p, of SUC2 (invertase) expression by high levels of glucose; binds to Mig1p-binding sites in SUC2 promoter; Protein containing zinc fingers very similar to zinc fingers in Mig1p | Null mutant is viable; a strain that contains a double disruption of MIG1 and MIG2 is defective in glucose repression of SUC2 expression |
| Mig3 | sc | | Probable transcriptional repressor involved in response to toxic agents such as hydroxyurea that inhibit ribonucleotide reductase; phosphorylation by Snf1p or the Mec1p pathway inactivates Mig3p, allowing induction of damage response genes | viable exhibits growth defect on a non-fermentable (respiratory) carbon source |
| Mth1 | sc | BPC1, DGT1, HTR1 | Negative regulator of the glucose-sensing signal transduction pathway, required for repression of transcription by Rgt1p; interacts with Rgt1p and the Snf3p and Rgt2p glucose sensors; phosphorylated by Yck1p, triggering Mth1p degradation | Null mutant is viable; mth1 mutants are deficient in glucose update and transcription of glucose transporters; multicopy expression of HXT genes suppresses some defects of mth1 mutants |
| Pak1 | sc | | Upstream kinase for the SNF1 complex; partially | null mutant is viable and sensitive to caffeine |

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|---|---|---|---|---|
| Rbx1 | sc | ROC1, HRT1, HRT2 | RING finger containing subunit of Skp1-Cullin-F-box ubiquitin protein ligases (SCF) | null mutant is inviable |
| Reg1 | sc | HEX2, PZF240, SPP43, SRN1 | Regulatory subunit of type 1 protein phosphatase Glc7p, involved in negative regulation of glucose-repressible genes; involved in RNA processing | The null mutant is viable but will not grow on galactose and non-fermentalbe carbon sources. Null mutants overacumulate glycogen, grow slowly on maltose and sucrose, are intolerant of nitrogen starvation, and are larger than wild-type cells. |
| Rgt2 | sc | | Plasma membrane glucose receptor, highly similar to Snf3p; both Rgt2p and Snf3p serve as transmembrane glucose sensors generating an intracellular signal that induces expression of glucose transporter (HXT) genes | Dominant mutant suppresses growth defect of snf3 mutants on low concentrations of glucose or fructose |
| Roc1 | sc | RBX1, HRT1, HRT2 | RING finger containing subunit of Skp1-Cullin-F-box ubiquitin protein ligases (SCF) | null mutant is inviable |
| Sic1 | sc | SDB25 | inhibitor of Cdc28p-Clb5p protein kinase complex | Null mutant is viable, shows increased frequency of broken and lost chromosomes; sic1 deletion mutant rescues lethality of cln1 cln2 cln3 triple mutant. |
| Sip1 | sc | | Alternate beta-subunit of the Snf1p kinase complex, may confer substrate specificity; vacuolar protein containing KIS (Kinase-Interacting Sequence) and ASC (Association with Snf1 kinase Complex) domains involved in protein interactions | Null mutant is viable, exhibits a slight increase in GAL gene expression |
| Sip2 | sc | SPM2 | Member of a family of proteins, including Sip1p and Gal83p, that interact with Snf1p and Snf4p and are involved in the response to glucose starvation; component of Snf1 protein complex involved in response to glucose starvation | null mutant is viable |

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| Skp1 | sc | MGO1 | Evolutionarily conserved kinetochore protein that is part of multiple protein complexes, including the SCF ubiquitin ligase complex, the CBF3 complex that binds centromeric DNA, and the RAVE complex that regulates assembly of the V-ATPase | Null mutant is inviable, temperature-sensitive mutations in SKP1 arrest in G1 or G2 |
| Skp2 | hs | FBXL1 | Substrate recognition component of the SCFubiquitin ligase complex. Specifically recognizes phosphorylated CDKN1B/p27kip and is involved in regulation of G1/S transition. Degradation of CDKN1B/p27kip also requires CKS1. | |
| Snf1 | sc | CAT1, CCR1, GLC2, HAF3, PAS14 | Protein serine/threonine kinase | equired for release from glucose repression, invertase expression, sporulation, and for expression of catabolite-repressed genes when glucose is limiting; regulates Adr1p-dependent transcription primarily at the level of chromatin binding | Null mutant is viable, sensitive to heat stress and starvation and fails to accumulate glycogen during growth in rich medium; sucrose nonfermenting, |
| Snf3 | sc | | | glucose sensor | Null mutant is viable, defective in high affinity glucose transport, unable to grow on low glucose media, unable to grow on raffinose; |
| Snf4 | sc | CAT3, SCI1 | | involved in release from glucose repression, invertase expression, and sporulation; associates with Snf1p | Null mutant is viable, sucrose nonfermenting; |
| Srb8 | sc | GIG1, NUT6, SSN5, YCR080W, MED12 | | RNA polymerase II mediator complex subunit | null mutant is viable. exhibits growth defect on a non-fermentable (respiratory) carbon source |
| Srb10 | sc | GIG2, NUT7, SRB10, UME5, RYE5, CDK8 | | Component of RNA polymerase II holoenzyme, involved in RNA pol II carboxy-terminal domain phosphorylation | null mutant is viable, exhibits set of phenotypes common to strains defective in SSN6/TUP1-mediated transcriptional repression. Other mutations show unscheduled meiotic gene expression (derepression of early meiotic genes), suppression of SNF1. |

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|---|---|---|---|---|---|
| Srb11 | sc | GIG3, NUT9, SSN8, UME3, RYE2 | CycC | Component of RNA polymerase II holoenzyme, involved in RNA pol II carboxy-terminal domain phosphorylation | null mutant is viable, exhibits set of phenotypes common to strains defective in SSN6/TUP1-mediated transcriptional repression. Other mutations show unscheduled meiotic gene expression (derepression of early meiotic genes), suppression of SNF1. |
| Ssn6 | sc | CRT8,CYC8 | | General transcriptional co-repressor, acts together with Tup1p; also acts as part of a transcriptional co-activator complex that recruits the SWI/SNF and SAGA complexes to promoters | Null mutant is viable; high level constitutivity for invertase, clumpiness, temperature-sensitive growth, alpha-specific mating defects and failure of homozygous diploids to sporulate |
| Std1 | sc | MSN3, SFS3 | | Protein that interacts with the Snf1p protein kinase and Spt15p in two-hybrid and in in vitro binding studies | Null mutant is viable, no defects in mating or sporulation. Suppressor of TBP deletion; multicopy suppressor of SNF; std1-mth1 has defective glucose derepression and sporulation |
| SUC2 | sc | | invertase (sucrose hydrolyzing enzyme) | | Null mutant is viable but cannot ferment sucrose |
| Tor1 | sc | DRR1 | phosphatidylinositol kinase homolog | Involved in cell cycle signaling and meiosis; phosphatidylinositol kinase homolog | Null mutant is viable, grows slowly; rapamycin resistance, tor1 tor2 double mutant is inviable |
| Tup1 | sc | AAR1, AER2, AMM1, CRT4, CYC9, FLK1, ROX4, SFL2, UMR7 | | General repressor of transcription (with Cyc8p), mediates glucose repression; exhibits similarity to beta subunits of G proteins | Null mutant is viable; exhibits flocculent colony morphology |
| URA3 | sc | | orotidine-5'-phosphate decarboxylase | | uracil auxotroph |
| Yck1/2 | sc | CKI2 | casein kinase | membrane-bound casein kinase I homolog | Null mutant is viable; yck1 yck2 double deletion mutants are inviable; yck1 point mutants suppress defective Snf1p kinase activity in snf4 strains |

# Appendix IV: Glossary of Acronyms and Terms

| Term | Stands for: | Definition |
|---|---|---|
| 3AT | 3-Amino Trizole | chemical used to reduce the number of false-positives of yeast interactors when grown using the HIS3 reporter construct. |
| 5FOA | 5-Fluoro-orotic acid | chemical used to select against yeast cells that are able to express URA3 |
| APC/C | anaphase promoting complex/ cyclosome | ubiquitin ligase similar to the SCF complex that promotes the G2-M transition and in exit from mitosis |
| CBP | calmodulin binding peptide | part of the calcium-dependent protein, calmodulin and used as a purification tag in the TAP tag |
| Cdk | Cyclin dependent kinase | kinase critical for cell division; partners with cyclins to form an active kinase and drives the cell cycle forward. Several exist in higher eukaryotes; only one in *Saccharomyces cerevisiae* - Cdc28. |
| CKI | Cyclin dependent kinase inhibitor | any of several proteins that inhibit the cyclin dependent kinase. Commonly known ones include p21 and p27 in humans and Sic1 and Far1 in *Saccharomyces cerevisiae* . |
| Cullin | | protein to recruit the E2 enzyme to the ligase. Known as Cul1 in humans and as Cdc53 in *Saccharomyces* |
| CYC | cytochome C | protein important for electron transport in the mitochodria. CYC1 is isoform 1 of the protein and is a well-defined gluose repressed gene that is active in the presence of glucose, but at lower levels than in non-glucose media. |
| F box protein | | a variable protein component of the SCF complex. F box proteins contain the F box motif and generally a C-terminal domain responsible for substrate interaction. |
| F box motif | | a defined motif contained within the F box proteins. The F box motif is named for its first family member, cyclin F, and all known F box motif-containing proteins interact with Skp1 of the SCF complex. |
| Fbl | F box-Leucine Rich Repeats | An F box protein containing a Leucine Rich Repeat domain as its substrate interaction domain |

| | | |
|---|---|---|
| Fbw | F box-WD-40 | An F box protein containing a WD-40 domain as its substrate interaction domain |
| Fbx | F box-unknown domain | An F box protein containing a domain as its substrate interaction domain that is not either the LRR or the WD-40. |
| GAL | Galactose utilization family member | Any one of several genes or proteins required for the utilization of Galactose as a carbon source |
| HECT | Homologous to E6 C-terminus | A family of ubiquitin ligases that have similarity to the C-terminus of the viral protein E6. These proteins receive the ubiquitin molecule from the E2 enzyme directly and then pass the ubiquitin molecule directly to the substrate. |
| HXT | hexose transporters | Any of a family of genes/proteins required for the transport of hexoses. Initially, glucose sensing molecules such as Snf3 were thought to be hexose transporters, so there is some nomenclature confusion in older literature. |
| IgG | immunoglobulin domain | part of the immune system protein immunoglobulin G and used as a purification tag in the TAP tag. Binds protein A |
| LRR | Leucine Rich Repeat | A protein-protein interaction motif commonly found in F box proteins that interact with the substrate selected to be ubiquitinated |
| MAL | Maltose utilization family member | Any one of several genes or proteins required for the utilization of Maltose as a carbon source |
| PEST | Proline-Glutamic acid Serine-Threonine | A motif consisting of acidic residues that is generally thought to indicate an unstable protein, though there are exceptions |
| PHD | Plant homeodomain | One newly defined type of ubiquitin ligase family named for its U box domain. This domain is similar to the zinc finger motif found in RING finger proteins |
| RING family | Really Interesting New Gene | A type of ubiquitin ligase family containing a RING finger protein in order to recruit an E2 enzyme. |

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|---|---|---|
| SCF | Skp1-Cullin-F box | ubiquitin ligase that is critical for the G1-S transition and for ubiquitination of a number of critical substrates. It is a member of the RING family of ligases and consists of Skp1, a Cullin, a variable F box protein, and a RING finger protein. |
| SUC | Sucrose utilization family member | Any one of several genes or proteins required for the utilization of Sucrose as a carbon source |
| TAP | Tandem Affinity Protein | A tag that allows for dual purification of selected proteins. In this thesis, it is defined as having the CBP peptide-TEV protease cleavage site-IgG binding peptide epitopes. Other TAP tags may make use of other epitopes, including poly-hisitidine tags. |
| TEV | | protease cleavage sie in the TAP tag allowing for dual purification of tagged proteins. |
| U box | Ubiquitin box | One newly defined type of ubiquitin ligase family named for its U box domain. This domain is similar to the zinc finger motif found in RING finger proteins |
| ubiquitin | | A small (76 aa) protein. Covalent attachment of ubiquitin to a substrate alters the status of that protein. Ubiquitin can form chains by forming a bond with a lysine residue on the substrate or another ubiquitin. |
| WD-40 | | protein-protein interaction motif defined by approximately 40 amino acid residues containing a significant portion of Tryptophan and Aspartic Acid residues |

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| MAL | Maltose utilization family member | Any one of several genes or proteins required for the utilization of Maltose as a carbon source |
| PEST | Proline-Glutamic acid Serine-Threonine | A motif consisting of acidic residues that is generally thought to indicate an unstable protein, though there are exceptions |
| PHD | Plant homeodomain | One newly defined type of ubiquitin ligase family named for its U box domain. This domain is similar to the zinc finger motif found in RING finger proteins |
| RING family | Really Interesting New Gene | A type of ubiquitin ligase family containing a RING finger protein in order to recruit an E2 enzyme. |

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|---|---|---|
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| SUC | Sucrose utilization family member | Any one of several genes or proteins required for the utilization of Sucrose as a carbon source |
| TAP | Tandem Affinity Protein | A tag that allows for dual purification of selected proteins. In this thesis, it is defined as having the CBP peptide-TEV protease cleavage site-IgG binding peptide epitopes. Other TAP tags may make use of other epitopes, including poly-hisitidine tags. |
| TEV | | protease cleavage sie in the TAP tag allowing for dual purification of tagged proteins. |
| U box | Ubiquitin box | One newly defined type of ubiquitin ligase family named for its U box domain. This domain is similar to the zinc finger motif found in RING finger proteins |
| ubiquitin | | A small (76 aa) protein. Covalent attachment of ubiquitin to a substrate alters the status of that protein. Ubiquitin can form chains by forming a bond with a lysine residue on the substrate or another ubiquitin. |
| WD-40 | | protein-protein interaction motif defined by approximately 40 amino acid residues containing a significant portion of Tryptophan and Aspartic Acid residues |

# *Appendix V*

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# The ISG15 Isopeptidase UBP43 Is Regulated by Proteolysis via the $SCF^{Skp2}$ Ubiquitin Ligase*

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**The Skp2 oncoprotein belongs to the family of F-box proteins that function as substrate recognition factors for SCF (Skp1, cullin, F-box protein) E3 ubiquitin-ligase complexes. Binding of the substrate to the $SCF^{Skp2}$ complex catalyzes the conjugation of ubiquitin molecules to the bound substrate, resulting in multi-ubiquitination and rapid degradation by the 26 S proteasome. Using Skp2 as bait in a yeast two-hybrid screen, we have identified UBP43 as a novel substrate for Skp2. UBP43 belongs to the family of ubiquitin isopeptidases and specifically cleaves ISG15, a ubiquitin-like molecule that is induced by cellular stresses, such as type 1 interferons (IFN), nephrotoxic damage, and bacterial infection. UBP43 was originally identified as an up-regulated gene in knock-in mice expressing an acute myelogenous leukemia fusion protein, AML1-ETO, as well as in melanoma cell lines treated with IFN-β. The phenotype of UBP43 knockout mice includes shortened life span, hypersensitivity to IFN, and neuronal damage, suggesting that tight regulation of ISG15 conjugation is critical for normal cellular function. In this study, we demonstrate that UBP43 is ubiquitinated *in vivo* and accumulates in cells treated with proteasome inhibitors. We also show that Skp2 promotes UBP43 ubiquitination and degradation, resulting in higher levels of ISG15 conjugates. In Skp2−/− mouse cells, levels of UBP43 are consistently up-regulated, whereas levels of ISG15 conjugates are reduced. Our results demonstrate that the $SCF^{Skp2}$ is involved in controlling UBP43 protein levels and may therefore play an important role in modulating type 1 IFN signaling.**

Modification of proteins by ubiquitin and ubiquitin-like (Ubl)[1] molecules, including SUMO, Nedd8, and ISG15, has emerged as a critical regulatory process in eukaryotes, controlling pathways such as the cell cycle, cellular stress response, intracellular signaling, development, and the immune response (1–5). Deregulation of ubiquitin or Ubl modification can cause autoimmune and neurodegenerative diseases, developmental abnormalities, and cancer.

Conjugation of ubiquitin and Ubls involves a three-step mechanism initially demonstrated for ubiquitin as follows. A single E1 ubiquitin-activating enzyme activates the Ubl molecule via formation of a thioester bond. Activated Ubl is then transferred to one of a large family of E2 ubiquitin-conjugating enzymes. In most cases, E2 enzymes are targeted to appropriate substrates by a class of substrate receptor complexes termed E3 ubiquitin ligases. Together, the E2 and E3 enzymes catalyze the formation of isopeptide bonds between ubiquitin and lysine residues on the target proteins. In the case of multiubiquitination, additional ubiquitin molecules are added to form ubiquitin chains. The multiubiquitinated proteins are then recognized and rapidly degraded into short peptides by the 26 S proteasome (3, 6). Modification by ubiquitin and Ubl molecules is a reversible process mediated by a large family of isopeptidases that exist to remove these molecules from their substrates. Although the function and regulation of these isopeptidases is poorly defined, the few that have been studied indicate an important role in growth and development (7, 8).

Specificity of the ubiquitin and Ubl pathways is conferred by the nature and activity of the E3 complexes. One of the best-studied E3 ligase complexes is the SCF complex. SCF complexes are composed of four subunits: the three core components Skp1, a Cullin family member, the RING finger protein Rbx1/Roc1, and one variable component, the F-box protein, that acts as the substrate recognition factor. A large number of F-box proteins have been identified in organisms ranging from yeast to humans. The mammalian F-box protein Skp2 may play a pivotal role in oncogenesis and has been implicated in degradation of several key regulators of cell proliferation including the tumor suppressor proteins $p27^{Kip1}$, $p57^{Kip2}$, (9) and p130 (10) and the replication factor, hORC1 (11).

We used the yeast two-hybrid technique to isolate potential Skp2-interacting proteins. We identified seven proteins, including UBP43, which will be described in this report. UBP43 belongs to the family of ubiquitin isopeptidases, one of the two classes of ubiquitin and Ubl deconjugating enzymes (12). UBP43 was identified as strongly expressed mRNA in hematopoietic tissue from an acute myeloid leukemia mouse model (13). Thereafter, UBP43 was identified as an up-regulated gene in melanoma cells treated with IFN-β and the protein kinase C activator MEZ. This treatment causes cells to irreversibly lose proliferation potential and begin progression toward terminal differentiation (14). UBP43 functions as an isopeptidase re-

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[1] The abbreviations used are: Ubl, ubiquitin-like; SCF, Skp1, cullin, F-box protein; LPS, lipopolysaccharide; IFN, interferon; MEF, mouse embryonic fibroblast; LRR, leucine-rich repeat; HEK, human embryonic kidney; HA, hemagglutinin; $SCF^{Skp2}$, Skp1-Cul1-F-box protein Skp2.

sponsible for cleaving the ubiquitin-like protein ISG15 from substrates (15). Upon treatment of cells with type I IFN or lipopolysaccharide (LPS), ISG15, UBP43, and the ISG15 E1 enzyme, UBE1L, are rapidly up-regulated (16). It seems that tight regulation of both the ISG15 conjugation and deconjugation pathways is required to ensure proper response to cellular stress. In UBP43 knockout mouse embryonic fibroblasts (MEFs), ISG15 cleavage from cellular substrates is strongly reduced, suggesting that UBP43 is the major ISG15 isopeptidase (17). UBP43−/− MEFs demonstrate prolonged STAT1 signaling and IFN hypersensitivity, which is in accordance with data showing that signal transducer and activator of transcription 1 is an ISG15-modified protein (18). These observations and the identification of UBP43 in the two tumor models suggest that UBP43 plays an important role in cellular proliferation and differentiation and that UBP43 levels need to be carefully controlled in cells. Indeed, UBP43 protein levels are regulated at the level of transcription (16) and post-translationally, as we show here, via the $SCF^{Skp2}$-mediated ubiquitin pathway.

## MATERIALS AND METHODS

*DNA Constructs*—Skp1, Skp2, and UBP43 were PCR-amplified using a human lymphocyte cDNA library (HL4006AE; BD Biosciences Clontech). Skp1 and Skp2 were cloned into MatchMaker (BD Biosciences Clontech) yeast two-hybrid vectors pGADT7 and pGBKT, respectively. All other plasmids were created with the GATEWAY cloning system using vectors from Invitrogen following manufacturer's protocols. The C-terminally V5-tagged UBP43 adenovirus construct was made using the pADeasy system (Qbiochem). Amplified clones were verified by sequencing.

*Antibodies*—The Skp2 antibody used for immunoprecipitation was purchased from Zymed Laboratories Inc.. Skp2 (His-435) and Cdk2 (Glu-119) antibodies used in immunoblotting analysis were purchased from Santa Cruz. His, HA, and V5 antibodies were purchased from Amersham Biosciences, Babco, and Invitrogen, respectively. The affinity-purified polyclonal ISG15 antibody was kindly supplied by Dr. Arthur Haas and does not cross-react with ubiquitin (19).

*Cell Extracts*—Unless stated otherwise, cells were harvested in mammalian lysis buffer (150 mM NaCl, 0.1% Nonidet P-40, and 50 mM Tris-HCl, pH 7.5) by scraping, incubating on ice for 10 min, and then centrifuging at 14,000*g* (40° C) for 15 min; the supernatant was retained.

*Yeast Two-hybrid Screen*—The MatchMaker (BD Biosciences Clontech) yeast two-hybrid system was used to screen for Skp2-interacting proteins. Yeast strain AH109 was transformed with plasmid pGBKT-Skp2 containing full-length Skp2 fused to the GAL4 DNA-binding domain. Transformed cells were streaked on plates lacking tryptophan to select for single clones. This strain was then transformed with a human lymphoma cDNA library (HL4006AE; Invitrogen) cloned into the vector pACT1 that fuses cDNAs to the GAL4 activating domain. $2 \times 10^6$ clones were screened on plates lacking histidine (+10 mM 3AT) or adenine and histidine (+10 mM 3AT). Interaction of positive clones was verified by retransforming clones into the original Skp2 bait strain and assaying for interaction. Skp2 and UBP43 constructs were then cloned into yeast expression vectors compatible with the ProQuest two-hybrid system from Invitrogen. Constructs were transformed into AH109 and screened on plates lacking leucine, tryptophan, or histidine (+30 mM 3AT).

*Production of Recombinant Proteins in Bacteria and GST Purification—Escherichia coli* BL21 (Invitrogen) cells were used to express the vector, pDEST15 (Invitrogen) containing GST or a GST-Skp2 fusion protein. One liter of each strain was induced with 100 mM isopropyl β-D-thiogalactoside for 2 h. Protein was isolated using a 3× freeze-thaw protocol with a dry ice and ethanol bath. Extracts were purified over glutathione-Sepharose 4B matrix (Amersham Biosciences) according to manufacturer's instructions. Extracts from HEK 293 cells transfected with indicated plasmids were added to the matrix. Bound fractions were analyzed by Western blotting using the indicated antibodies.

*Immunoprecipitation*—80% confluent A549 cells on a 10-cm plate were infected with adenoviruses expressing Skp2 with an N-terminal FLAG tag, and UBP43 with a C-terminal V5 tag. Infection was confirmed with GFP. 36 h after infection, cells were harvested in mammalian lysis buffer. The lysate was subjected to a FLAG column (Sigma), and bound proteins were eluted with 1.5× FLAG peptide (Sigma) and subjected to Western blot analysis.

*Ubiquitination*—Skp2 was subcloned into pDEST31 (Invitrogen) containing an N-terminal $His_6$ epitope. UBP43 was subcloned into pDEST40 (Invitrogen) containing C-terminal V5 and $His_6$ epitopes. HA-ubiquitin and $His_6$-ubiquitin constructs were a gift from Dr. Rosalie Sears (Oregon Health and Sciences University, Portland, OR). A549 cells, transfected with indicated plasmids, were treated for 5 h with 10 μM MG132 (Calbiochem).

*$^{35}S$ Pulse-chase*—REF52 and A549 cells were infected with UBP43-V5 adenovirus for 20 h. Skp2 wild-type (+/+) and Skp2 knockout (−/−) primary MEFs were infected in the presence of Effectene reagent. Cells were then labeled for 30 min with 7 mCi of [$^{35}$S]methionine, chased with media containing 5 mM methionine and 3 mM cysteine, washed, and collected at 0, 15, 30, 60, 120, and 180 min. Cell extracts were denatured and then renatured for V5 immunoprecipitation with anti-V5 antibody from Invitrogen. Proteins were separated by SDS-PAGE and visualized by autoradiography. Proteasome inhibitors (5 μM MG132 + 5 μM lactacystin) were added 5 h before the pulse and maintained throughout the pulse and chase phases.

*IFN-β Treatment*—Skp2 wild-type and Skp−/− MEFs were treated with 1000 units/ml mouse IFN-β, and extracts were harvested 24 h later. For cell growth measurements, $3.6 \times 10^4$ immortalized MEF cells were plated in six-well plates, treated with 500 units/ml of mouse IFN-β, and harvested in duplicate. For each time point, total viable cell number was assessed by counting with a hemacytometer. Trypan blue staining was used to identify dead cells. A minimum of 150 cells was counted per sample.

## RESULTS

*UBP43 Interacts with Skp2*—To identify Skp2-interacting proteins, we performed a yeast two-hybrid screen and obtained eight clones coding for putative Skp2 interacting proteins (see "Materials and Methods"). Sequencing of the cDNA inserts revealed two previously identified and five novel Skp2 interactors. In validation of our screen, we isolated Skp1 (two clones) and Cks1. Skp1 is the scaffold protein that anchors Skp2 to the SCF complex via the Skp2 F-box domain (18). Cks1 is a protein recently identified to interact with Skp2 and facilitate substrate recognition of p27 (20, 21). Of the other five clones isolated, we focused our attention on a clone coding for a C-terminal portion of UBP43. Both the UBP43 C terminus (amino acids 121–373) that was isolated in the original screen and full-length UBP43, but not the N terminus (amino acids 1–121), were able to interact with Skp2, as assayed by production of β-galactosidase and growth on plates lacking histidine (Fig. 1*A*). To further isolate the Skp2-interacting region of UBP43, we made successive C-terminal truncations of the 121–373 fragment (Fig. 1*B*). We located a region between amino acids 183 and 352 of UBP43 that seems to be involved in its interaction with Skp2. The deletion construct containing the entire C-terminal region (121–373) as well as a construct lacking the last 21 amino acids, fragment 121–352, demonstrated robust interaction. However, a construct containing amino acids 121–285 showed markedly reduced interaction. When fragment 121–183 was expressed, interaction with Skp2 was completely abrogated. We therefore conclude that the region between amino acids 121 and 285 of UBP43 is required for the interaction between UBP43 and Skp2.

*Skp2 Interaction with UBP43 Requires the Skp2 Leucine-rich Repeat (LRR) Domain*—Next, we analyzed the interaction of UBP43 and Skp2 *in vivo*. Fig. 1*C* demonstrates that full-length Skp2 can co-immunoprecipitate UBP43 ectopically expressed in the human lung cancer cell line A549. A construct lacking the LRR and C terminus showed no binding to UBP43. However, constructs lacking the N terminus or both the N terminus and F-box regions but retaining the LRR and C terminus did associate with UBP43. The LRR region of Skp2 has been implicated in substrate binding. Our data thus suggest that UBP43 is a substrate of $SCF^{Skp2}$.

*Skp2 and UBP43 Interact in Vitro*—To verify the interaction of UBP43 with Skp2, we performed *in vitro* binding experiments. *Escherichia coli* that expressed GST-Skp2 or GST alone

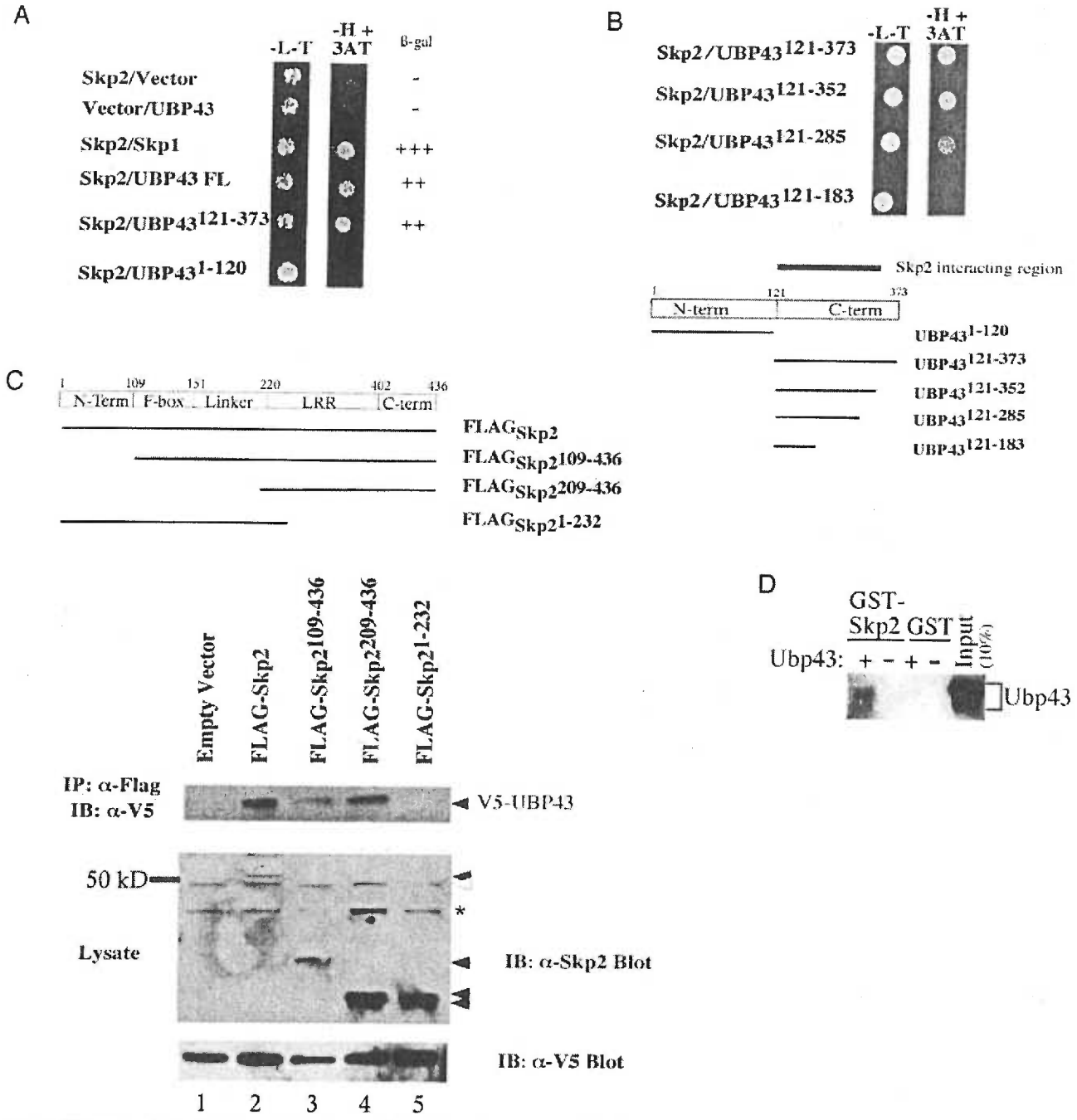


FIG. 1. **UBP43 and Skp2 interact *in vivo* and *in vitro*.** *A*, yeast two-hybrid screen. Transformants were analyzed for transcriptional activation of the *GAL4* promoter driven reporter constructs. *Left*, growth on −Leu−Trp plates (*-L-T*) or on −His plates containing 30 mM 3-amino-1,2,4-triazole (3-AT) (*-H+3-AT*) to test expression of the *HIS3* reporter gene (*-His+3-AT*). *Right*, β-galactosidase activity. *B*, yeast strains expressing the indicated constructs were grown on −H+3-AT plates to assess protein interaction as above. *C*, mammalian co-immunoprecipitation. Human lung carcinoma A549 cells were infected with adenovirus expressing UBP43-V5 and various FLAG-Skp2 constructs. Cell extracts were immunoprecipitated with anti-FLAG beads, separated by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. The *asterisk* indicates a background band. Endogenous Skp2 is denoted by a *white arrowhead*, ectopic Skp2 by *black arrowheads*. The proteasome inhibitor lactacystin was added to a final concentration of 10 μM 5 h before harvest. *IB*, immunoblot; *IP*, immunoprecipitation. *D*, GST-Skp2 binds UBP43 *in vitro*. GST-Skp2 (*lanes 1* and *2*) or GST (*lanes 3* and *4*) were expressed in *E. coli* and purified over glutathione beads. Beads were incubated with HEK293 cell extracts expressing UBP43 (*lanes 1* and *3*) or empty vector (*lanes 2* and *4*).

were bound to glutathione beads, followed by incubation with extract from human embryonic kidney (HEK) 293 cells that expressed ectopic UBP43. Fig. 1*D* shows that GST-Skp2, but not GST, binds to UBP43 *in vitro*.

*Levels of UBP43 Are Modulated by Skp2 and by Proteasome Inhibitors*—Our finding that UBP43 interacts with the LRR domain of Skp2 suggested that UBP43 is a substrate of $SCF^{Skp2}$. Therefore, UBP43 protein levels could be controlled by protein degradation. To address this question, we expressed Skp2 and UBP43 in HEK293 cells. UBP43 levels were strongly reduced when Skp2 was expressed (Fig. 2*A*, *lane 2*) compared with when UBP43 was expressed alone (*lane 1*). Because Skp2 functions in an E3 ligase complex that targets substrates for degradation by the 26 S proteasome, we next assayed whether UBP43 accumulates in cells that have been treated with the proteasome inhibitor MG132. Indeed, under these conditions,

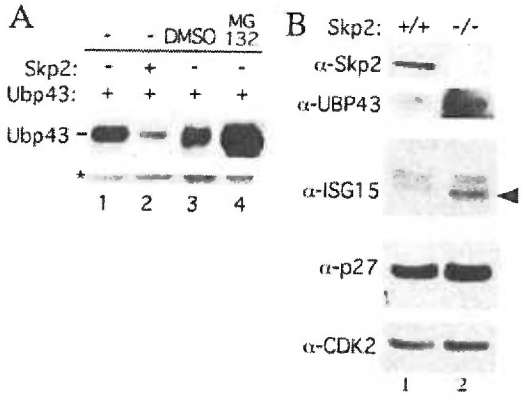


FIG. 2. **UBP43 steady state levels are modulated by Skp2 and by proteasome inhibitors.** *A*, HEK293 cells were transfected with UBP43 and either control vector (−) or Skp2 and subjected to Western blot analysis. Proteasome inhibitor MG132 (10 μM) or $Me_2SO$ (*DMSO*) as control was added 7 h before cell harvest. The *asterisk* indicates a loading control. *B*, absence of Skp2 results in increased UBP43 levels. Extracts from asynchronous low passage primary Skp2 wild-type (+/+) or knockout (−/−) MEFs were immunoblotted with antibodies specific to the indicated proteins. The *arrowhead* denotes free ISG15.

robust UBP43 accumulation can be observed (Fig. 2*A*, *lane 4*). Together, these results suggest that UBP43 is degraded in the proteasome most likely by $SCF^{Skp2}$-mediated ubiquitination.

*Levels of UBP43 and ISG15 Are Altered in Skp2−/− Cells*—To test whether levels of UBP43 are increased in cells that are devoid of Skp2, we performed immunoblotting of primary, low passage MEF extracts derived from wild-type or Skp2−/− mice. As shown in Fig. 2*B*, UBP43 levels are significantly increased in Skp2−/− extracts (*lane 2*) compared with wild type (*lane 1*). Note that UBP43 levels are very low in unstimulated wild-type cells and are hardly detectable with the available antibody. We also observed a significant increase in steady-state UBP43 levels in Skp2−/− cells stimulated with LPS (data not shown). In addition to the increase in UBP43 levels in Skp2−/− cells, an increase in free ISG15 levels was observed (*lane 2*, *arrowhead*). This suggests that in the absence of Skp2, UBP43 levels are up-regulated, resulting in increased cleavage of ISG15 from substrates (see Fig. 5*B*) and a concomitant increase in free ISG15.

*Skp2 Enhances UBP43 Ubiquitination in Vivo*—Proteasomal degradation of proteins is triggered by multi-ubiquitination of targeted polypeptides. To determine whether UBP43 is ubiquitinated *in vivo*, we transfected HEK293 cells with plasmids encoding Skp2, V5-tagged UBP43, and HA-tagged ubiquitin. UBP43, in the presence of overexpressed Skp2, appeared in higher molecular mass forms consistent with ubiquitination (Fig. 3*A*). These high molecular mass bands were intensified upon addition of the proteasome inhibitor lactacystin. Next, we performed immunoprecipitation with anti-V5 antibodies to capture UBP43 protein. High molecular mass bands were observed that were immunoreactive against anti-HA antibodies, indicating that these bands represented ubiquitinated UBP43 (Fig. 3*B*, *lanes 2* and *3*). Ubiquitination was enhanced by ectopic Skp2 (*lane 3*) and was absent if either V5-UBP43 or HA-ubiquitin was omitted (*lanes 1* and *4*). If these high molecular mass bands correspond to ubiquitinated UBP43 species, then inhibition of the proteasome should result in an increase and possibly a shift to even higher molecular mass bands. This is indeed the case, as shown in Fig. 3*C*, *lane 3*. In this experiment, His-V5-tagged UBP43 was captured on nickel-nitrilotriacetic acid beads under denaturing conditions, followed by immunoblotting against the HA tag on ubiquitin. Because in these experiments, the amount of transfected UBP43 was high compared with Skp2, we did not observe a significant reduction in UBP43 steady-state levels upon Skp2 co-transfection. We conclude that UBP43 is ubiquitinated *in vivo*. To test whether Skp2 can increase the amount of ubiquitinated UBP43, we infected normal rat embryo fibroblast (REF52) cells with V5-tagged UBP43 expressed from an adenovirus together with increasing levels of adeno-Skp2 virus. Fig. 3*D* shows that under proteasome inhibition conditions (MG132, *lanes 3*, *5*, and *7*), UBP43 ubiquitination is enhanced by increasing levels of Skp2. We kept the level of Skp2 at or near endogenous levels (data not shown), whereas UBP43 levels were higher to allow detection of ubiquitinated species. In conclusion, these experiments demonstrate that UBP43 is ubiquitinated *in vivo* by Skp2 and is then degraded in a proteasome-dependent manner.

*Skp2 Promotes Degradation of UBP43 in Vivo*—Our results suggested that UBP43 levels were controlled by $SCF^{Skp2}$-mediated degradation via the ubiquitin-proteasome pathway. To measure UBP43 degradation rates *in vivo*, we performed pulse-chase experiments in adenovirus-UBP43 infected rat fibroblast (REF52) cells. UBP43 has a half-life of ~60 min under unstressed conditions (Fig. 4*A*, *lanes 2–5*). Again, UBP43 is very hard to detect under these conditions; however, the GFP control lane (*lane 1*) clearly demonstrates the specificity of the UBP43 band. When cells were treated with a proteasome inhibitor (Fig. 4*A*, *lanes 6–9*), UBP43 was stabilized significantly. We obtained similar results in human A549 cells (data not shown). In addition, cycloheximide treatment of cultures followed by analysis of UBP43 protein levels confirmed the pulse-chase results (data not shown). Ectopic Skp2 expression resulted in accelerated degradation of UBP43 (data not shown). We repeated the pulse-chase analysis in low passage Skp2+/+ and Skp−/− primary MEFs. The half-life of UBP43 was ~50 min in Skp2 wild-type cells compared with 120 min in knockout cells (Fig. 4, *B* and *C*). When Skp2 was re-expressed in the Skp2−/− cells, levels of UBP43 dropped dramatically at the 15-min time point and then remained at that low level for the rest of the chase period. We conclude that Skp2 can initiate rapid degradation of UBP43 via the ubiquitin-proteasome pathway.

*Skp2 Activity Modulates the ISG15 Conjugation Pathway*—ISG15 conjugation to substrates is induced upon IFN-β stimulation. To determine whether Skp2 has an effect on ISG15 conjugation by way of its regulation of UBP43, we overexpressed Skp2 in the A549 human lung carcinoma cell line. After treatment with LPS, cells overexpressing Skp2 showed a marked increase in ISG15 conjugation compared with cells transfected with empty vector (Fig. 5*A*). On the other hand, Skp2−/− MEFs induced with IFN-β displayed a reduction in ISG15 conjugation (Fig. 5*B*) and an increase in free ISG15 (Fig. 2*B*) compared with wild-type MEFs. We conclude that Skp2 can modulate the level of ISG15 conjugates, most likely via degradation of UBP43.

Cells from UBP43−/− mice exhibit increased levels of ISG15 conjugates; these animals are hypersensitive to induction of the type I IFN pathway (17). Therefore, we would expect that Skp2−/− cells, which have reduced levels of ISG15 conjugates, might be more resistant to the growth-inhibiting effects of IFN-β treatment. To test this, we analyzed the sensitivity of Skp2−/− cells toward IFN-β. Skp2 wild-type cells showed a marked reduction in cell growth 24 h after IFN-β reduction (Fig. 5*C*). In contrast, the growth rate of Skp2−/− cells was unaffected by IFN-β induction at 24 h. These data suggest that absence of Skp2 decreases or delays the response to IFN-β, most likely because of the higher levels of UBP43 observed (Fig. 2*B*).

## DISCUSSION

The biological function of ISG15 modification is not well understood. It is clear, however, that carefully controlled

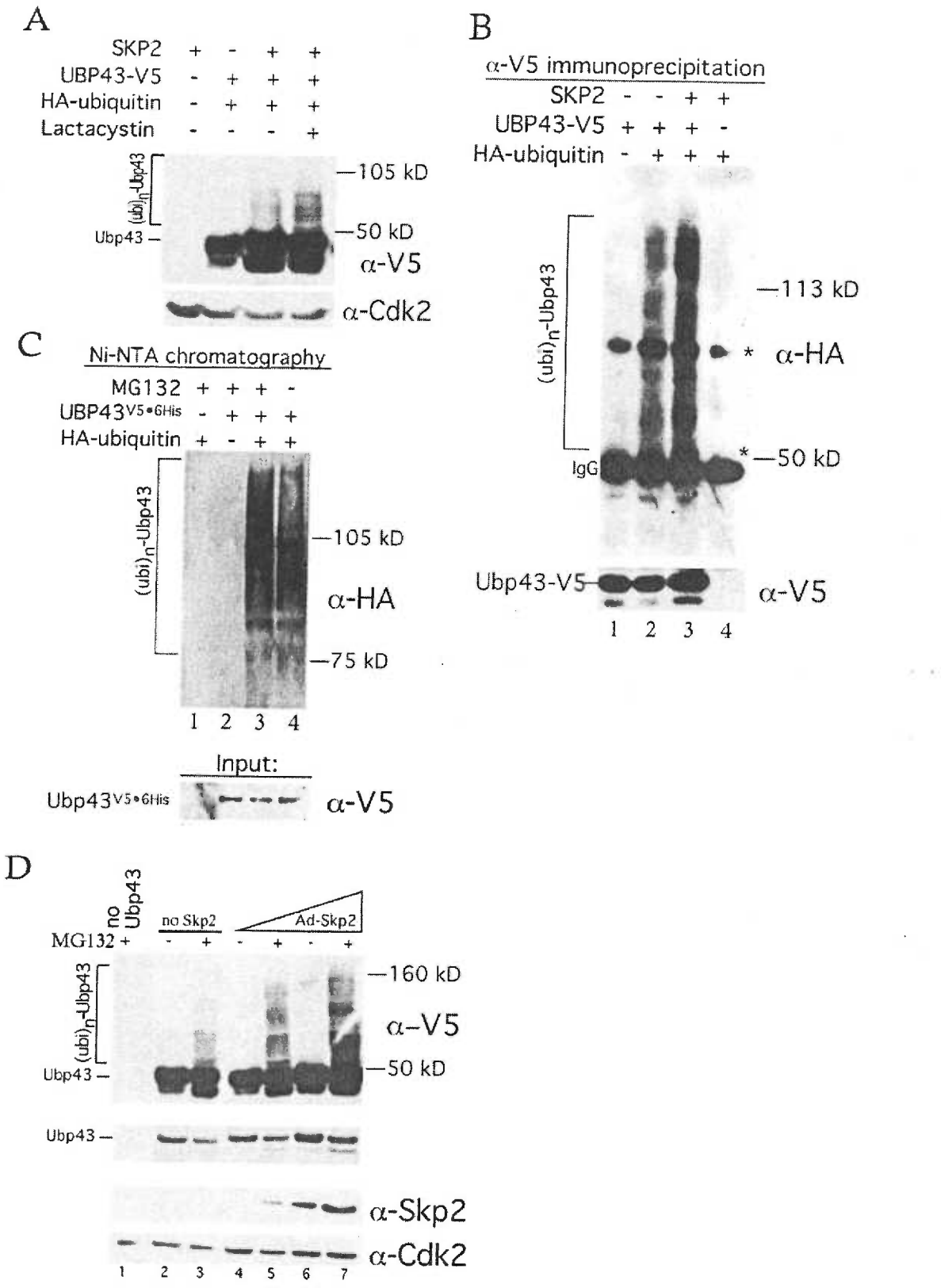


FIG. 3. **Skp2 increases UBP43 ubiquitination *in vivo*.** *A*, extracts from HEK293 cells were transfected with UBP43-V5 (*lanes 2–4*), Skp2 (*lanes 1, 3, 4*), and HA-ubiquitin (*lanes 2–4*), separated by SDS-PAGE, and analyzed with the indicated antibodies. Lactacystin (10 μM) was added 5 h before harvest. B, extracts from HEK293 cells transfected with UBP43-V5 (*lanes 1–3*), Skp2 (*lanes 3* and *4*), and HA-ubiquitin (*lanes 2–4*) were immunoprecipitated with anti-V5 antibody, separated by SDS-PAGE, and analyzed with the indicated antibodies. *Asterisks* mark heavy and light IgG bands. *C*, A549 cells were transfected with $UBP43^{V5\text{-}6HIS}$ (*lanes 2–4*) and HA-ubiquitin (*lanes 1, 3,* and *4*). Proteasome inhibitor, 5 μM MG132, and/or 5 μM lactacystin (*lanes 1–3*) was added 5 h before harvest. Extracts were purified using nickel-nitrilotriacetic acid (Ni-NTA) beads under denaturing conditions (8 M urea) to capture $UBP43^{V5\text{-}6HIS}$. Eluates, separated by SDS-PAGE, were analyzed by Western blotting with the indicated antibodies. *D*, REF52 cells were infected with constant amounts of adenovirus expressing UBP43-V5 or GFP and increasing amounts of adenovirus expressing Skp2 (*Ad-Skp2*) in the presence or absence of proteasome inhibitor (5 μM MG132, 5 μM lactacystin). Lysates were subjected to Western blot analysis.

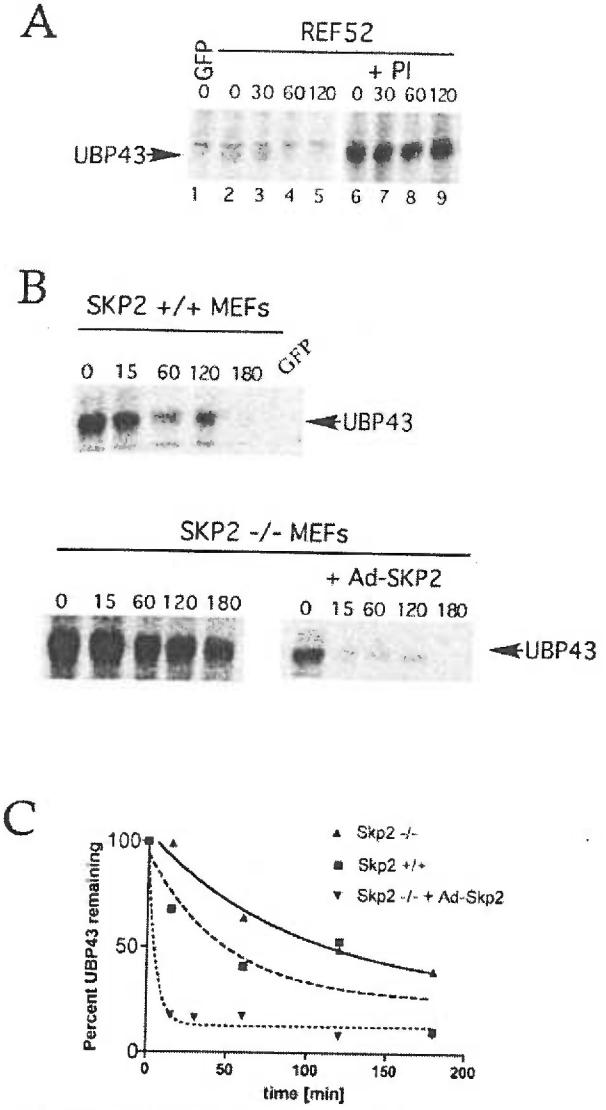


FIG. 4. **UBP43 stability.** *A*, proteasome inhibitors increase UBP43 protein stability. Normal rat embryo fibroblast REF52 cells were infected with UBP43-V5 adenovirus for 20 h and subjected to pulse-chase analysis in the presence or absence of proteasome inhibitors (PI, 5 $\mu$M MG132, 5 $\mu$M lactacystin). *GFP*, control infection with green fluorescent protein virus. *B*, UBP43 has increased stability in Skp2−/− MEFs. Skp2+/+ and Skp2−/− MEFs (passage 4) were infected with UBP43 adenovirus and, where indicated, with Skp2 adenovirus, followed by pulse-chase analysis. *C*, graphical representation of *B*. *Squares, dashed line*, Skp2+/+ MEFs; *triangles, solid line*, Skp2−/− MEFs; *inverted triangles, dotted line*, Skp2−/− MEFs infected with Adeno-Skp2.

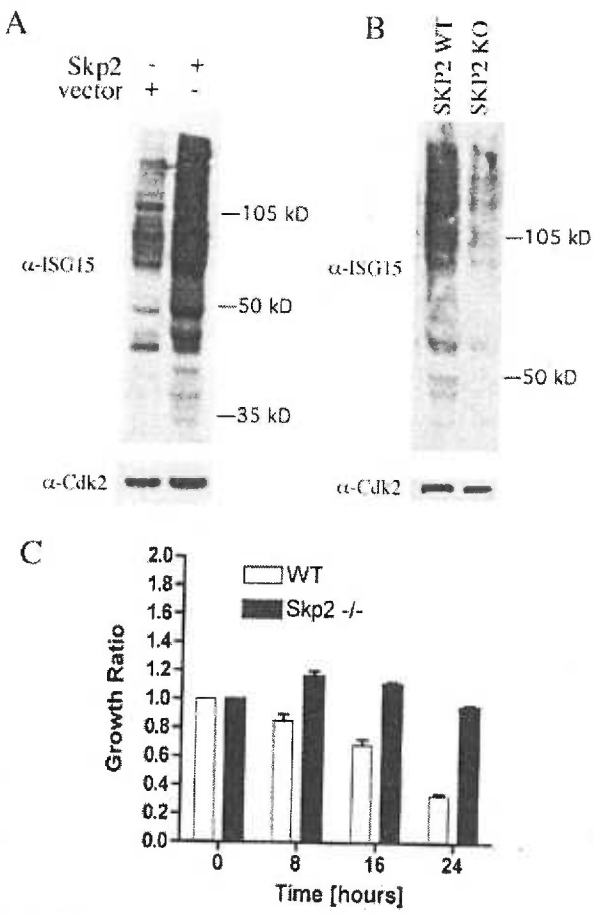


FIG. 5. **Skp2 interferes with IFN signaling.** *A*, Skp2 affects ISG15 conjugate levels. A549 cells were transfected with indicated vectors and extracts analyzed for ISG15 conjugation using an affinity-purified polyclonal $\alpha$-ISG15 antibody (gift from Arthur Haas). *B*, asynchronously growing wild-type or Skp2−/− MEFs were treated with 1000 units/ml of mouse IFN-$\beta$ for 24 h, and extracts were analyzed for ISG15 conjugation. *C*, Skp2−/− cells are resistant to IFN. MEFs were treated with 500 units/ml mouse IFN-$\beta$ and viable cells were counted at the indicated time points. Growth ratio is defined as number of cells with interferon treatment divided by number of cells without interferon treatment.

ISG15 conjugation and deconjugation to substrates is crucial for the health of a cell and of an organism. This suggests that the level and activity of enzymes that control ISG15 modification, including UBE1L and UBP43, need to be tightly regulated. Indeed, mice lacking UBP43 are short-lived, develop neuronal injury, exhibit hypersensitivity to IFN, and demonstrate increased apoptosis in hematopoietic tissues (17). On the other hand, ectopic expression of UBP43 blocks monocyte differentiation in cell culture (13). In addition, the E1 enzyme for ISG15, UBE1L, is absent in all 14 lung cancers examined for UBE1L expression, suggesting that the lack of ISG15 conjugation contributes to malignant transformation. Cellular levels of UBP43 are controlled at the level of transcription by LPS and IFN type 1 induction (14, 16). We demonstrate here that the $SCF^{Skp2}$ ubiquitin ligase controls the UBP43 protein level by ubiquitin-mediated degradation via the proteasomal pathway. Our data demonstrate that Skp2 binds to UBP43 and initiates its multi-ubiquitination, resulting in UBP43 degradation via the proteasome. In MEFs lacking Skp2, levels of free ISG15 are high, and ISG15 conjugates are low, consistent with increased UBP43 levels. On the other hand, high levels of Skp2 result in an increase in ISG15-conjugated proteins. It was interesting that upon LPS treatment, UBP43 protein was stabilized, an effect that was countered by high Skp2 levels (data not shown). The coordinated induction of both ISG15 conjugating and deconjugating pathways suggests that ISG15 modification is a dynamic process that needs to be carefully controlled for normal cellular function and viability. Indeed, ectopic expression of ISG15 in various cell types initiates apoptosis.[2] In this context, Skp2-mediated degradation might play a fine-tuning role to adjust the levels of UBP43 according to the growth and stress conditions of a cell. Skp2 itself is regulated at the level of transcription and protein degradation (22, 23). Skp2 protein is absent in $G_0$ and early $G_1$ cells, rises as cells enter S phase, and declines in mitosis. We have observed an inverse correlation between Skp2 and ectopic UBP43 levels in synchronized A549 cells (data not shown), consistent with the role of Skp2 in

[2] S. Lanker, unpublished observations.

degrading UBP43. We have not yet been able to follow endogenous UBP43 levels through the cell cycle, mainly because UBP43 levels are very low in cells not treated with IFN or LPS, and the available antibody does not detect endogenous UBP43. Our data do not exclude the possibility that more than one F-box protein participates in the degradation of UBP43; indeed, in Skp2−/− cells, UBP43 is still fairly unstable, with a half-life of about 2 h (Fig. 4*C*). However, UBP43 steady-state levels are greatly increased in Skp2−/− cells, and Skp2 reexpression reduces the levels back to normal, arguing that Skp2 does have a major effect on UBP43 protein levels.

The recognition of substrates by SCF complexes is often catalyzed by substrate phosphorylation at particular residues (24–31). This is also true for $SCF^{Skp2}$ (9, 10, 22, 32, 33). In addition, the adapter protein Cks1 was shown to be required for efficient targeting of p27 and p130 (20, 21). We have preliminary evidence that UBP43 is phosphorylated, but whether phosphorylation is important for binding and whether Cks1 is needed for efficient interaction with Skp2 are under investigation.

ISG15 conjugation plays an important role in stress response, and is also implicated in controlling cell proliferation and differentiation. It is noteworthy that influenza virus proteins inhibit ISG15 expression and conjugation, suggesting that ISG15 mediates antiviral activity (34). It is intriguing that Skp2 controls the levels of cell cycle regulators and, as we show here, a factor in stress response. A connection between cell cycle control and stress response at the level of SCF-mediated degradation has been documented. For example, the yeast $SCF^{Cdc4}$ complex controls the CDK inhibitors Sic1 and Far1 as well as the transcription factor Gcn4 involved in the response to amino acid starvation. $SCF^{Grr1}$ degrades $G_1$ cyclins and has an important role in the cellular response to glucose starvation. It will be interesting to understand the molecular mechanism that ties Skp2 to cellular stress and the role of ISG15 conjugation in this process. Dissecting how Skp2 connects cell cycle control and cellular stress signaling will serve as a paradigm for similar pathways that integrate cell division and external signals.

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# *Appendix VI*

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# Transferable Domain in the $G_1$ Cyclin Cln2 Sufficient To Switch Degradation of Sic1 from the E3 Ubiquitin Ligase $SCF^{Cdc4}$ to $SCF^{Grr1}$

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**Degradation of *Saccharomyces cerevisiae* $G_1$ cyclins Cln1 and Cln2 is mediated by the ubiquitin-proteasome pathway and involves the SCF E3 ubiquitin-ligase complex containing the F-box protein Grr1 ($SCF^{Grr1}$). Here we identify the domain of Cln2 that confers instability and describe the signals in Cln2 that result in binding to Grr1 and rapid degradation. We demonstrate that mutants of Cln2 that lack a cluster of four Cdc28 consensus phosphorylation sites are highly stabilized and fail to interact with Grr1 in vivo. Since one of the phosphorylation sites lies within the Cln2 PEST motif, a sequence rich in proline, aspartate or glutamate, serine, and threonine residues found in many unstable proteins, we fused various Cln2 C-terminal domains containing combinations of the PEST and the phosphoacceptor motifs to stable reporter proteins. We show that fusion of the Cln2 domain to a stabilized form of the cyclin-dependent kinase inhibitor Sic1 (ΔN-Sic1), a substrate of $SCF^{Cdc4}$, results in degradation in a phosphorylation-dependent manner. Fusion of Cln2 degradation domains to ΔN-Sic1 switches degradation of Sic1 from $SCF^{Cdc4}$ to $SCF^{Grr1}$. ΔN-Sic1 fused with a Cln2 domain containing the PEST motif and four phosphorylation sites binds to Grr1 and is unstable and ubiquitinated in vivo. Interestingly, the phosphoacceptor domain of Cln2 binds to Grr1 but is not ubiquitinated and is stable. In summary, we have identified a small transferable domain in Cln2 that can redirect a stabilized $SCF^{Cdc4}$ target for $SCF^{Grr1}$-mediated degradation by the ubiquitin-proteasome pathway.**

The precise temporal and spatial control of key biological regulatory proteins via degradation by the ubiquitin-proteasome machinery is a critical regulatory process in eukaryotes. Due to its flexibility and specificity, the ubiquitination machinery controls many diverse regulatory pathways, including the cell cycle, cellular stress responses, intracellular signaling, and development. Flexibility is brought about by the modular nature of the ubiquitination process, involving three classes of enzymes, termed E1, E2, and E3 (reviewed in reference 16). A single ubiquitin-activating enzyme, E1, activates the 76-amino-acid (76-aa) polypeptide ubiquitin via formation of a high-energy thiolester bond. Activated ubiquitin is then transferred to one of a large family of E2 ubiquitin-conjugating enzymes; the relatively unspecific E2 enzymes are targeted to an appropriate substrate by a class of substrate receptor complexes termed E3 ubiquitin ligases. Specificity of the ubiquitin pathways is conferred by the nature and activity of the E3 complexes. Together, the E2 and E3 enzymes catalyze the formation of isopeptide bonds between ubiquitin and lysine residues on the target proteins. Additional ubiquitin molecules are added to form multiubiquitin chains, and the multiubiquitin proteins are recognized and rapidly degraded into short peptides by the 26S proteasome. One key to understanding the regulation of ubiquitin-mediated degradation is to elucidate the molecular nature of substrate recognition. Here we describe the identification of a transferable domain in a model substrate, the $G_1$ cyclin Cln2. We show that this domain, when activated by a phosphorylation signal, can interact with a specific E3 enzyme, the $SCF^{Grr1}$ complex, resulting in rapid ubiquitin-mediated degradation.

Two E3 ubiquitin ligase complexes, the anaphase-promoting complex (APC) and the SCF (Skp1/Cdc53/F-box protein) complex, have attracted considerable attention in recent years due to their prominent role in controlling the cell cycle. Both factors are highly conserved among eukaryotes and function in a modular fashion to recognize different classes of substrates (reviewed in reference 16). However, the two complexes differ in their mode of regulation. The APC appears to be activated by posttranslational mechanisms and by association with two specific adapter molecules. In contrast, the SCF complex is proposed to be constitutively active; substrate phosphorylation promotes recognition by the SCF complex, which is followed by ubiquitination and degradation of the substrate. Target proteins that bind to the SCF complex only in their phosphorylated state include the $G_1$ cyclins Cln1 and Cln2 (24, 31, 39, 45), the cyclin-dependent kinase (CDK) inhibitors Sic1 (11, 39) and p27 (5, 41), and the transcription factor NF-κB inhibitor IκBα (reviewed in reference 21). Using in vivo mutational analysis and in vitro ubiquitination assays, degradation of each of these substrates has been demonstrated to depend both on a specific F-box protein and on the phosphorylation state of the substrate. Thus, ubiquitination of specific targets not only depends upon the identity of that target but also upon its state.

Functional SCF complexes, composed of Skp1, Cdc53/cullin, Rbx1/Roc1, and an F-box protein, associate with the E2 enzyme Ccd34 to mediate targeting and ubiquitination of specific

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substrate proteins (20, 30, 39, 40, 42). The SCF complex is a critical regulator of the $G_1$/S transition, and subunit composition and function are highly conserved among eukaryotes (1, 20, 26, 28, 32, 48). A variety of SCF complexes exist, each with a different F-box protein. A large number of F-box proteins have been identified; the *Saccharomyces cerevisiae* genome contains at least 17 proteins, and database searches and two-hybrid screens have revealed more than 50 F-box proteins in plants and animal cells and more than 60 in the nematode *Caenorhabditis elegans* (1, 32, 46). This suggests that the SCF system controls a large number of regulatory processes in eukaryotes. However, the vast majority of F-box proteins have yet to be assigned a function. Three F-box proteins in yeast cells (Grr1, Cdc4, and Met30) and two in mammalian cells (Skp2 and βTrCP) have been studied in detail. $SCF^{Cdc4}$ is required for degradation of the CDK inhibitors Sic1p (11, 39) and Far1 (15), the replication factor Cdc6 (9), and the transcription factor Gcn4 (23). $SCF^{Grr1}$ targets the $G_1$ cyclins Cln1 and Cln2 and the putative Cdc42 effector Gic2 (18) for ubiquitination and degradation (2, 24, 31, 39).

The transition from $G_1$ to S phase controls entry into and exit from the cell cycle and is, therefore, a crucial regulatory point. In the yeast *S. cerevisiae*, this transition requires the activity of the CDK Cdc28 complexed with the $G_1$-specific cyclins, Cln1, Cln2, and Cln3. The cyclic accumulation of Cln1 and Cln2 in $G_1$ phase and rapid decay once cells enter S phase are controlled by periodic transcription of Cln1 and Cln2 and regulated protein degradation, respectively. The determinant for $G_1$ cyclin degradation lies in the C-terminal 170 aa since truncations of this domain cause stabilization of Cln2. The C terminus contains a PEST domain as well as six of the seven Cdc28 consensus phosphorylation sites. The PEST domain, found in all three yeast $G_1$ cyclins, was originally identified as a potential determinant of protein instability on the basis of its frequent occurrence in unstable proteins (33) but has yet to be functionally defined. Deletions in the C terminus of Cln2 and Cln3 that include the PEST motifs result in phenotypes consistent with hyperactivation of $G_1$ cyclins and partially stabilize the mutant proteins (36, 49). However, it is not clear whether the PEST sequences per se or other aspects of the PEST-containing region are the relevant determinants. We have shown that one of those determinants is phosphorylation. Thus, a yeast $G_1$ cyclin mutated in the seven potential Cdc28 phosphorylation sites is highly stabilized and renders cells insensitive to nutrient- and growth-inhibitory signals (24). Our present model suggests that the instability of the $G_1$ cyclins derives from Cdc28-dependent autophosphorylation of the cyclin subunit, which targets the cyclin for degradation (24, 37, 45, 49) by the $SCF^{Grr1}$. A similar model was proposed for mammalian cells based on findings reported for the $G_1$ cyclins E and D1 (6, 8, 48). These observations strongly suggest that precise expression and destruction of yeast $G_1$ cyclins are crucial for proper regulation of the cell cycle and that this mechanism is highly conserved from yeast to humans.

A key issue, however, remains unresolved: what is the molecular nature of the signal that promotes recognition by the F-box subunit of the SCF complex? Each of the known F-box proteins recognizes two or more substrates: Grr1 targets Cln1, Cln2, and Gic2 for degradation; Cdc4 recognizes Sic1, Far1, Gcn4, and Cdc6. In mammalian cells, the F-box protein Skp2 targets p27 and the transcription factor E2F1 (27) for destruction, whereas the F-box protein βTrCP targets IκBα (50) and β-catenin (14, 25, 26, 47). Yet there is little if any sequence homology between substrates recognized by the same F-box protein. Our recent observation that $SCF^{Grr1}$ interacts via the cationic surface of the Grr1 leucine-rich repeat (LRR) domain with phosphorylated substrates, including Cln2, begins to address this important question (17). Here, we extend these findings by demonstrating that a small domain within the carboxy terminus of Cln2, containing a PEST element as well as four crucial phosphorylation sites, converts a stable reporter protein into a substrate for the $SCF^{Grr1}$-mediated ubiquitin-proteasome pathway. Interestingly, while fusions of Cln2 domains to a glutathione *S*-transferase (GST) reporter protein render these chimerical proteins unstable, their degradation is phosphorylation and Grr1 independent. In contrast, fusions to stabilized Sic1 result in phosphorylation- and Grr1-dependent ubiquitination and degradation.

## MATERIALS AND METHODS

**Yeast strains and culture.** Strains were isogenic with 15Daub (*MAT*a *ade1 his2 leu2-3,112 trp1-1 ura3Δns bar1Δ* [34]) or with W303 (*MAT*a *ade2-1 his3-1,15 ura3-1 leu2-3,112 trp1-1 can1-100*). Culture conditions and medium were as indicated and were prepared by standard methods. Yeast strains were grown in selective medium or YAPD (1% yeast extract, 2% peptone, adenine [40 mg/liter], 2% glucose) at 23, 30, or 37°C as indicated. For expression of the Sic1 fusions, strains were grown overnight in glucose-containing selective medium, diluted to an optical density at 600 nm ($OD_{600}$) of 0.2 in YMRaff (yeast medium–2% raffinose) and grown for two generations. Then, galactose was added to a final concentration of 2% for 1 to 2 h. Expression of GST fusion proteins was induced with 500 μM $CuSO_4$ for 1 h.

**Plasmids.** Primer sequences and details about plasmid constructions are available upon request. Briefly, single and multiple point mutations in *CLN2* were generated by sequential PCR. PCR products were subcloned and sequenced to verify fidelity. To generate GST fusions, the appropriate Cln2 domains were amplified by PCR; for subcloning purposes, primers were designed to attach 5′ *Bam*HI and 3′ *Xho*I sites to the amplified fragments. The various Cln2 domains were ligated into the pYEX-4T1 vector (Clontech Labs), generating GST fusions under control of the *CUP1* promoter. To generate the ΔN-Sic1 fusions, the same *CLN2* fragments were subcloned into plasmid pSL276, resulting in fusions of *CLN2* domains with *SIC1* with N-terminal deletions followed by a hemagglutinin (HA) and a hexahistidine (6His) tag. Plasmid pSL276, derived from RDB597 (a generous gift from R. Verma) containing *SIC1-HA-6His* under control of the *GAL1* promoter, introduces an *Xho*I site at position 313 in the *SIC1* coding sequence. Cloning of the *CLN2* domains into *Bam*HI-*Xho*I-cut *SIC1* replaces the first 312 nucleotides of *SIC1*, which encode 104 aa, with *CLN2* sequences.

To generate chromosomally 6His-tagged *GRR1*, the 3′ end of *GRR1* was amplified by PCR and cloned into the pLEU2-6His vector (pSL212 [derived from pKAN-6His]; S. B. Haase, M. Wolff, and S. Reed, unpublished data), resulting in in-frame fusion of *GRR1* to 6His. pSL212 was cut with *Hpa*I for targeted integration into the *GRR1* locus.

To create N-terminally 6His-tagged *GRR1* for expression in SF9 insect cells, wild-type full-length *GRR1* was cloned into pFASTBacHTc (Gibco BRL-Life Technologies), and recombinant baculovirus was generated according to the manufacturer's conditions.

PEST scores were determined with the program PESTFIND developed by Martin C. Rechsteiner and Scott W. Rogers (35) (http://www.at.embnet.org/embnet/tools/bio/PESTfind/).

**Baculovirus expression and purification of Grr1.** For the expression of 6His-Grr1 in insect cells, SF9 cells were infected with recombinant baculovirus encoding the full-length Grr1 for 48 h. Cells were lysed by resuspension in SF9 lysis buffer (phosphate-buffered saline containing 0.5% NP-40, 5 mM β-mercaptoethanol, protease inhibitors {0.2 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], 1 mM EDTA, 20 μM leupeptin, 1 μM pepstatin}, and phosphatase inhibitors [10 mM NaF, 10 mM glycerol phosphate]) followed by incubation on ice for 20 min. Extracts were clarified by centrifugation for 10 min at 14,000 rpm (20,000 × *g*) in an Eppendorf 5810R microcentrifuge, aliquoted, and snap-frozen in liquid nitrogen. 6His-Grr1 was batch purified by incubation of SF9 extracts

adjusted to 500 mM NaCl with Ni-nitrilotriacetic acid (Ni-NTA) beads for 2 h at 4°C, followed by washes containing 20, 50, 80, 120, and 200 mM imidazole. The 80 mM imidazole fraction was used for binding studies.

**Immunoprecipitation and immunoblotting.** Yeast protein extracts were made with glass beads in lysis buffer (50 mM Tris-HCl [pH 7.5], 0.1% NP-40, 250 mM NaCl) containing protease inhibitors and phosphatase inhibitors (5 mM EDTA, 5 mM EGTA, 0.1 mM orthovanadate). Cells were lysed with glass beads by 5 to 10 cycles of vortexing for 1 min followed by a 1-min incubation on ice. Extracts were collected after centrifugation for 15 min at 14,000 rpm (20,000 × *g*) in an Eppendorf 5810R microcentrifuge. The protein concentrations of the lysate were determined by the Bio-Rad protein assay.

Extracts were fractionated on sodium dodecyl sulfate (SDS) gels and transferred onto polyvinylidene difluoride membrane (PolyScreen; NEN) by semidry blotting. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline–0.25% Tween and incubated with primary antibodies (1:1,000 in Tris-buffered saline–1% milk–0.25% Tween unless otherwise indicated) for 2 h to overnight followed by secondary antibodies (horseradish peroxidase-conjugated) (1:10,000; Bio-Rad) for 1 h. Antibodies used were anti-HA (mouse monoclonal; Babco), anti-Myc (mouse monoclonal 9E10) (1:250; Santa Cruz), anti-Cdc28 (goat, yC-20) (1:250; Santa Cruz), and anti-GST (rabbit polyclonal; Santa Cruz). Signals were detected on films by enhanced chemiluminescence (SuperSignal-Pico; Pierce). Anti-HA or anti-GST beads were made by covalently coupling the antibody (rabbit polyclonal anti-GST antibody [Santa Cruz] or mouse anti-HA ascites [Babco]) to protein A-Sepharose with dimethylpimelimidate.

For coimmunoprecipitations, extracts containing 1 to 2 mg of total proteins were incubated with the appropriate resin (anti-GST beads or anti-HA beads) in lysis buffer containing protease and phosphatase inhibitors for 2 h at 4°C. The beads were washed three times with lysis buffer and boiled with 2× SDS sample buffer, and bound proteins, along with extracts and flowthroughs, were analyzed by immunoblotting. For analysis of the interaction of $Cln2^{4T3S}$, $Cln2^{M46}$, or Cln2-Sic1 fusions with Grr1, the various Cln2 constructs were transformed into a yeast strain carrying chromosomally integrated *GRR1-6His* (or into a wild-type yeast strain as negative control). Extracts were made and coimmunoprecipitations performed using low-salt lysis buffer (100 mM NaCl). Total proteins (10 mg) were immunoprecipitated, and 9 of 10 of the bound fractions were analyzed for Grr1 and 1 of 10 was analyzed for Cln2-Sic1 fusions.

**Analysis of phosphorylation.** The phosphorylation status of the recombinant fusion proteins was analyzed by treatment with calf intestine phosphatase (CIP) (10 U/μl; New England Biolabs) in the presence or absence of phosphatase inhibitors. Extracts (300 μg) were bound on anti-HA beads in lysis buffer with or without phosphatase inhibitors for 2 h at 4°C. After being washed three times with buffer with or without phosphatase inhibitors, the beads were incubated with 50 μl of alkaline buffer (50 mM Tris-HCl [pH 8.5], 150 mM NaCl) containing 20 U of CIP in the presence or absence of phosphatase inhibitors at 30°C for 60 min on a rotating support. Beads were washed twice with buffer and boiled in 2× SDS sample buffer. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-HA antibody.

**Detection of ubiquitination.** Yeast strains containing the Cln2-Sic1 constructs were transformed with a plasmid carrying a tagged mutant ubiquitin (CUP1-Ubi-HIS-MYC-RA). This polyhistidine- and Myc-tagged K48R, G76A mutant ubiquitin competes with endogenous ubiquitin and is incorporated into ubiquitin chains, but the K48R substitution prevents further polymerization of ubiquitin and the G76A substitution inhibits hydrolysis by ubiquitin isopeptidases (24). Transformants were grown in selective glucose-containing medium and then diluted to an $OD_{600}$ of 0.2 in raffinose-containing medium and grown to an $OD_{600}$ of 0.5. Protein expression was induced with 250 μM $CuSO_4$ for 4 h and with 2% galactose for two additional hours. Glass bead extracts (7.5 mg) made in buffer G (6 M guanidine hydrochloride, 0.1 M $NaH_2PO_4$, 20 mM Tris-HCl, pH 8.0) were incubated with $Ni^{2+}$-NTA Agarose (Qiagen) for 1 h at room temperature. Beads were washed three times with 2.5 ml of buffer G and four times with 5 ml of buffer C (50 mM Tris-HCl [pH 8.0], 500 mM NaCl). Bound proteins were eluted with 25 μl of buffer E (100 mM Tris-HCl [pH 6.8], 1% SDS, 100 mM dithiothreitol, 100 mM EDTA) for 10 min at room temperature on a rotating support, and the eluates were analyzed by SDS-PAGE and immunoblotting with anti-HA antibodies.

**Determination of protein degradation and half-life.** Strains containing the various Cln2-Sic1 fusions under control of the *GAL1* promoter were grown in glucose-containing medium, diluted to an $OD_{600}$ of 0.25 in raffinose-containing medium, and grown for two generations. Expression of the fusion proteins was induced by addition of galactose to a final concentration of 2% for 30 min to 1 h. At time point 0, cells were filtered and resuspended in glucose-containing medium to repress transcription. Samples were removed at the time points indicated by filtering 20 ml of culture on ice, washing the pellets briefly with water, and freezing the cell pellets in liquid nitrogen. Extracts were made with glass beads, and protein content was normalized using the Bio-Rad assay and analyzed by immunoblotting with monoclonal anti-HA antibodies or polyclonal anti-Cln2 antibodies (data not shown). Band intensities were quantified using PhosphorImager technology and ImageQuant software (Molecular Dynamics) or LabWorks software (UVP). For accurate calculations, we routinely included internal controls with serial dilutions of proteins and normalized the samples to the Cdc28 loading control. Protein half-lives were estimated by best-fit analyses of degradation curves.

**Pulse-chase analysis.** Logarithmically growing cells expressing GST-Cln2 domain fusions from the *CUP1* promoter were induced with $CuSO_4$ for 2 h, washed, resuspended in methionine-free medium, and labeled by incubation with 1 mCi of $^{35}$S-protein labeling mix (Easy Tag Express-[$^{35}$S]-protein labeling mix; NEN) for 5 min at 30°C. Excess cold methionine and cysteine were added, and samples were collected at the time points indicated. Extracts were made with glass beads and subjected to denaturing immunoprecipitation with anti-GST beads. Beads were boiled with 2× SDS sample buffer and proteins bound were fractionated on SDS gels and visualized by PhosphorImager analysis as described above.

## RESULTS

We have shown previously that Cdc28-mediated phosphorylation of Cln2 promotes its rapid degradation by the ubiquitin-proteasome pathway (24, 45). A replacement of the seven Cdc28 consensus phosphorylation sites in Cln2 by alanine (termed $Cln2^{4T3S}$) stabilized Cln2 eightfold. However, the Cln2 domain(s) responsible for recognition by the SCF degradation machinery is not defined. Therefore, we set out to delineate the domain(s) in Cln2 important for instability.

**A cluster of four phosphorylation sites destabilizes Cln2.** First we asked whether all seven Cdc28 phosphorylation sites in Cln2 were important for degradation. We had previously observed that (i) alanine substitutions at sites 3 and 5 (mutant D35; Cdc28 phosphorylation sites are labeled 1 through 7 [Fig. 1A]) increased the half-life of Cln2 to 35 min, (ii) substitution at site 7 had no effect on Cln2 stability, and (iii) deletion of residues 376 to 514 (eliminating sites 2 through 6) resulted in a highly stabilized Cln2 (24) (Fig. 1 and Table 1). These results suggested that phosphorylation sites 3 and 5 in combination with any or all of sites 2, 4, and 6 are important for Cln2 destabilization. We, therefore, created all possible permutations of alanine substitutions of sites 2, 4, and 6; combined those with the D35 mutant (Fig. 1A); and analyzed the half-life of the mutant proteins (Fig. 1B). Mutation of site 2 slightly but consistently destabilized Cln2. One particular mutant, termed $Cln2^{M46}$, that had sites 3, 4, 5 and 6 mutated, was stabilized to a degree comparable to that for the seven-site mutant $Cln2^{4T3S}$. Interestingly, these sites cluster in a 35-aa domain (the D domain) that overlaps marginally (9 aa) with the PEST domain, pointing to the D domain as an important determinant of Cln2 instability. When introduced in single copy into the yeast genome and expressed under its own promoter, the mutant $Cln2^{M46}$ resulted in higher protein steady-state levels and in elimination of some of the low-mobility phosphorylation forms of Cln2 (Fig. 2 and data not shown). The mean cell size of strain SLY349, containing a single copy of $Cln2^{M46}$ as its sole source of $G_1$ cyclin activity, was reduced by 40% compared to that of the isogenic $Cln2^{wt}$ strain SLY156, suggesting a hyperactive Cln2/Cdc28 kinase due to high steady-state levels of $Cln2^{M46}$ (data not shown). $Cln2^{M46}$ was slightly but consistently more hyperactive in mating pheromone and cell size assays than was the double mutant $Cln2^{D35}$ (data not shown). Consistent with hyperactive $G_1$ cyclin kinase, $Cln2^{M46}$ cells

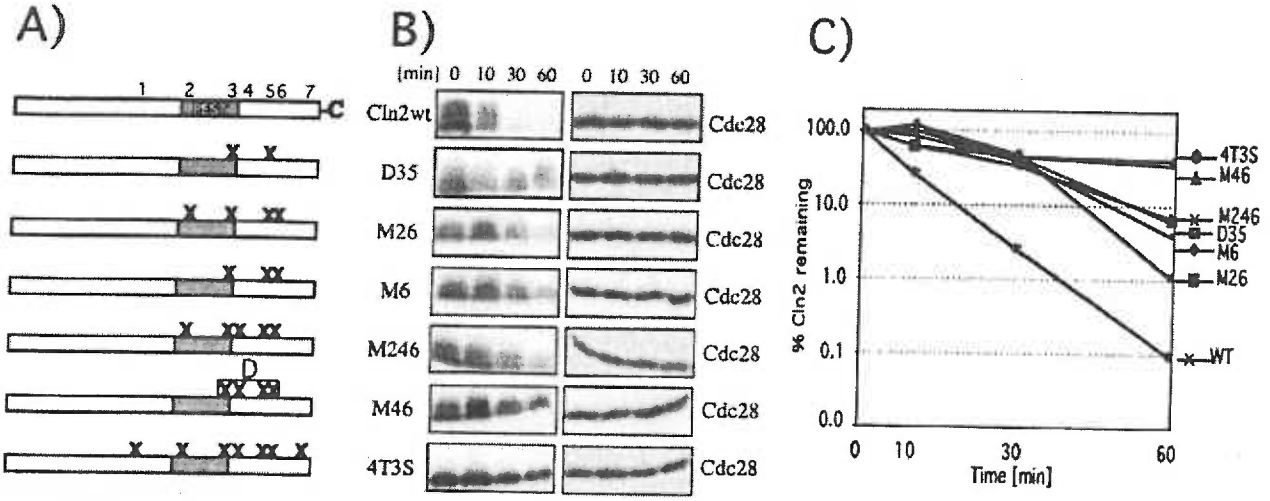


FIG. 1. A cluster of four phosphorylation sites destabilizes Cln2. (A) Cln2 protein coding region. The seven Cdc28 phosphorylation sites are numbered (1, T311; 2, T381; 3, S396; 4, T405; 5, S427; 6, T430; 7, S518). The PEST domain is indicated as hatched box. Abbreviations: X, amino acid substitution to alanine; D, the region in mutant M46 harboring the four relevant substitutions that render Cln2 stable. (B) Stability of wild-type and mutant Cln2. Wild-type Cln21 and mutant Cln2 tagged with an HA epitope were pregrown under noninducing conditions (2% raffinose). *GAL1-CLN2* or its derivatives were expressed for 45 min by addition of 2% galactose and then repressed by addition of 2% glucose. Extracts were prepared from samples taken at the indicated times and analyzed by immunoblotting using anti-HA antibodies. Equal loading of samples was verified by incubating the blots with anti-Cdc28 antibodies. (C) Graph representing degradation rates from at least two independent experiments. Values were normalized to Cdc28 as internal loading control and to a dilution series (see Materials and Methods).

continued to proliferate in the presence of the growth inhibitor alpha factor (data not shown). Interestingly, overexpression of the stabilized mutants $Cln2^{M46}$ and $Cln2^{4T3S}$, but not wild-type Cln2, from the *GAL1* promoter is lethal, arresting cells in late M phase (C. Wittenberg and S. Lanker, unpublished). In conclusion, we have identified a cluster of four phosphorylation sites in Cln2 that are essential for its rapid degradation.

**Grr1 interacts with phosphorylated Cln2 in vivo, but not**

TABLE 1. Genotypes and phenotypes of relevant *CLN2* mutants and *CLN2* domain fusions

| Genotype | Name | No. of sites mutated | Half-life (min)[a] | Binding | |
|---|---|---|---|---|---|
| | | | | Grr1 | Cdc28 |
| Cln2 mutants | | | | | |
| *CLN2* | Wild type | 0 | 8 | ++ | + |
| *CLN2(S518A)* | | 1 | 6 | ND[c] | + |
| *CLN2(S396,427A)* | D35 | 2 | 35 | ND | + |
| *CLN2(Δ376–514)* | ΔHN | 5[b] | 55 | ND | + |
| *CLN2(Δ376–545)* | ΔC | 6[b] | 60 | ND | + |
| *CLN2(T311,381,405,430A S396,427,518A)* | 4T3S | 7 | 60 | – | + |
| *CLN2(S396,427A T405,430A)* | M46 | 4 | 55 | – | + |
| *CLN2(S396,427D T405,430D)* | M4D | 4 | 55 | ND | ND |
| *cln2Δxs* | Cln2Δxs | 0 | 30 | ND | – |
| *cln2Δxs(T311,381,405,430A S396,427,518A)* | ClnΔxs(4T3S) | 0 | 30 | ND | – |
| GST-Cln2 fusions | | | | | |
| *GST* | GST | na | 166 | – | – |
| *GST-CLN2C(376–545)* | GST-2C(wt) | 0 | 41 | – | – |
| *GST-CLN2(376–545; T405,430A S396,427A)* | GST-2C(M46) | 6 | 38 | – | – |
| *GST-CLN2(376–431)* | GST-PD | 0 | 93 | – | – |
| *GST-CLN2(396–431)* | GST-D | 0 | 134 | – | – |
| Cln2-ΔN-Sic1 fusions | | | | | |
| *CLN2C(376–545)–ΔN105-SIC1* | 2C(wt)–ΔN-Sic1 | 0 | 18 | ++ | + |
| *CLN2C(376–545; T405,430A S396,427A)–ΔN105-SIC1* | 2C(M46)–ΔN-Sic1 | 4 | ~180 | – | + |
| *CLN2C(396–431)–ΔN105-SIC1* | D-Sic1 | 0 | 150 | + | + |
| *CLN2C(376–403)–ΔN105-SIC1* | P-Sic1 | 0 | 90 | – | + |
| *CLN2C(396–431)–ΔN105-SIC1* | PD-Sic1 | 0 | 41 | ++ | + |

[a] Cln2 half-life was determined by densitometric analysis.
[b] Number of sites deleted.
[c] ND, not determined.

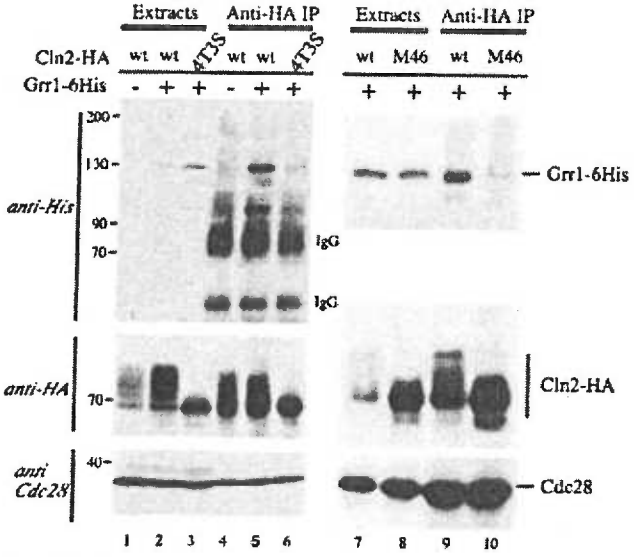


FIG. 2. Grr1 interacts with phosphorylated Cln2 but not with hypophosphorylated $Cln2^{4T3S}$ or $Cln2^{M46}$. Lysates from strains expressing either untagged *GRR1* (lanes 1 and 4) or chromosomally 6His-tagged *GRR1* (lanes 2, 3, 5, 6, and 7 to 10) from its endogenous promoter and expressing wild-type (wt) *CLN2HA* (lanes 1, 2, 4, 5, 7, and 9), *CLN*$^{24T3S}$*-HA* (lanes 3 and 6), or *CLN2*$^{M46}$*-HA* (lanes 8 and 10) from the *GAL1* promoter were immunoprecipitated (IP) with a monoclonal anti-HA antibody (lanes 4 to 6, 9, and 10). Immunoprecipitates were immunoblotted with anti-His antibody to detect Grr1-6His, with 12CA5 antibody to detect Cln2HA, or with anti-Cdc28 antibody. IgG, immunoglobulin G.

**with hypophosphorylated $Cln2^{4T3S}$ or $Cln2^{M46}$.** Cln2 is ubiquitinated by $SCF^{Grr1}$ in complex with the E2 Cdc34. The F-box protein Grr1 has been shown to interact with phosphorylated $G_1$ cyclins Cln1 and Cln2 in vitro (39, 40); in vivo interaction has been demonstrated using overexpression of both Grr1 and Cln2 (17, 22). We evaluated whether the stabilization of the $Cln2^{4T3S}$ and $Cln2^{M46}$ mutants was due to loss of interaction with Grr1. The abundance of Grr1 is very low (12); to preclude potential artifacts associated with overexpression of proteins, we constructed by targeted integration strains that carry C-terminally tagged Grr1 in single copy under control of the native Grr1 promoter. Here we demonstrate, using coimmunoprecipitation experiments, an in vivo interaction between endogenous Grr1 and phosphorylated Cln2 (Fig. 2, upper panel, lanes 5 and 9). Importantly, this interaction was strongly reduced when the mutants $Cln2^{4T3S}$ and $Cln2^{M46}$ were expressed (lanes 6 and 10). However, wild-type as well as both mutant forms of Cln2 bound equally well to Cdc28 (Fig. 2, lower panel, lanes 4, 5, 6, 9, and 10), demonstrating that $Cln2^{4T3S}$ and $Cln2^{M46}$ are folded correctly and maintain the ability to interact with and activate Cdc28. We conclude that Grr1 interacts in vivo with phosphorylated Cln2 and that mutations in phosphorylation sites that stabilize Cln2, $Cln2^{4T3S}$, and $Cln2^{M46}$ significantly reduce this interaction.

**Destabilization of GST-Cln2 domain fusions does not depend on phosphorylation.** Previous studies have suggested that the PEST domain is necessary but not sufficient for Cln2 instability (36). Since we have implicated the phosphorylated D domain as an important protein instability determinant, we wanted to establish the relationship between the PEST and the D domains and ultimately identify the domain(s) necessary and sufficient to promote Cln2 degradation.

We fused the following Cln2 domains to the stable GST polypeptide (Fig. 3A): the Cln2 C terminus (2C; aa 376 to 545), the C terminus harboring the substitutions in each of the four essential Cdc28 phosphorylation sites ($2C^{M46}$), the D domain encompassing the four phosphoacceptor sites (aa 396 to 431), and the PD domain (aa 376 to 431). We investigated the stability of the GST fusions by pulse-chase analysis (data not shown). Quantification of at least two independent experiments indicated a half-life of about 170 min for GST and the control GST-SKP1, of about 40 min for GST-$2C^{wt}$ and GST-$2C^{M46}$, of 90 min for GST-PD, and of 135 min for GST-D (Table 1). These data show that the C-terminal domain of Cln2 and, to a lesser degree, the PD domain, but not the D domain alone, are able to confer significant instability to GST. However, the observation that GST-$2C^{wt}$ and GST-$2C^{M46}$ have similar half-lives indicates that, unlike the case for wild-type Cln2, degradation of these fusion constructs is not controlled by phosphorylation. We hypothesized that an $SCF^{Grr1}$-independent pathway, which is independent of substrate phosphorylation, degrades Cln2 domains fused to GST. In agreement with this, GST-Cln2 fusions did not interact with Cdc28 in vivo, were not phosphorylated in vivo or in an in vitro phosphorylation assay using insect cell-expressed active Cdc28/Cln2 kinase, and did not interact with Grr1 in vivo (data not shown).

**Construction of Cln2 domains fused to a stable ΔN-Sic1 reporter.** Sic1 binds to and inhibits B-type cyclin/Cdc28 (Clb/Cdc28) kinase complexes via its C terminus (44). Importantly, Sic1 is a well-characterized substrate of $SCF^{Cdc4}$; the N-terminal 105 aa are necessary and sufficient for $SCF^{Cdc4}$-mediated ubiquitination and degradation (11, 39, 44). Based on our GST fusion experiments we hypothesized that fusions of Cln2 domains to stabilized Sic1 lacking the N terminus would target the Cln2-Sic1 fusions for $SCF^{Grr1}$-dependent degradation. Accordingly, we decided to fuse the following Cln2 domains—$2C^{wt}$, $2C^{M46}$, PD, and D—as well as the PEST domain (P; aa 376 to 403) to an N-terminally truncated Sic1 (ΔN-Sic1; aa 106 to C terminus) containing an HA-6His double tag. The fusion genes were put under control of the inducible *GAL1* promoter to allow for controlled expression and repression by galactose and glucose, respectively.

**The PD-ΔN-Sic1 fusion is rapidly degraded.** First, we tested the stability of Cln2-Sic1 fusions by promoter shutoff experiments. Cells were grown in raffinose-containing medium; expression of the fusion proteins was induced with galactose for 1 h and then repressed with glucose. Samples were collected at the time points indicated after glucose addition. As shown previously (43), wild-type Sic1 was very unstable, with a half-life of less than 5 min, whereas a Sic1 phosphorylation site mutant is extremely stable (Fig. 3B, top two panels). Grafting the 2C domain onto the ΔN-Sic1 protein destabilized it dramatically, with a half-life of approximately 18 min (Fig. 3B, third panel). Importantly, however, the mutant $2C^{M46}$ was highly stable (Fig. 3B, fourth panel; half-life more than 180 min). The PD fusion was rapidly degraded (Fig. 3B, last panel; half-life about 41 min), suggesting that we have identified a domain within Cln2 sufficient to confer significant instability to ΔN-Sic1. In contrast, the D and the P fusion proteins were

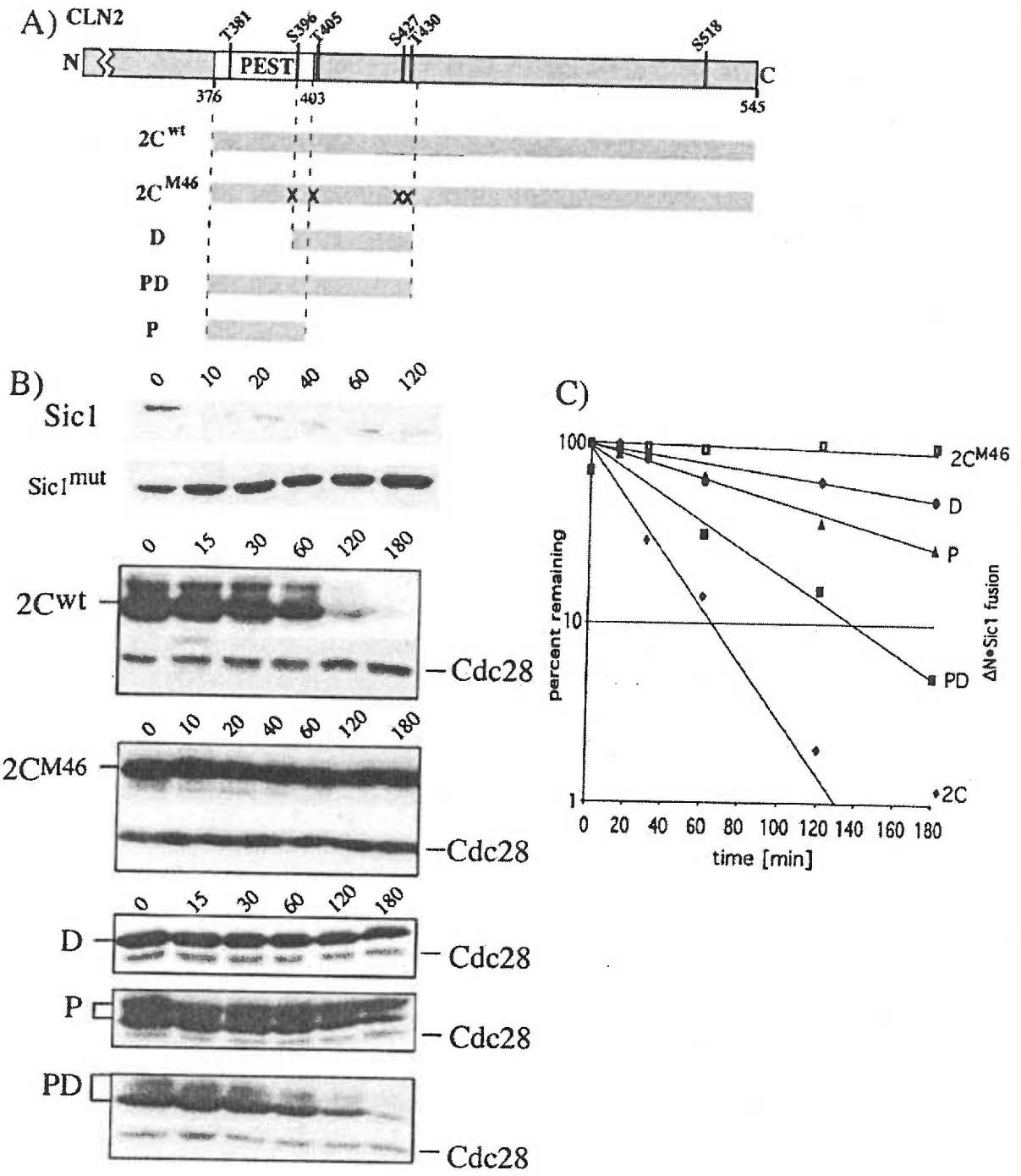


FIG. 3. The Cln2-2C and -PD domains confer phosphorylation-dependent instability upon a stable ΔN-Sic1 reporter protein. (A) Domains of Cln2 fused to reporter proteins. The top bar shows the *CLN2* C terminus containing the PEST domain (open box) and six Cdc28 phosphorylation sites. Below are the Cln2 domains that were fused to the reporter proteins; mutations of serine or threonine residues to alanines are marked (X). (B) Wild-type Sic-HA, stabilized Sic$^{mut}$-HA harboring mutations in four phosphorylation sites, or ΔN-Sic1-HA fused to the indicated Cln2 domains was subjected to *GAL1* promoter shutoff experiments as described in Fig. 1. Extracts from samples taken at the indicated time points after repression of the *GAL1* promoter were analyzed by immunoblotting using anti-HA antibodies. As a loading control, anti-Cdc28 antibodies were used. (C) Graph representing the degradation rates of the various fusions.

stable (Fig. 3B, fifth and sixth panels) with half-lives of about 150 and 90 min, respectively. Since 2C$^{wt}$ and PD carrying four intact phosphorylation sites were rapidly degraded whereas nonphosphorylatable 2C$^{M46}$ was highly stabilized, we suggest that degradation of these fusion proteins is phosphorylation dependent.

**2C$^{wt}$, P, and PD fusions are phosphorylated.** We have demonstrated previously that Cln2 is phosphorylated in vivo and in

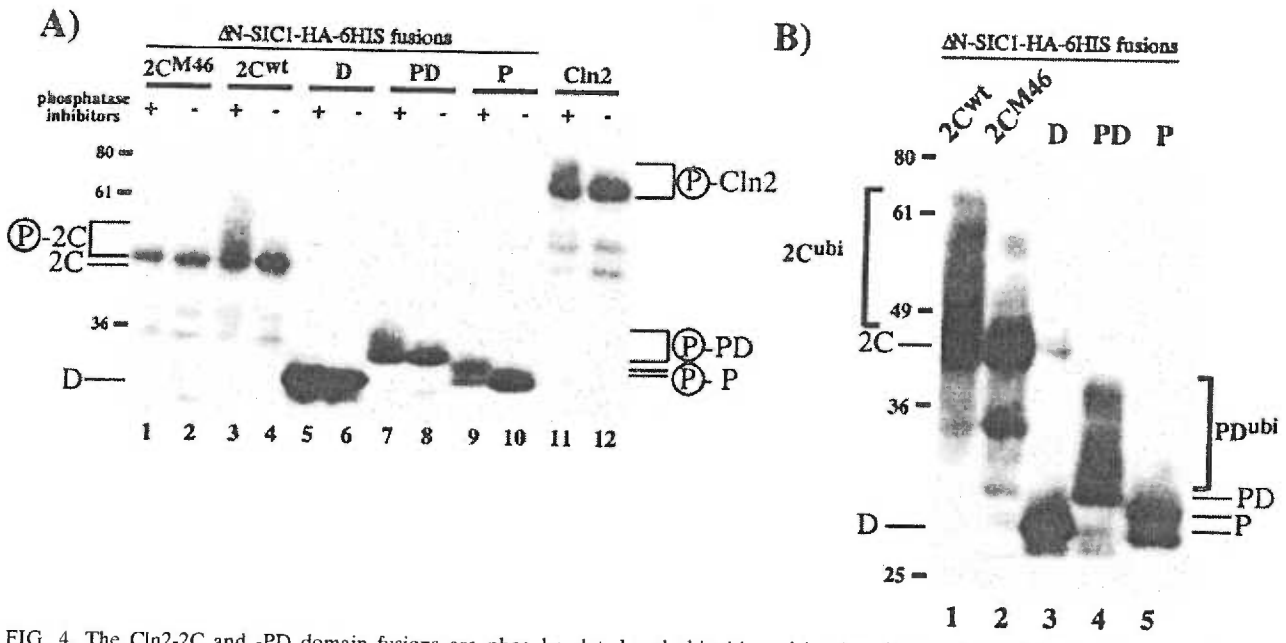


FIG. 4. The Cln2-2C and -PD domain fusions are phosphorylated and ubiquitinated in vivo. (A) Cln2-ΔN-Sic1-HA fusion proteins were captured on anti-HA beads and incubated for 30 min with CIP in the presence (+) or absence (−) of phosphatase inhibitors, followed by immunoblotting using anti-HA antibodies. The encircled "P" designates phosphorylated species. (B) Extracts prepared from cells expressing the indicated Cln2-ΔN-Sic1-6His-HA fusions and 6His-Myc-tagged K48R, G76A mutant ubiquitin (6His-MYC-UBI-RA) were chromatographed over Ni-NTA beads in a buffer containing 6 M guanidine hydrochloride. Bound proteins and ubiquitin conjugates were analyzed by immunoblotting using anti-HA antibodies.

vitro in a Cdc28-dependent manner (24). Cln2 phosphorylation was manifested on SDS-polyacrylamide gels as a series of low-mobility bands that collapsed into a single band upon treatment with calf intestine phosphatase. We also showed that phosphorylation of the stable M46 mutant Cln2 is reduced more than 90% compared to that of the wild type and runs as a single band on SDS-polyacrylamide gels. Therefore, we analyzed the electrophoretic behavior of the fusion proteins. The low-mobility bands of fusion proteins $2C^{wt}$ and PD were eliminated upon treatment with CIP in the absence of phosphatase inhibitors (Fig. 4A, lanes 4 and 8) but not in the presence of inhibitors (Fig. 4A, lanes 3 and 7). The P domain produced a double band in the presence of phosphatase inhibitors (Fig. 4A, lane 9). Upon treatment with CIP, most of the lower-mobility forms were converted to higher-mobility forms; the double bands persisted, suggesting that the P fusion contains an additional modification (Fig. 4A, lane 10). Both $2C^{M46}$ and D did not exhibit any low-mobility forms (Fig. 4A, lanes 1 and 5) and were unchanged upon CIP treatment (Fig. 4A, lanes 2 and 6). This experiment demonstrates that the fusion proteins $2C^{wt}$ and PD and, to a lesser degree, P are modified by phosphorylation. As expected, the $2C^{M46}$ did not display any modified forms. In this assay, the D fusion appears to be unphosphorylated; however, we cannot exclude the possibility that the D fusion is modified by phosphorylation that does not result in a shift on SDS-PAGE.

**2C- and PD-ΔN-Sic1 fusions are ubiquitinated.** Our previous result showed that phosphorylated Cln2 was ubiquitinated in vivo in a Cdc53- and Cdc34-dependent manner (45), consistent with Cln2 degradation via the $SCF^{Grr1}$ pathway. Here we have examined whether the Cln2-ΔN-Sic1 fusions were ubiquitinated in vivo. To be able to detect in vivo polyubiquitinated proteins, we coexpressed a polyhistidine- and Myc-tagged K48R, G76A mutant ubiquitin gene (Ubi-6His-MYC-RA) together with the Cln2-Sic1–HA–6His fusions. Ubi-6His-MYC-RA competes with endogenous ubiquitin and is incorporated into ubiquitin chains, but the K48R substitution prevents multiubiquitin chain formation; proteins that contain short chains of ubiquitin (up to three ubiquitin moieties) are poor substrates for the proteasome. The G76A substitution inhibits hydrolysis by ubiquitin isopeptidases (24). Therefore, the double-mutant tagged ubiquitin was expected to enrich for ubiquitinated species in vivo. Denatured extracts from strains expressing the fusion constructs and Ubi-6His-MYC-RA were incubated with $Ni^{2+}$-NTA beads, and after extensive washing, bound proteins were eluted and analyzed by Western blotting with anti-HA antibody. Note that under these conditions, ubiquitinated Sic1 fusion species will be detected as a lower-mobility smear above the nonubiquitinated fusion proteins. Consistent with their instability, the Sic1 fusions $2C^{wt}$ and PD displayed extensive ubiquitination (Fig. 4B, lanes 1 and 4), while $2C^{M46}$, D, and P did not show significant ubiquitination (Fig. 4B, lanes 2, 3, and 5). We conclude that $2C^{wt}$ and, in particular, the PD domain contain signals sufficient to promote ubiquitination most likely via $SCF^{Grr1}$ (see below). Neither the PEST domain P, although phosphorylated to some degree, nor the D domain alone is sufficient to attract the ubiquitination machinery.

**Grr1, but not Cdc4, binds to 2C-, PD, and D-ΔN-Sic1 fusions.** SCF specificity relies on the nature of the F-box protein: Sic1 is targeted for degradation via binding of Cdc4 to its phosphorylated N terminus, while Cln2 is targeted by Grr1. To

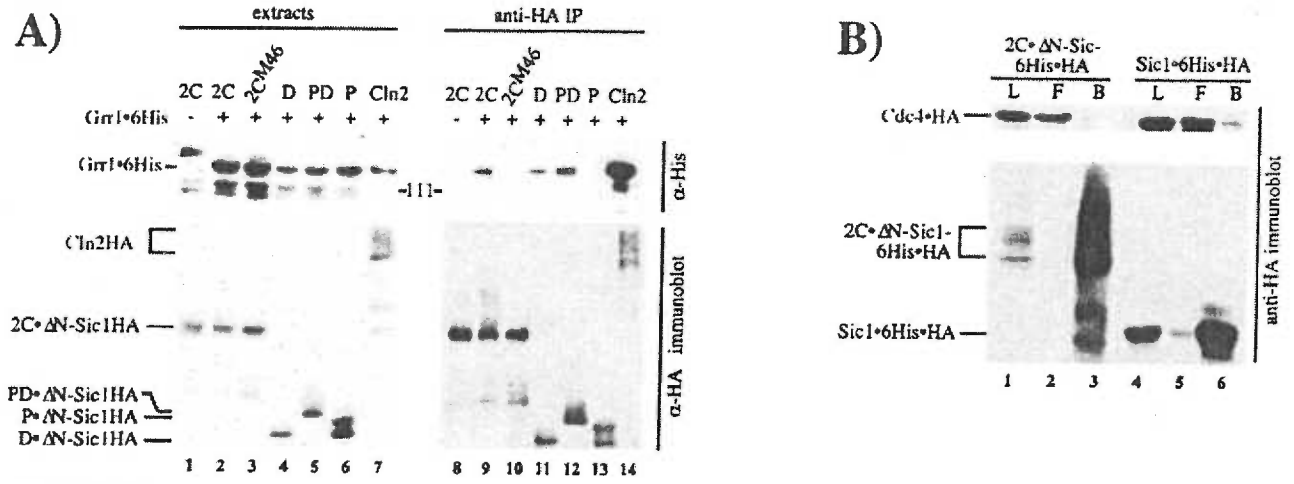


FIG. 5. Grr1 interacts with Cln2-2C, D, and PD fused to ΔN-Sic1-HA-6His. (A) Lysates from strains expressing either untagged *GRR1* (lanes 1 and 8) or chromosomally 6His-tagged *GRR1* (lanes 2, 3, 5, 6, and 7 to 10) from its endogenous promoter and those expressing wild-type *CLN2HA* (lanes 7 and 14), or the indicated Cln2 domain fusions to ΔN-Sic1-HA-6His from the *GAL1* promoter, were immunoprecipitated (IP) with a monoclonal anti-HA antibody (α-HA) (lanes 8 to 14). Immunoprecipitates were immunoblotted with anti-His antibody (α-His) to detect Grr1-6His or with anti-HA antibody to detect Cln2HA. (B) Cdc4 fails to interact with 2C-ΔN-Sic1-HA-6His. Lysates from strains expressing chromosomally HA-tagged *CDC4* from its endogenous promoter and expressing 2C-ΔN-Sic1-HA-6His (lanes 1 to 3) or Sic1-HA-6His (lanes 4 to 6) from the *GAL1* promoter were incubated with $Ni^{2+}$-NTA beads. Extracts (L), flowthrough (F), and bound proteins (B) were analyzed by immunoblotting with anti-HA antibody.

investigate whether Grr1 would recognize the various Cln2 domains fused to ΔN-Sic1, the fusion constructs were transformed into yeast strains that carried C-terminally 6His-tagged Grr1 in single copy under control of the native *GRR1* promoter. Expression of the HA-tagged fusion proteins was induced briefly with galactose, and coimmunoprecipitation with anti-HA beads was performed followed by SDS-PAGE and Western blotting. Grr1-6His bound to $2C^{wt}$ but not to $2C^{M46}$ (Fig. 5, lanes 9 and 10). Grr1-6His coprecipitated with the PD domain (Fig. 5, lane 12) and, unexpectedly, the D domain (Fig. 5, lane 11) but not with the P domain (Fig. 5, lane 13). These results show that, as with full-length Cln2, phosphorylation of the 2C-Sic1 fusion is required for its interaction with Grr1, since the mutation of the phosphorylation sites in the fusion protein $2C^{M46}$ abolished the interaction. Interestingly, the D domain was sufficient for binding to Grr1, albeit with reduced efficiency compared to PD (Fig. 5, compare lanes 11 and 12). In contrast, we were not able to detect an interaction between Cdc4 and 2C-ΔN-Sic1 fusions (Fig. 5B). We conclude that the 2C and the PD fusions to ΔN-Sic1 target these chimeras for recognition by Grr1, thus switching the F-box protein specificity for Sic1 from Cdc4 to Grr1.

**Degradation of Cln2-ΔN-Sic1 fusions does not depend on Clb/Cdc28 kinase.** What kinase(s) is involved in degradation of the Cln2-ΔN-Sic1 fusions? Our previous experiments demonstrated that functional Cdc28 as well as $G_1$ cyclins is essential for degradation of Cln2 (24, 37); however, another report suggested that Clb/Cdc28 kinase can phosphorylate $G_1$ cyclins, thus targeting them for degradation (4). To address the role that Clb/Cdc28 kinase might play in degradation of the Cln2-ΔN-Sic1 fusions, we first verified that the Cln2-ΔN-Sic1 fusions interacted with Cdc28, most likely via the Sic1 C terminus. Cln2-ΔN-Sic1 fusions were precipitated with anti-HA beads, and proteins bound were analyzed by Western blotting using anti-HA and anti-Cdc28 antibodies. All Cln2-ΔN-Sic1 fusion proteins were able to bind Cdc28 (Fig. 6A, lanes 6, 9, 12, 15, and 18), while the negative control Cln2Δxs (Cln2 with a deletion in the cyclin box [7]) was unable to interact with Cdc28 (lane 3).

To address whether Clb/Cdc8 binding to the Sic1 C terminus was necessary for Cln2-ΔN-Sic1 degradation (43), we deleted the Clb/Cdc28 binding domain in the C terminus of the various Sic1 fusions (Sic1-ΔC) (44) (Fig. 6B) and analyzed the stability of the resulting deletion mutants. Degradation of 2C-ΔN-Sic1-ΔC fusions was unaltered (Fig. 6C), while the PD-ΔN-Sic1-ΔC fusions were slightly stabilized (data not shown), suggesting that C-terminal binding to Clb/Cdc28 is not essential for rapid degradation of the Cln2-ΔN-Sic1 fusions.

Is Clb/Cdc28 kinase essential for phosphorylation and degradation of Cln2-ΔN-Sic1 fusions in *trans*? If so, then inhibition of Clb/Cdc28 kinase by expression of undegradable Sic1 ($Sic1^{4A}$) (43) should alter the degradation rate of the Cln2-ΔN-Sic1 fusions. However, we observed at most a slight increase in stability of the Cln2-ΔN-Sic1 fusions when coexpressed with $Sic1^{4A}$ (data not shown), suggesting that, as is the case for wild-type Cln2, the $G_1$ cyclin/Cdc28 kinase is the major kinase that phosphorylates the Cln2-ΔN-Sic1 fusions, initiating their ubiquitination and degradation.

**Cdc28 is not required for interaction of phosphorylated Cln2 with Grr1 in vitro.** We envision two plausible explanations accounting for the observed dependence of Cln2 degradation on Cdc28: (i) Cdc28 might be necessary solely for the phosphorylation step, but not for subsequent steps including Grr1 binding; (ii) alternatively, interaction with Cdc28 might be important for recognition by Grr1 and, possibly, later steps including ubiquitination and degradation. To distinguish between these models, we tested whether the presence of Cdc28 in a complex with the fusion protein is required for the inter-

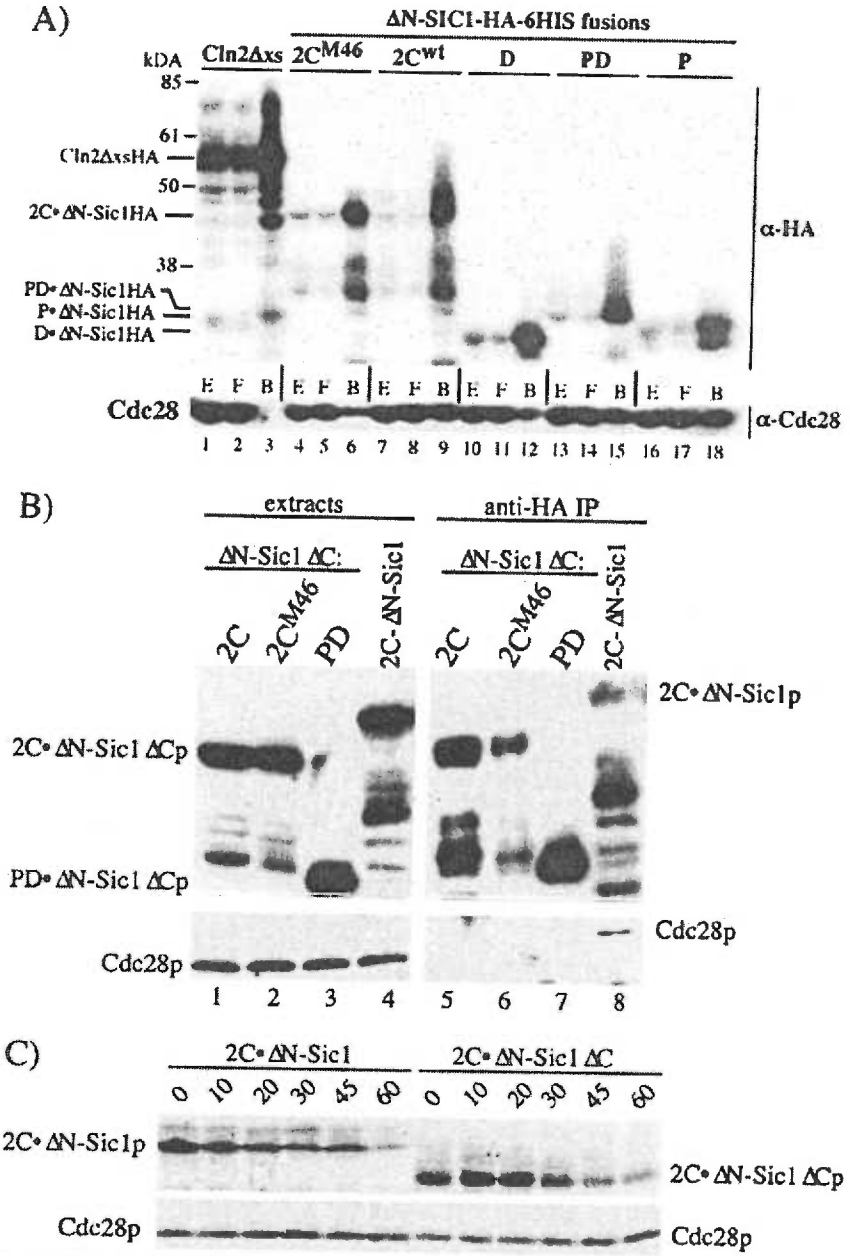


FIG. 6. (A) Cdc28 binds to Cln2-ΔN-Sic1 fusions. Strains expressing the indicated fusions (lanes 4 to 18) or a mutant Cln2Δxs lacking its cyclin box were subjected to coimmunoprecipitation and immunoblotting as described in the legend to Fig. 5, except that anti-Cdc28 antibody (α-Cdc28) was used to detect bound Cdc28. Abbreviations: E, extract; F, flowthrough; B, bound; α-HA, anti-HA antibody. (B) Deletion of the Sic1 C terminus results in loss of Clb-Cdc28 binding. Strains expressing 2C-ΔN-Sic1 (lanes 4 and 8) or Cln2 domains fused to ΔN-Sic1 lacking its C terminus (lanes 1 to 3 and 5 to 7) were subjected to coimmunoprecipitation (IP) and immunoblotting as described in the legend to Fig. 5, except that anti-Cdc28 antibody was used to detect bound Cdc28. (C) Deletion of the Sic1 C terminus does not affect the stability of 2C-ΔN-Sic1. 2C-ΔN-Sic1-HA and 2C-ΔN-Sic1-HA with a C-terminal deletion of the Clb/Cdc28 binding site were subjected to *GAL1* promoter shutoff experiments as described in the legend to Fig. 1. Extracts from samples taken at the indicated time points after repression of the *GAL1* promoter were analyzed by immunoblotting using anti-HA antibodies. As a loading control, anti-Cdc28 antibodies were used.

action between Grr1 and Cln2 in vitro. Cln2HA was expressed from a Gal-inducible promoter and precipitated with anti-HA beads (Fig. 7, lanes 1 and 3, lower panel). The beads were washed either with lysis buffer or with buffer containing 1% SDS to wash off the bound Cdc28 (Fig. 7, lanes 2 and 4) and incubated with extracts from insect cells expressing Grr1-6His. Grr1 was bound to Cln2 independently of whether Cdc28 was present or absent (Fig. 7, lanes 1 and 2, top panel). The negative controls with mock insect cell extract (Fig. 7, lanes 3 and 4) or mock yeast extract (Fig. 7, lanes 5 to 8) did not retain significant amounts of Grr1-6His. SCF-mediated degradation is a highly conserved process, and it is known that insect cells

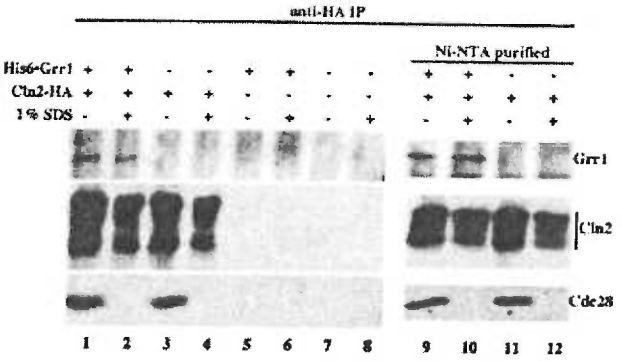


FIG. 7. Release of Cdc28 from phosphorylated Cln2 does not affect binding to Grr1. Extracts from strains expressing Cln2HA (lanes 1 to 4 and 9 to 12) or not expressing Cln2HA (lanes 5 to 8) were incubated with anti-HA beads for 1 h and washed two times under low-stringency (lysis buffer; lanes 1, 3, 5, 7, 9, and 11) or high-stringency (lysis buffer + 1% SDS) conditions, followed by incubation in extracts from insects cells expressing 6His-Grr1 (lanes 1, 2, 5, and 6), mock extracts (lanes 3, 4, 7, and 8), Ni-NTA-purified 6His-Grr1 (lanes 9, 10), or mock-purified extracts (lanes 11 and 12) for 1 h at 4°C. Beads were washed and boiled in 2× SDS sample buffer, and bound proteins were analyzed by immunoblotting using anti-His antibody to detect 6His-Grr1, anti-HA antibody to detect Cln2HA, and anti-Cdc28 antibody. IP, immunoprecipitation.

can complement missing yeast components of the core SCF complex (20, 40). To avoid the possibility that an insect cell-derived Cdc28 homolog might bind to Cln2, we purified Grr1-6His over Ni-NTA beads and repeated the experiment (Fig. 7, lanes 9 to 12). Again, Grr1 interacted with column-bound Cln2 irrespective of the presence or absence (Fig. 7, lanes 9 and 10, respectively) of Cdc28. These results show that Grr1 can interact with phosphorylated Cln2 in vitro in the absence of Cdc28.

**Cln2-ΔN-Sic1 fusions depend on functional $SCF^{Grr1}$ but not on functional $SCF^{Cdc4}$.** Undegradable Sic1 is lethal due to inhibition of S-phase initiation (43). We showed that fusion of Cln2 domains to undegradable ΔN-Sic1 destabilized ΔN-Sic1. Therefore, we hypothesized that fusions of Cln2 domains to ΔN-Sic1 should alleviate the lethality of ΔN-Sic1. Indeed, cells expressing 2C-ΔN-Sic1, PD-ΔN-Sic1, or wild-type Sic1 from the *GAL1* promoter were able to grow on galactose-containing plates (Fig. 8A), whereas cells expressing stabilized Sic1 or $2C^{M46}$-ΔN-Sic1 were not. In addition, since Cln2-ΔN-Sic1 fusion degradation is dependent on $SCF^{Grr1}$, Grr1 should become essential in cells expressing Cln2-ΔN-Sic1 fusions, whereas Grr1 is dispensable in wild-type cells. Indeed, we observed that *grr1*Δ strains expressing 2C-ΔN-Sic1 or PD-ΔN-Sic1 from the *GAL1* promoter were not able to grow on galactose plates (Fig. 8B).

The finding that the Cln2-ΔN-Sic1 fusions bind to Grr1 (Fig. 5) suggested that their degradation was no longer dependent on $SCF^{Cdc4}$ but rather was dependent on functional $SCF^{Grr1}$. To test this, we expressed the fusion constructs and wild-type Sic1 in strains bearing inactivated alleles of *grr1*, *cdc53*, and *cdc4*. The 2C- and PD-ΔN-Sic1 fusions were stable in strains with *grr1* deleted (*grr1*Δ) and in strains with a temperature-sensitive *cdc53* [*cdc53*(Ts)] allele grown at the restrictive temperature (37°C) (Fig. 8C and D). In contrast, stability of the Cln2-ΔN-Sic1 fusions was unaltered in *cdc4*(Ts) strains at 37°C. As expected (13), Sic1 was completely stable in *cdc4*(Ts) strains at 37°C (Fig. 8C). We conclude that fusion of 2C or PD to ΔN-Sic1 switches degradation of Sic1 from an $SCF^{Cdc4}$-dependent to an $SCF^{Grr1}$-dependent pathway.

## DISCUSSION

**Cln2 PD domain: transferable signal for $SCF^{Grr1}$-mediated degradation.** One of the key issues in ubiquitin-mediated degradation is substrate recognition. In this report, we have identified a domain in the Cln2 C terminus, the PD domain, which is sufficient to convert a stable reporter polypeptide, ΔN-Sic1, into an efficient substrate for $SCF^{Grr1}$-mediated degradation via the ubiquitin-proteasome pathway. The PD domain encompasses the PEST sequence (P) and four Cdc28 phosphorylation sites crucial for Cln2 instability (D) (Fig. 1 and 3) (24). Substrate phosphorylation has emerged as a hallmark for recognition by the SCF-dependent proteolysis (11, 24, 39, 44). We show that the PD-ΔN-Sic1 fusion and the $2C^{wt}$-ΔN-Sic1 fusion are unstable proteins that are phosphorylated and ubiquitinated in vivo and are recognized by Grr1. Thus, our data strongly suggest that, similarly to wild-type Cln2, ΔN-Sic1 fusions are degraded via the $SCF^{Grr1}$ pathway. In contrast, neither the phosphorylation mutant $2C^{M46}$ nor the P or D domain was sufficient to target the ΔN-Sic1 fusion for rapid degradation. Interestingly, the D domain is able to interact with Grr1 in a coprecipitation experiment, albeit to a lower degree than the PD or the 2C domain. However, the D fusion does not display a ubiquitin ladder in vivo, nor were we able to detect phosphorylation, although we do not rule out the possibility that the D fusion is phosphorylated in vivo without causing the characteristic shift in mobility on SDS-polyacrylamide gels. The P fusion is modified in vivo, most likely by phosphorylation, since phosphatase treatment reduces the size of the double band. Neither D nor P fusions are ubiquitinated in vivo, consistent with their inability to target ΔN-Sic1 for rapid degradation.

**PEST domain: protein instability motif?** The role of the PEST motif in protein degradation has been under investigation since its first description (33, 35). No clear function has yet been assigned to PEST domains. Indeed, one example, ubiquitin-mediated degradation of the yeast Matα2 repressor, clearly does not depend on the PEST motif (19). An earlier report investigating Cln2 protein turnover implicated the PEST domain as necessary but not sufficient for rapid degradation (36). The authors based their conclusion on the finding that elimination of a 37-residue segment encompassing the PEST domain stabilized Cln2 but that the isolated PEST motif was unable to confer instability when fused to a stable reporter protein. In agreement with their conclusion, we demonstrate here that the PEST motif alone is not sufficient to significantly destabilize ΔN-Sic1, although we do observe a slight reduction in the half-life of the P-ΔN-Sic1 fusion compared to that of stabilized Sic1 or D-ΔN-Sic1 (Fig. 5). In addition, the P domain fusion is not detectably ubiquitinated and does not bind to Grr1; thus, we conclude that the PEST motif is important but not sufficient for Cln2 degradation: it requires the phosphorylation-dependent signaling of the adjacent D domain to per-

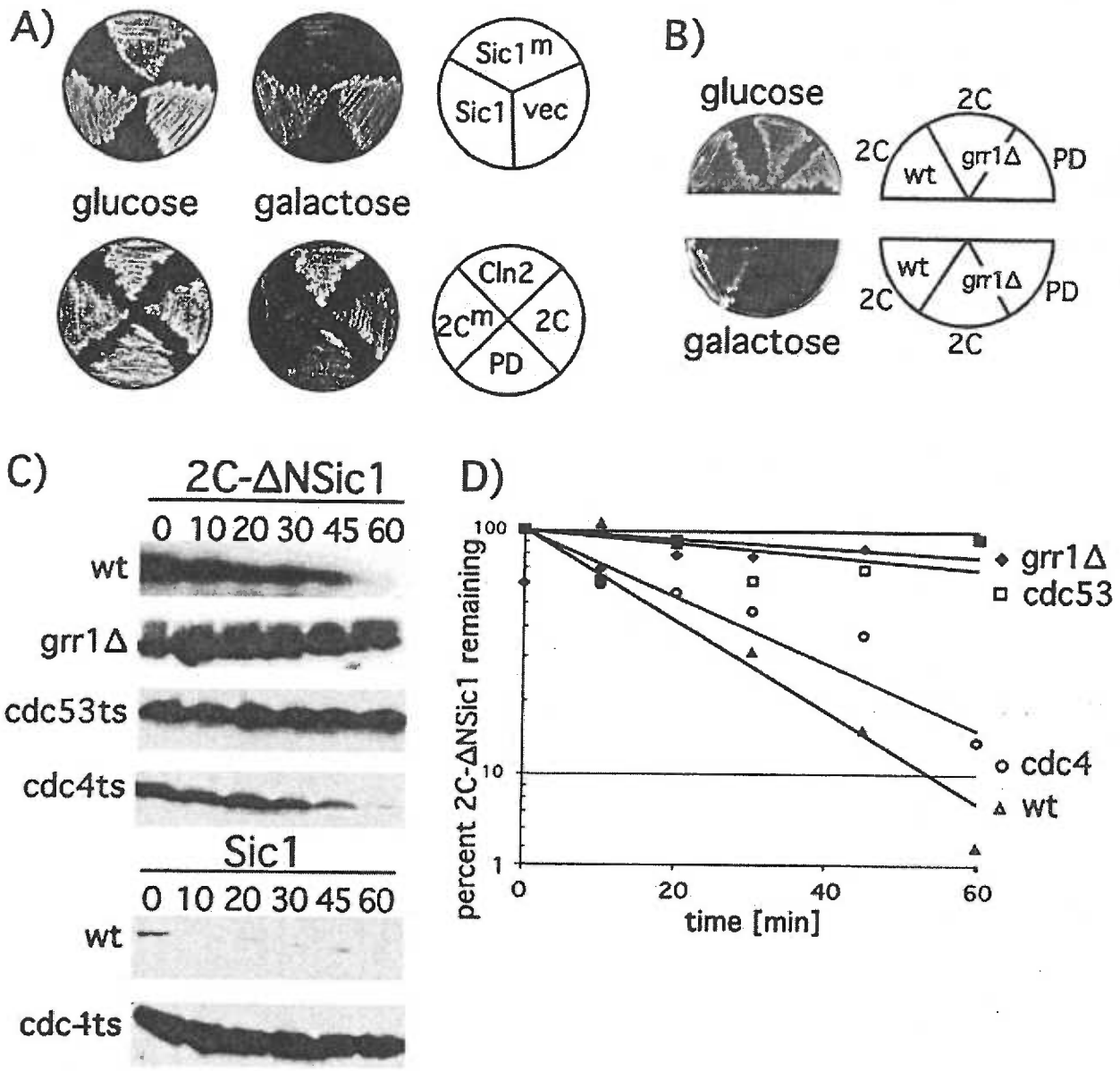


FIG. 8. Cln2-ΔN-Sic1 fusions are degraded by $SCF^{Grr1}$. (A) Cln2 fusions alleviate the lethality of stabilized Sic1. Strains expressing Sic1; stabilized $Sic1^{4A}$ ($Sic1^{m}$); empty vector (vec); Cln2; or fusions of stabilized Sic1 (ΔN-Sic1) with 2C (2C), mutant $2C^{M46}$ ($2C^{m}$), and PD (PD)—all under control of the *GAL1* promoter—were replica plated onto glucose- and galactose-containing plates and incubated at 30°C for 3 days. (B) Grr1 is essential in strains expressing Cln2-ΔN-Sic1 fusions. Wild type (wt) strains or strains with Grr1 deleted (grr1Δ) and expressing 2C-ΔN-Sic1 (2C), or PD-ΔN-Sic1 (PD) under control of the *GAL1* promoter, were replica plated onto glucose- and galactose-containing plates and incubated at 30°C for 3 days. (C) Degradation of 2C and PD fusions to ΔN-Sic1 depends on functional $SCF^{Grr1}$. 2C-ΔN-Sic1-HA or Sic1-HA was expressed in a wt strain, in a *grr1*Δ strain, or in temperature-sensitive strains (cdc53ts and cdc4ts) grown at 37°C and subjected to *GAL1* promoter shutoff experiments as described in the legend to Fig. 1. Extracts from samples taken at the indicated time points after repression of the *GAL1* promoter were analyzed by immunoblotting using anti-HA antibodies. (D) Graph representing the degradation rate of 2C-ΔN-Sic1 in the indicated strains.

form its function within the ubiquitin-dependent degradation pathway.

The PEST motif seems to be necessary for the degradation of some but not all SCF substrates. Since the PEST motif has never been demonstrated to be sufficient for signal-induced degradation, we hypothesize that the PEST domain represents a constitutive signal for degradation that can be modulated, often by phosphorylation, to become a signal-dependent motif for the SCF degradation machinery. Our data with the GST fusions demonstrate that the isolated PEST motif can destabilize reporter proteins, but the lack of proper phosphorylation results in $SCF^{Grr1}$-independent, intermediate instability. A similar conclusion was reached for PEST domain- and phosphorylation-dependent degradation of Cln3 (49). These authors concluded that PEST elements might function to confer phosphorylation dependence on degradation. We note, how-

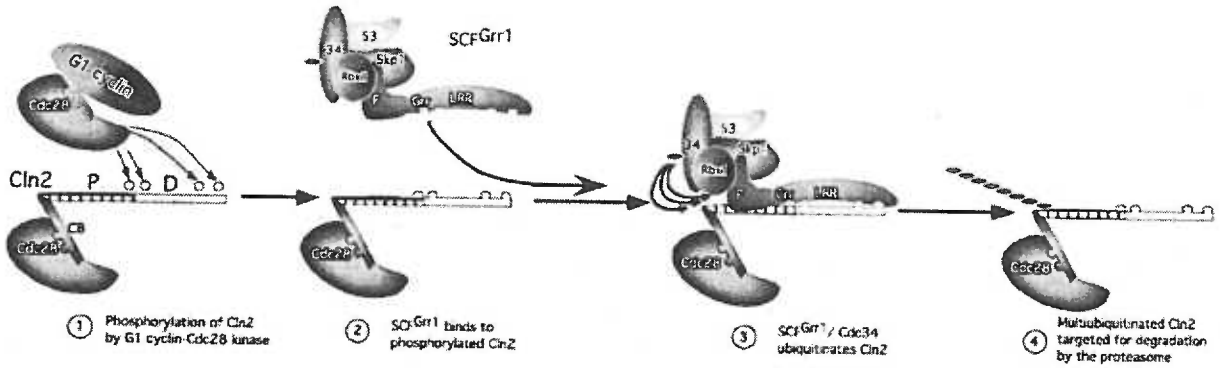


FIG. 9. Model of phosphorylation-induced binding of $SCF^{Grr1}$ to Cln2. For an explanation, see text. Abbreviations: CB, cyclin box; P, Cln2 PEST domain; D, Cln2 D domain; F, Grr1 F box; LRR, Grr1 LRR domain; 34, Cdc34; 53, Cdc53.

ever, that the Cln3 PEST domain appears to be phosphorylated when fused to beta-galactosidase, a protein that does not bind Cdc28; in contrast, our Cln2 PEST domain fusion to GST is not phosphorylated, a difference that awaits further experimentation to be understood.

**Phosphorylation-dependent recognition of Cln2 by Grr1.** While several models can be imagined to account for the results shown above, we favor the multisite phospho-epitope model for degradation of Cln2 (Fig. 9). According to this model, phosphorylated Cln2 residues participate directly in binding to Grr1. Initially, Grr1 (via the C terminus [see below]) might bind weakly to the hypophosphorylated D domain of Cln2/Cdc28 complexes, consistent with the observed residual interaction between the Cln2 phosphorylation mutants and Grr1 (Fig. 2). Subsequently, phosphorylation at multiple sites generates additional, high-affinity binding sites for Grr1, thus stabilizing the interaction. High-affinity binding allows the $SCF^{Grr1}$/Cdc34 complex to efficiently ubiquitinate Cln2. Multiubiquitinated Cln2 is finally recognized by the proteasome and rapidly degraded, freeing the bound Cdc28 subunit as well as the $SCF^{Grr1}$ complex.

We propose that multistep phosphorylation of Cln2 by Cln2/Cdc28 occurs in *trans*, setting a threshold level for $G_1$ cyclin kinase activity. This establishes a minimal $G_1$ phase duration, ensuring that essential $G_1$ phase events dependent on $G_1$ cyclin kinase activity have been initiated prior to the degradation of $G_1$ cyclins. A recent report analyzing recognition of phosphorylated Sic1 by Cdc4 proposed a very similar mechanism (29). These authors demonstrate that degradation of Sic1 requires multisite phosphorylation of Sic1, thereby establishing a minimal $G_1$ phase period required for proper DNA replication. They propose a model whereby multisite phosphorylation of Sic1 converts the gradual accumulation of Cln-Cdc28 kinase into a switch-like response for Sic1 degradation and onset of S phase. Such a switch-like loss of Cln2 would only be effective if Cln2 is phosphorylated in *trans*. In support of this idea, a gradual increase in Cln2 phosphorylation during $G_1$ has been documented (28). This model suggests that although Cln2 must bind Cdc28 for Cln2 to become phosphorylated, Cln2 in that complex is not a substrate for its bound Cdc28 activity. Yet we have shown that Cln2 lacking its cyclin box is not a good substrate for phosphorylation in vivo (24). Furthermore, we show here that Cln2 fusions to GST are not phosphorylated. We also show that, although fusions to ΔN-Sic1 or ΔN-Sic1 lacking the C terminus are phosphorylated in vivo, phosphorylation is not dependent upon binding of Clb/Cdc28 to Sic1. There are a number of possible explanations for these observations. It is possible that recognition of Cln2 as a substrate requires sequences outside the PD domain. We suppose that, in the context of intact Cln2, Cdc28 binding is required for that recognition, whereas Sic1 provides the appropriate context in the absence of bound CDK. The fact that Sic1 is a Cln2/Cdc28 substrate suggests that interaction with Cln2 can occur but does not lead to stable binding. We show here that Cdc28 binding to the inhibitory site on Sic1 is not required for degradation of Cln2/ΔN-Sic1 chimeras. However, that does not preclude a contribution of a distinct binding site on Sic1 for Cln2/Cdc28 kinase. It is unclear whether sequences outside the Sic1 N terminus might contribute to such an interaction. Clearly, further experimentation is needed to fully understand this issue. Cln2 C-terminal phosphorylation has been shown to regulate the cellular localization of Cln2 (10, 28). Interestingly, the $Cln2^{4T3S}$ phosphorylation mutant was shown to accumulate in the nucleus, raising the possibility that the stability of different Cln2 phosphorylation mutants is related to localization rather than to binding to Grr1. While this is an interesting mechanism, published data demonstrating that Grr1 is localized both in the nucleus and in the cytoplasm would argue against it (3). It will be of interest to correlate the localization of differently mutated Cln2 mutants and of the Cln2-ΔN-Sic1 fusions to their stability.

We have recently developed a structural model for the Grr1 LRR (17). We predicted a high density of positively charged residues on the concave surface of the LRR and hypothesized that certain basic residues on the concave surface are essential for interaction with phosphorylated Cln2. Indeed, when these basic residues were mutated to neutral or acidic residues, interaction with Cln2 was lost. As a result, Cln2 and Gic2, another $SCF^{Grr1}$ substrate (18), were strongly stabilized, demonstrating that Grr1 interacts with phosphorylated targets via basic residues on the concave surface of the Grr1 (LRR) domain (17). Thus, we suggest that electrostatic interactions between basic residues on Grr1 LRR and phosphorylated residues on Cln2 contribute a portion of the energy required for that interaction. In an attempt to create a constitutively unstable Cln2, we generated a mutant Cln2 that carried glutamic

acid substitutions in the four phosphoacceptor sites within the D domain ($Cln2^{4D}$). However, $Cln2^{4D}$ remained functional, failed to bind to Grr1 in vivo, and was highly stable (data not shown), suggesting that a negative charge at those four Cln2 residues is not sufficient for interaction with Grr1.

It is noteworthy that additional domains besides the Grr1 LRR domain appear to contribute to the interaction between Grr1 and Cln2. We have shown that the Grr1 C terminus is important for stable interaction with Cln2 (17), a finding that might be explained by the recent structural model of human Skp2, a Grr1 homolog (38). This model shows that the Skp2 C terminus wraps back along the entire LRR fold, thus covering the putative substrate interaction domain. Thus, by analogy to the Grr1 C terminus, the Skp2 C terminus might play an important role in substrate recognition.

As demonstrated in this work, four phosphorylated residues within 33 aa in the Cln2 C terminus serve as an essential recognition signal; similarly, multiple phosphorylations within the N terminus target Sic1 for degradation (29, 44). Therefore, binding of F-box proteins to their substrates often appears to depend on a cluster of several phosphorylated residues. In contrast, other phosphorylation-dependent protein-protein interactions often involve single phospho residues or short continuous phosphopeptide sequences, such as phosphoserine motifs recognized by 14-3-3 proteins or phosphotyrosine by SH2-containing proteins.

**SCF specificity: F-box hypothesis.** Bai et al. (1) proposed that F-box proteins serve to link different substrates to Skp1 and the SCF machinery. This hypothesis was confirmed in vitro by demonstrating that $SCF^{Cdc4}$ binds tightly to and ubiquitinates Sic1, but not Cln1 (11, 40), whereas $SCF^{Grr1}$ specifically binds to and ubiquitinates phosphorylated Cln1 and Cln2 but does not recognize Sic1. Here we have demonstrated that the F-box specificity for Sic1 can be switched in vivo from Cdc4 to Grr1 by exchanging the Sic1 signaling domain with the Cln2 PD domain. These results confirm the modularity concept of the SCF machinery and represent, to our knowledge, the first demonstration of a chimeric substrate for the SCF pathway. In a reciprocal approach, Zhou and coworkers (51) recently demonstrated that a mammalian F-box protein, βTrCP, fused to a pocket protein binding peptide ($\beta TrCP^{E7N}$), was able to bind and ubiquitinated stable pocket proteins, including pRB and p107, causing their rapid destruction by the proteasome. These results not only strengthen the F-box hypothesis but also demonstrate that substrate binding—often catalyzed by substrate phosphorylation—is likely the key requirement for degradation by the SCF machinery.

Most SCF-substrate interactions in yeast and mammalian cells have been shown to depend on phosphorylation. Our description of the determinants of Cln2-Grr1 interaction and Cln2 instability may serve as a general model for the study of substrate recognition by SCF complexes.

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# Chapter 8

# Figures and Figure Legends

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Figure 1- Mechanism of Ubiquitination
Figure from Burger et al., 2004

An E1 or ubiquitin-activating enzyme activates ubiquitin by the formation of an ATP-dependent thio-ester linkage between the E1 cysteine and ubiquitin (1). The E1 enzyme interacts with an E2 or ubiquitin conjugating enzyme and the ubiquitin molecule is passed from the E1 to the E2 (2). The activated ubiquitin molecule is now primed for attachment to a substrate. The E2 enzyme associates with an E3 or ubiquitin ligase. The ubiquitin ligase selects the substrate (blue oval) to be ubiquitinated. Ubiquitin ligases can then aid in the conjugating of the ubiquitin molecule to a lysine residue on the substrate (3). A ubiquitin chain is formed in an unclear process that may require an E4 enzyme (4). After the substrate has been ubiquitinated, it is traditionally targeted to the 26S proteasome to be degraded by the proteasomal proteases (5). The process of degradation leaves the ubiquitin molecules intact and allows them to be recycled (bottom right).

The proteasome exists as two parts- the 19S subunit (dark blue circles) can form a "cap" on either end of the "core," or the 20S subunit (purple circles). The ubiquitinated substrate interacts with proteins in the cap, then the substrate is unfolded and is translocated through the pore in the center of the 20S proteasome core where it is degraded by the internal proteases.

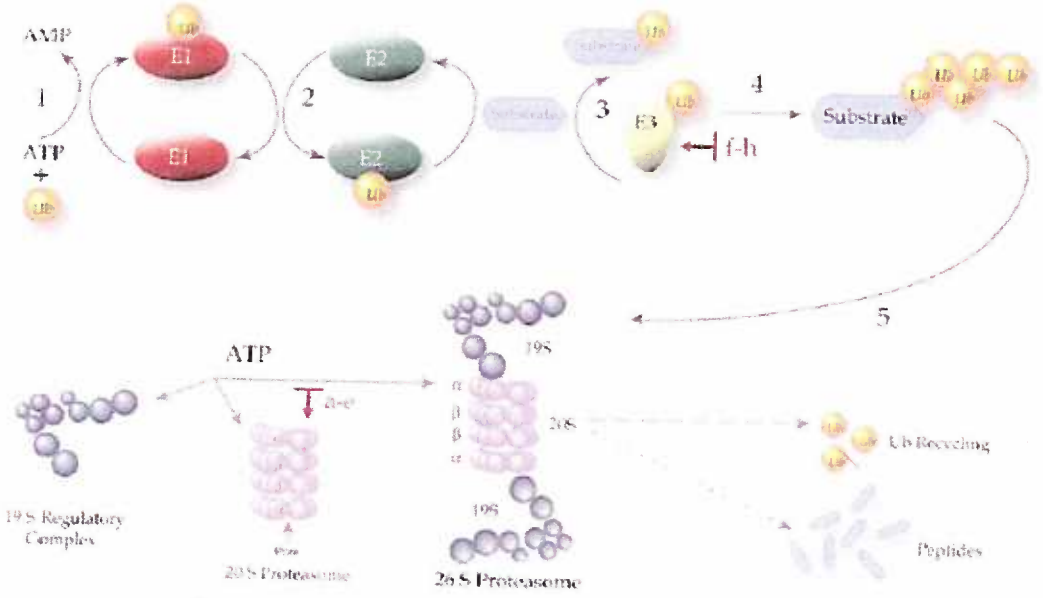

AMP
ATP
1
E1
E1
2
E2
E2
3
E3
f-h
4
Substrate
5
ATP
19S Regulatory Complex
20S Proteasome
19S
20S
19S
26 S Proteasome
Ub Recycling
Peptides

Figure 2- Families of Ubiquitin Ligases
Figure from Burger et al., 2004

As mentioned in the text, there are at least 4 defined types of ubiquitin ligases. The families are defined by their functional ligase domain. Shown here are the RING domain family (shown as two types, the monomeric and multi-subunit forms), the HECT domain family, the U box family, and finally the PHD family.

The RING-type E3's (left hand box, top row) pass the activated ubiquitin molecule from the E2 directly to the substrate. This can be seen in the first panel where Mdm2 and its RING finger (RF) interact with the E2 and the ubiquitin molecules are attached to p53. Alternatively, in the $SCF^{Skp2}$ (a human F box protein) complex (center box, top row), the RING finger (RF) protein, Roc1 binds the E2 and the ubiquitin molecule is attached to p27.

The HECT –type ligase (right hand box, top row), shown here as E6-AP binds the E2 and the ubiquitin molecule is accepted on to E6AP and then is attached to the substrate, shown here as p53.

The U box –type ligase (left hand box, bottom row), CHIP, interacts with its substrate, HER2/neu through the heat shock proteins HSP70/90. In this manner, HER2/neu can become ubiquitinated and targeted for destruction.

Similarly, the PHD-containing ligase (right hand box, bottom row), MEKK1, ubiquitinates it target, Erk1/2, as shown in the last panel.

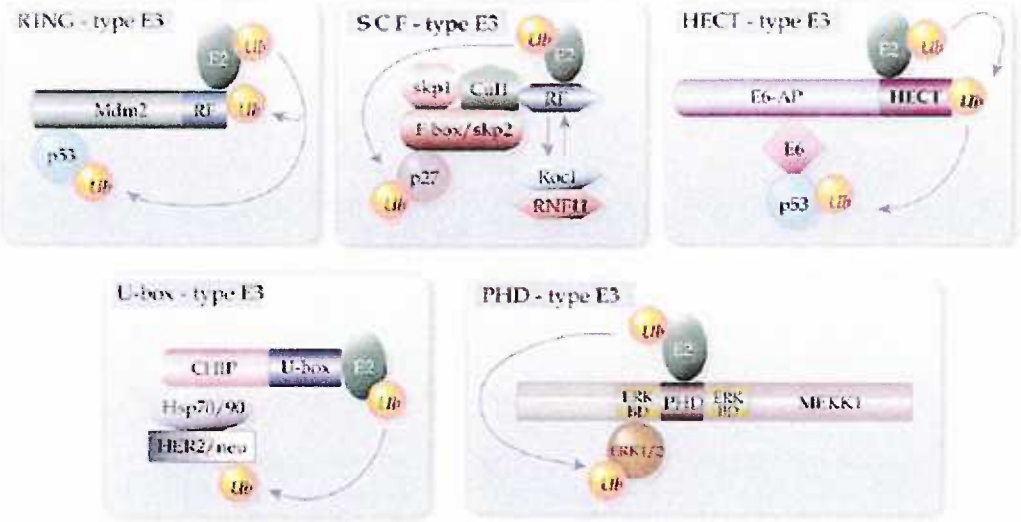

RING - type E3
E2
Ub
Mdm2
RI
p53
SCF - type E3
skp1
Cul1
F-box/skp2
p27
Koc1
RNF11
HECT - type E3
E6-AP
HECT
E6
p53
U-box - type E3
CHIP
U-box
Hsp70/90
HER2/neu
PHD - type E3
PHD
MEKK1
ERK1/2

Figure 3- SCF Complex
Figure from Zheng et al., 2002

The SCF (*S*kp1-*c*ullin-*F* box) complex is comprised of several proteins. Skp1 (blue) is a scaffolding protein and interacts with the large, rigid, cullin protein (green). The cullin binds the RING finger protein Hrt1/Roc1/Rbx1 (red). The cullin and Hrt1/Roc1/Rbx1 proteins work together to recruit the E2 enzymes (yellow) that can directly ubiquitinate the substrate selected by the F box protein (pink). The active cysteine can be seen facing the F box protein. Finally, the variable F box protein associates with Skp1 through its F box.

The F box protein confers substrate selection upon the SCF complex through a secondary protein-protein interaction motif. These motifs are varied and include WD40 repeats and shown here, the Leucine Rich Repeat motif. The F box protein displayed here is Skp2, a human F box protein that is structurally similar to the yeast protein, Grr1. This is a cartoon of several crystal structures together.

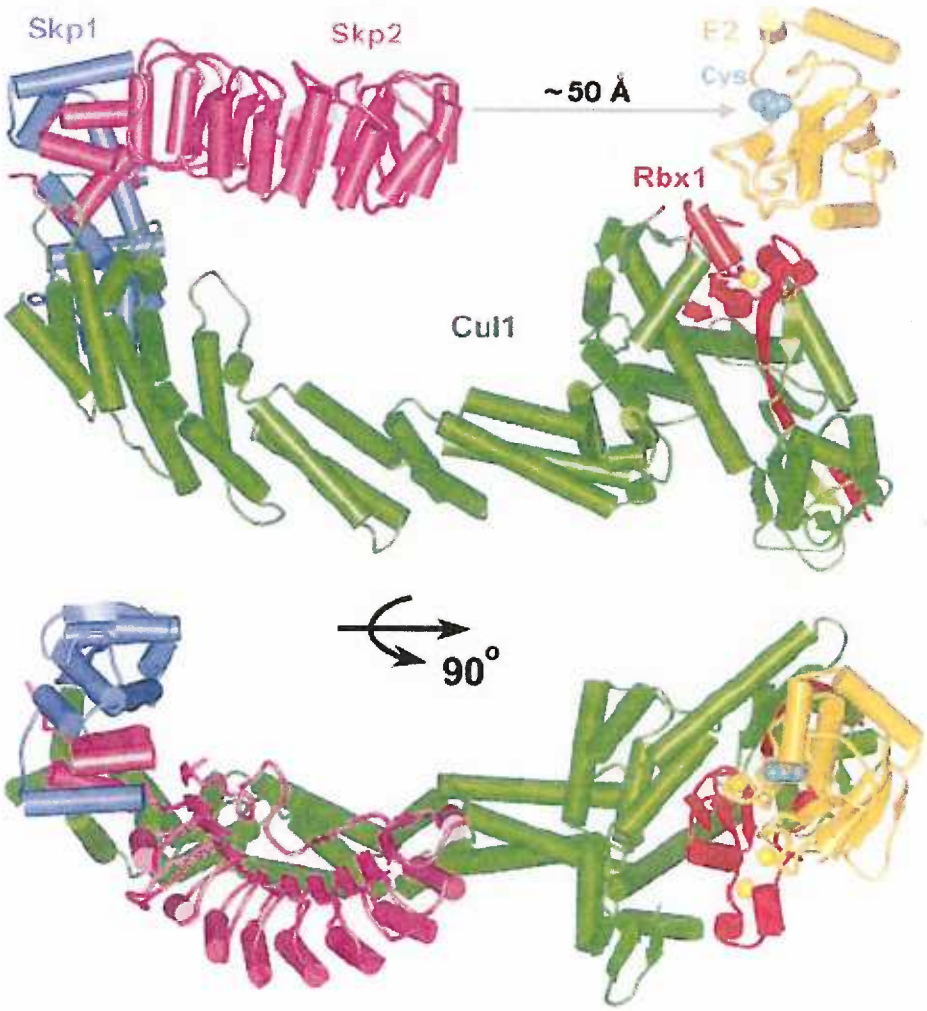

Skp1
Skp2
E2
Cys
~50 Å
Rbx1
Cul1
90°

Figure 4a- Glucose Repression –High Glucose
Figure adapted from Johnston, 1999

a. In high glucose conditions (demonstrated by the blue hexagons), Snf3 and Rgt2 sense the level of glucose and communicate this level to the inside of the cell. Casein kinase, which associates with the C-terminal tails of Snf3 and Rgt2, phosphorylates the paralogues, Mth1 and Std1. Phosphorylated Mth1 is ubiquitinated by $SCF^{Grr1}$ (at least shown for Mth1). It is subsequently degraded and unable to stimulate Rgt1 and keep it in its hypo-phosphorylated, promoter-bound state. Rgt1 is unable to prevent transcription of the glucose transporters (HXTs). Thus, the glucose transporters are induced upon the presence of glucose. Simultaneously, other repressors inhibit expression of the glucose-repressed transcripts. Mig1 is shown here as an example. Not shown is that both Rgt1 and Mig1 coordinate with the co-repressors Ssn6 and Tup1 for repression.

# High Glucose

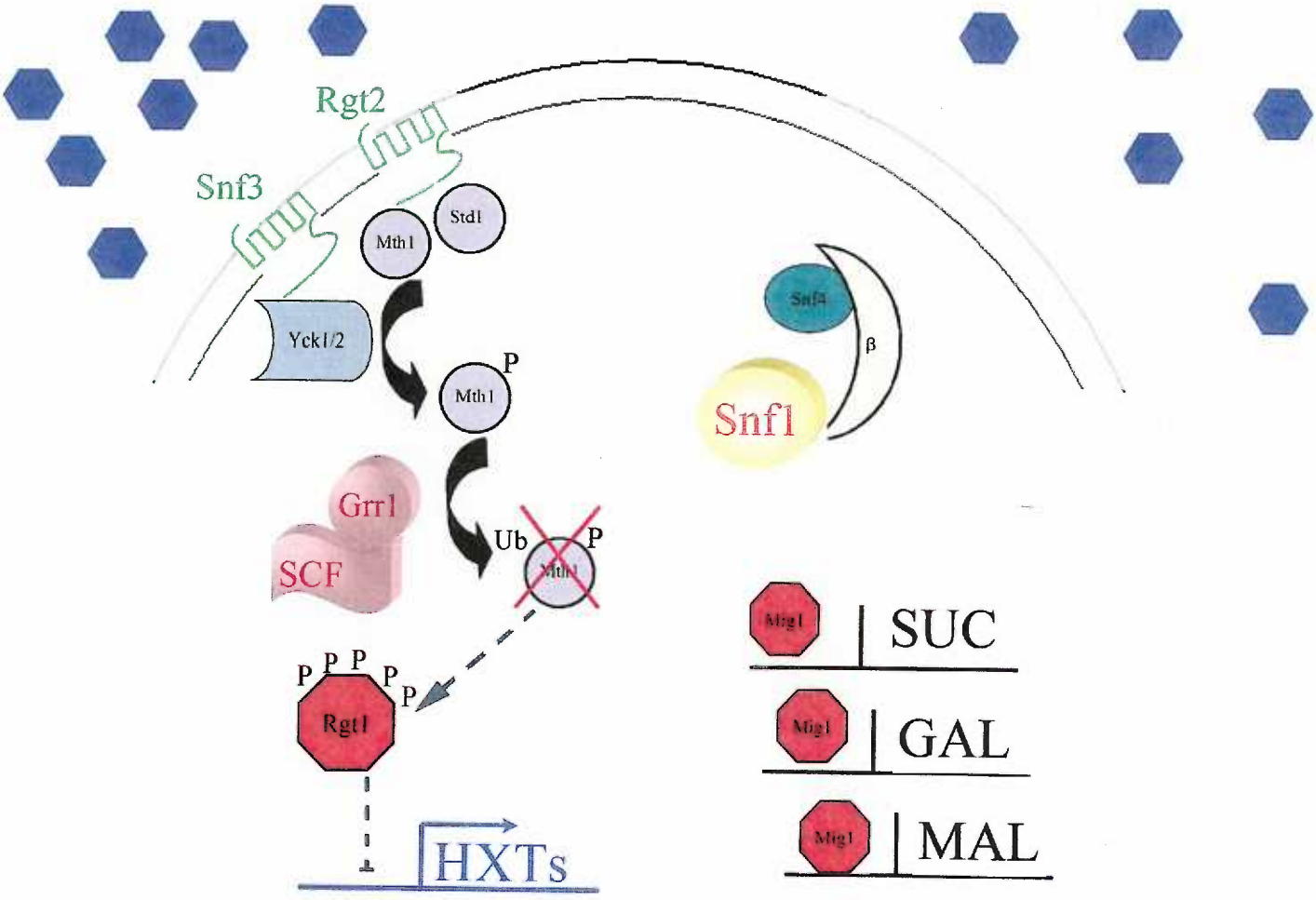


Figure 4b- Glucose Repression –Low Glucose (Active Snf1)
Figure adapted from Johnston, 1999

b. When levels of glucose are low (blue hexagons), the cell mobilizes to use other carbon sources (shown as yellow and green hexagons). Mth1 and Std1 are able to maintain Rgt1 in its hypo-phosphorylated state, and Rgt1 inhibits transcription of a majority of glucose transporters. Snf1 kinase is activated by phosphorylation (not shown) and associates with Snf4 to form the active kinase complex. The active complex phosphorylates a number of substrates (directed by its β-subunit). For example, phosphorylation of Mig1 excludes it from the nucleus and the glucose-repressed transcripts can now be transcribed.

# Low or No Glucose

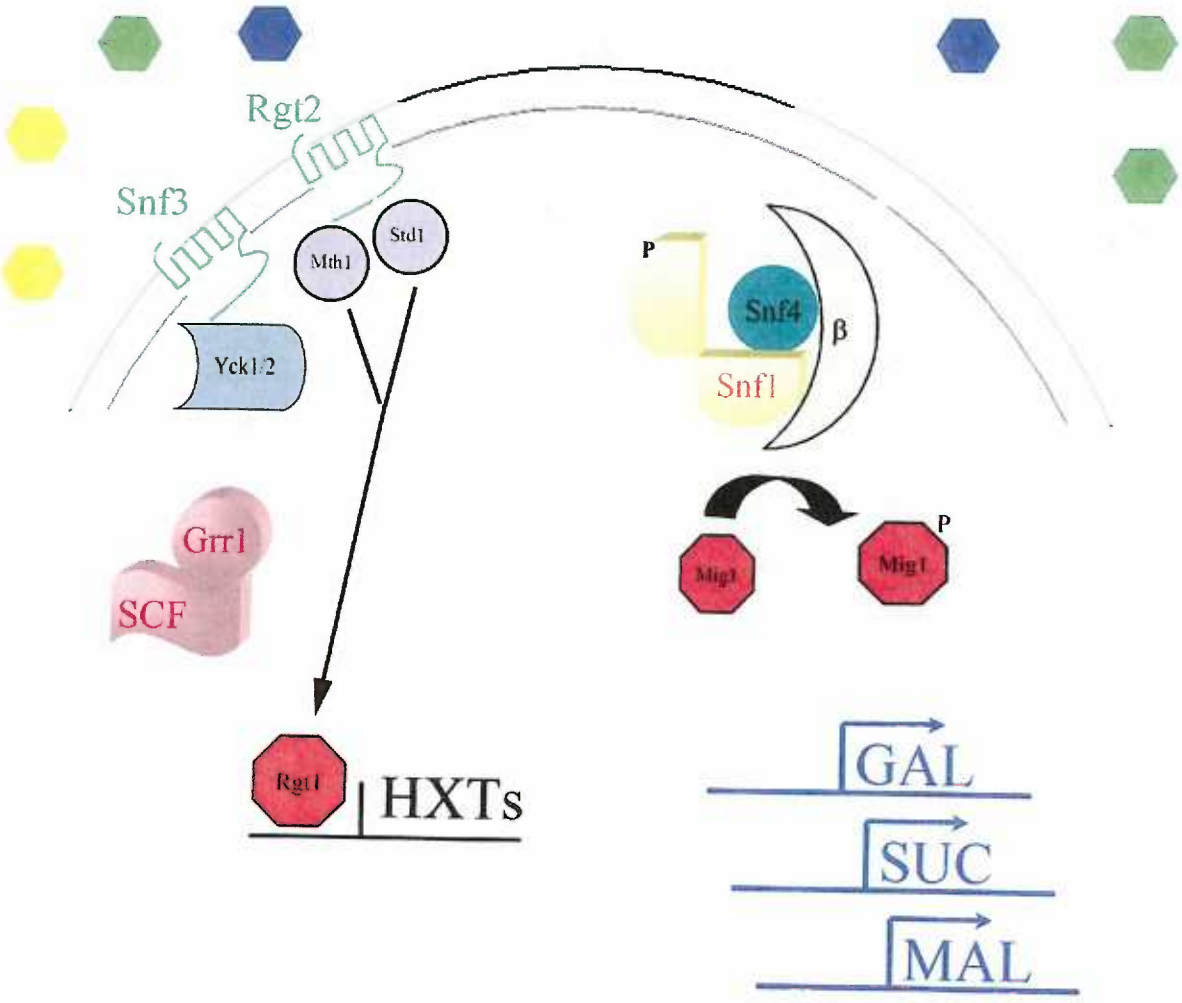


Figure 5- Snf1 kinase complex (Active vs. Inactive)
Figure adapted from Kuchin et al., 2003

When inactive, the kinase domain (KD) and regulatory domain (RD) of Snf1 interact and exclude Snf4 from binding to Snf1. However, both Snf4 and Snf1 are still present in the complex, tethered by one of several β-subunits. Activation of Snf1 comes in the form of phosphorylation of residue T210 by Elm1, Tor1, or Pak1. Phosphorylation releases the domains and the kinase domain no longer interacts with the regulatory domain. Snf4 is now present to prevent re-association of the KD and the RD. Inactivation of the Snf1 complex occurs through the protein phosphatase Glc7 and its subunit, Reg1.

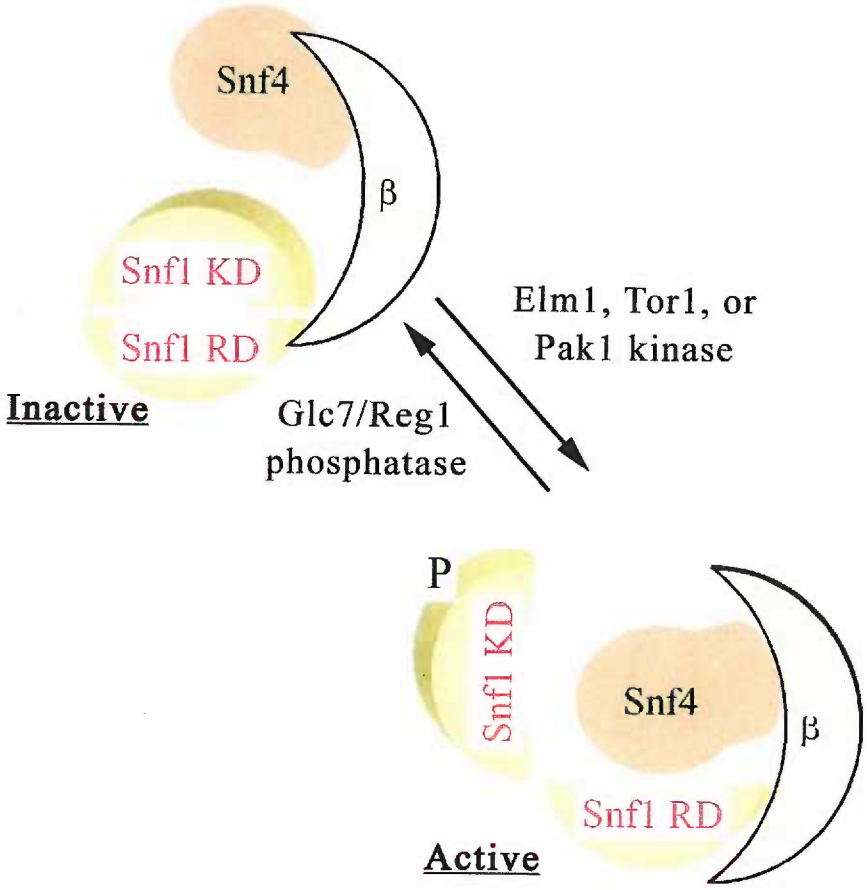
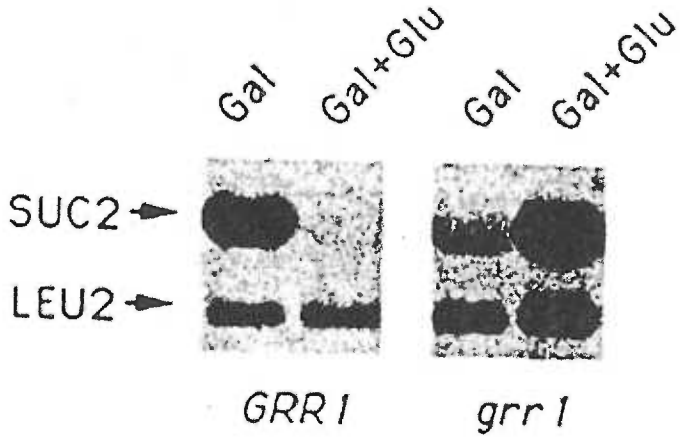

Snf4
β
Snf1 KD
Snf1 RD
Inactive
Elm1, Tor1, or
Pak1 kinase
Glc7/Reg1
phosphatase
P
Snf1 KD
Snf4
β
Snf1 RD
Active

Figure 6- Grr1Δ alters SUC2 transcription
Flick and Johnston, 1991

In an early paper characterizing Grr1, Flick and Johnston showed that inactivation of Grr1 altered the expression of glucose repressed genes. Shown here are the mRNA levels of SUC2 from a Northern blot. When Grr1 is intact, the levels of SUC2 are inhibited by the presence of glucose. When glucose is absent, the levels of SUC2 increase. However, when Grr1 is inactivated, not only are the levels of SUC2 expressed in galactose lower, but glucose appears to stimulate, rather than inhibit, SUC2 expression.

# Figure 6




Flick JS, Johnston M., Mol Cell Biol, 1991

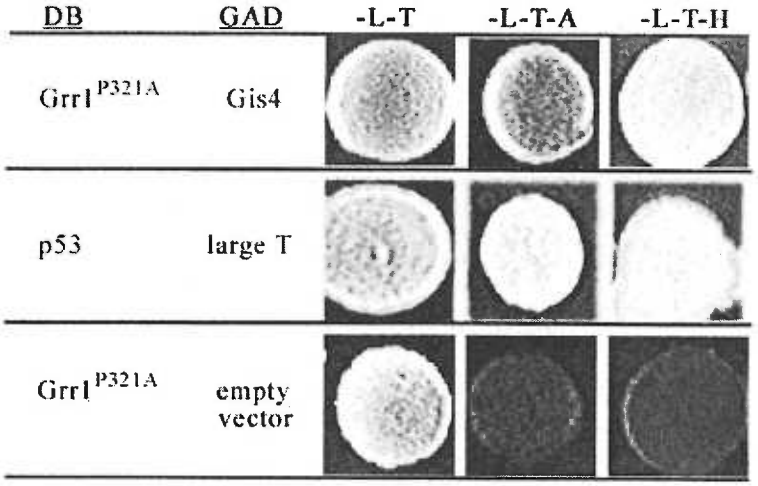
Figure 7 – Grr1 Interacts with Gis4

a. Identification of Grr1 Interactors, Specifically Gis4: Plasmids containing indicated proteins or empty vectors were transformed into SLY477 (yeast strain AH109). Interactions were determined by growth on media lacking leucine, tryptophan, adenine or histidine (plus 2.5 mM 3-amino-trizole). P53 and the large T antigen interaction is shown here as a positive control. "DB"= DNA binding domain, "GAD"= GAL4 Activation Domain

b. Gis4 interacts with Grr1 *in vivo*: 1 mg of whole cell extract from cells containing Gis4-TAP, 6His-Grr1, or both were subjected to affinity purification using calmodulin resin and examined via Western Blot. 10 μg of lysate was loaded (lanes 1-3) to show cellular protein levels. 100% of protein bound to beads was loaded (lanes 4-6) to demonstrate protein interaction with the beads and the co-precipitation. 6His-Grr1 is co-precipitated when Gis4 is pulled down. Gis4 bands present in lane 4 are due to spill over from lane 5 during loading. The α-PAP antibody is specific to the protein A element of the TAP tag.

# Figure 7

A


DB
GAD
-L-T
-L-T-A
-L-T-H
Grr1P321A
Gis4
p53
large T
Grr1P321A
empty vector

B

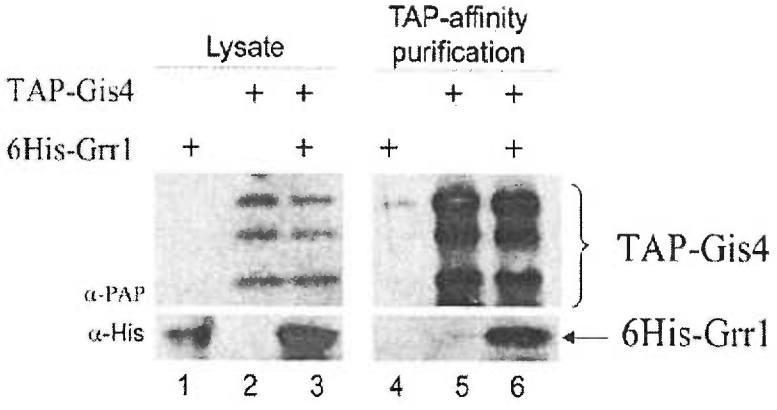

Lysate
TAP-affinity purification
TAP-Gis4
+ + + +
6His-Grr1
+ + + +
TAP-Gis4
α-PAP
α-His
6His-Grr1
1 2 3 4 5 6

Figure 8- Gis4 Regions

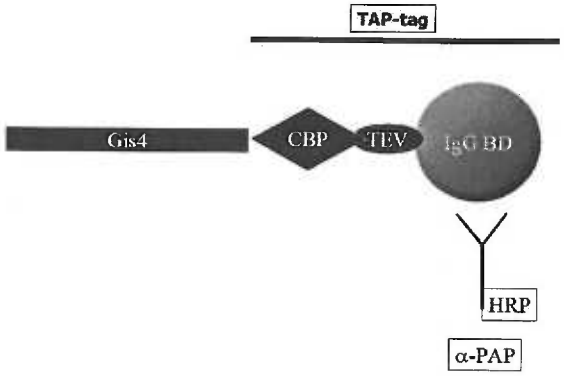
a. Cartoon of Gis4 and the TAP tag: Gis4 was C-terminally tagged at the chromosome. The TAP tag (Tandem Affinity Purification) consists of a Calmodulin Binding Peptide (CBP) fused to a TEV (Tobacco Etch Virus) protease cleavage site fused to the IgG Binding Domain of Protein A (IgG BD). The IgG BD can be used in immunoassays when the anti-PAP (Peroxidase Anti-Peroxidase) antibody is used. This antibody is generally HRP conjugated.

b. Substrate Sequence Analysis: PEST domains predicted (square boxes) for the substrates; the predicted score is below the region. Scores above 5 units are considered strong PEST domains and anything above 0 is of possible interest. Asterisks (*) are potential and defined phosphorylation sites. Numbers shown are amino acid residues.
   - Gis4: The sequence of Gis4 does not predict any catalytic domains. There are four potential PEST domains predicted and there are four potential phosphorylation sites (asterisks) based on the customary serine or threonine followed by a proline consensus sequence for cyclin dependent kinases or the Snf1 kinase (a S/T protein kinase). The C-terminus ends in –CAIM, a predicted sequence for modification by farnesylation. The region containing the S represents a region of Gis4 consisting of many serine residues.
   - Cln1 and Cln2: Both are G1 cyclins and contain the cyclin domain: a helical domain present in cyclins and TFIIB (twice) and Retinoblastoma (once). It is a protein recognition domain functioning in cell-cycle and transcription control [Schultz, 1998 #392;Letunic, 2002 #393]. Each also has a PEST domain. The proposed region of interaction with Grr1 is shown for Cln2 [Berset, 2002 #129].
   - Gic2: contains a CRIB (Cdc42/Rac Interactive Binding) domain important for its interaction with Cdc42. It is also known as a PDB domain. It has a weakly defined PEST domain that contains part of the CRIB domain [Schultz, 1998 #392;Letunic, 2002 #393].
   - Mth1: contains a Casein Kinase Phosphorylation Domain (CKPD) important to its role in glucose sensing. It also contains a weak PEST domain.

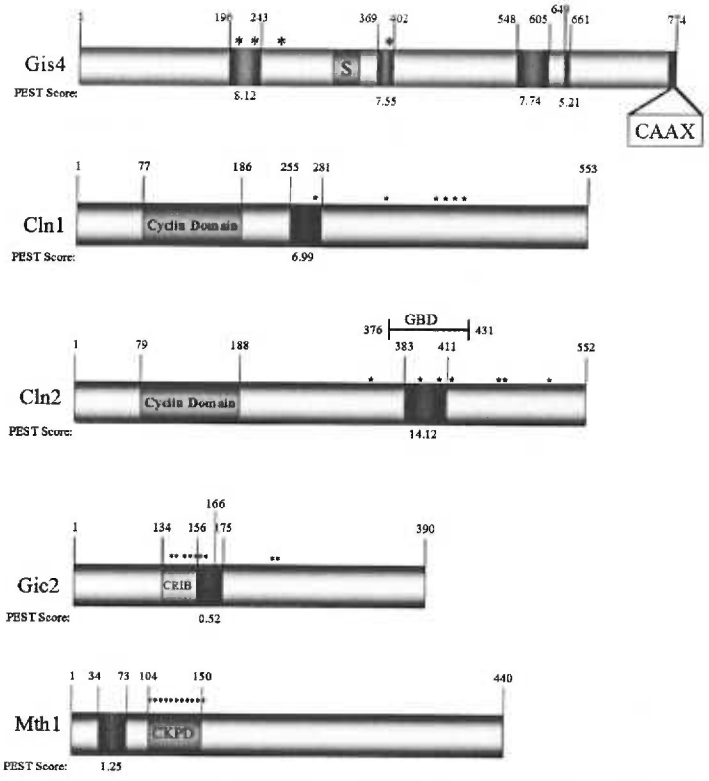
c. Deletion Constructs: Plasmids containing indicated deletion constructs and Grr1 or empty vectors were transformed into SLY476. Interactions were determined by lacZ activity on filter lifts. (FL= full length Gis4, ML= Middle,long section, MS=middle, short section, ΔC=lacking the C-terminal half of the protein, ΔN= lacking the N-terminal half of the protein)

# Figure 8

A.


TAP-tag
Gis4
CBP
TEV
IgG BD
HRP
α-PAP

B.


Gis4
PEST Score:
S
CAAX
Cln1
Cyclin Domain
Cln2
GBD
Gic2
CRIB
Mth1
CKPD

C.

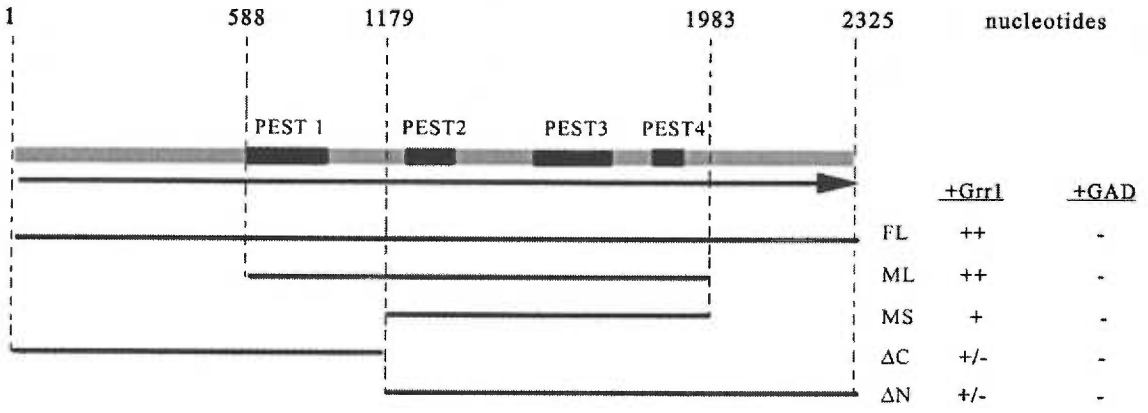

1
588
1179
1983
2325
nucleotides
PEST 1
PEST2
PEST3
PEST4
+Grr1
+GAD
FL ++ -
ML ++ -
MS + -
ΔC +/- -
ΔN +/- -

Figure 9- Gis4 protein levels are directly proportional to Grr1 protein levels

a. Gis4 levels correlate with Grr1 levels: Wild type cells (lanes 1-4), cells containing Gis4-TAP were extracted and loaded at 10, 20, 30, and 40 μg to compare levels of Gis4 via Western Blotting. Extracts from lanes 9-12 over-express Grr1 from a plasmid via the (Alcohol Dehydrogenase 1) ADH1 promoter. Extracts from lane 5-8 are from *grr1Δ* cells.

# Figure 9

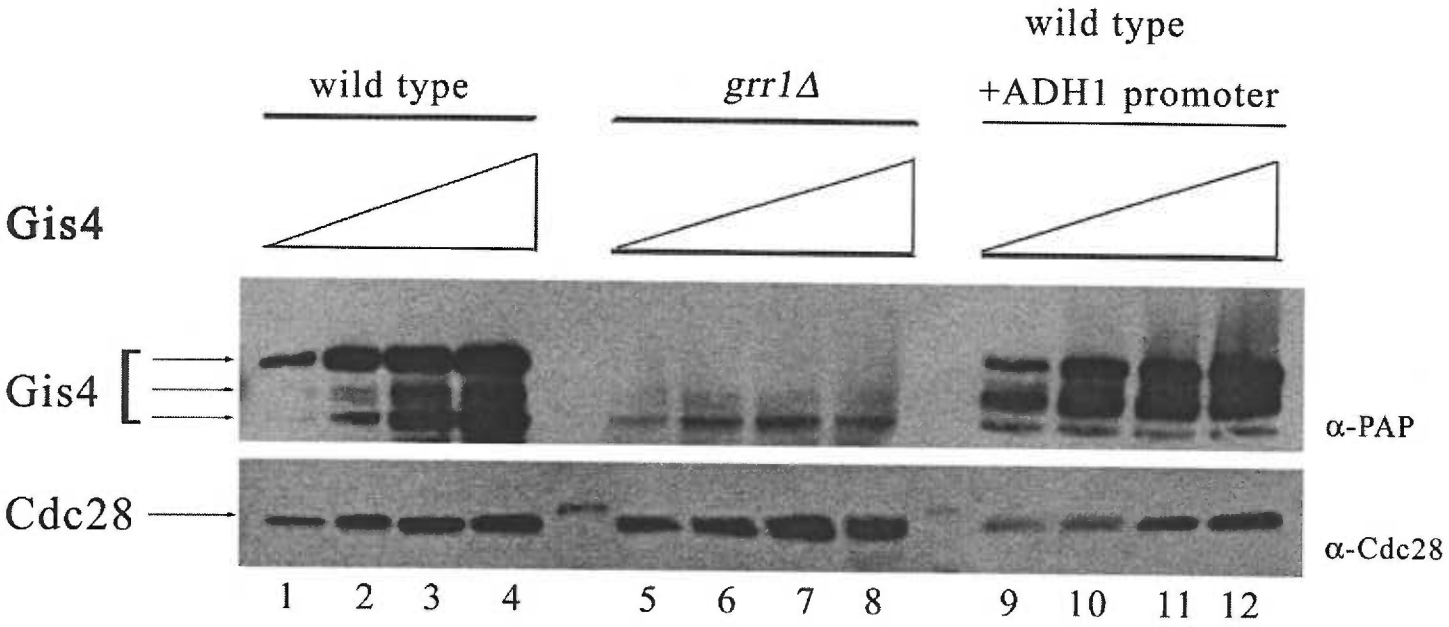

wild type
grr1Δ
wild type
+ADH1 promoter
Gis4
Gis4
Cdc28
α-PAP
α-Cdc28
1 2 3 4 5 6 7 8 9 10 11 12

Figure 10- Gis4 is a stable protein

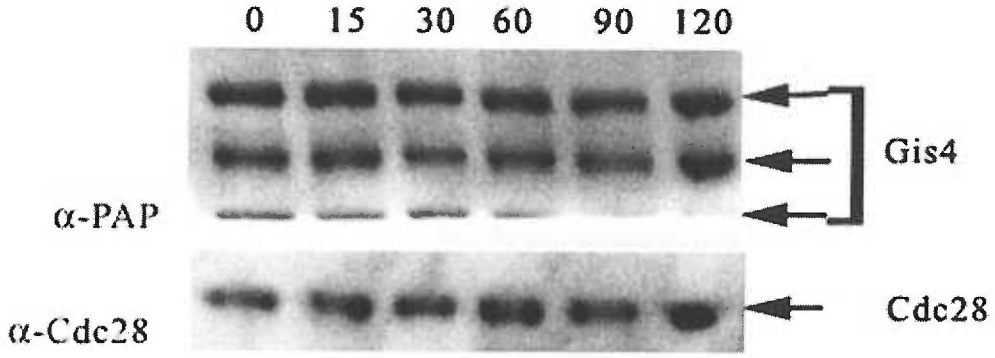
a. Gis4 is stable when treated with CHX in wild type cells: Cycloheximide was added at a final concentration of 10 μg/mL and Gis4-TAP containing cells were harvested at indicated times. Cdc28 is included as a loading control.

b. Grr1 is an unstable protein, indicating that the CHX is effective: Cells containing TAP-tagged Grr1 were treated with CHX at a final concentration of 10 μg/mL and harvested at indicated times. Cdc28 is included as a loading control.

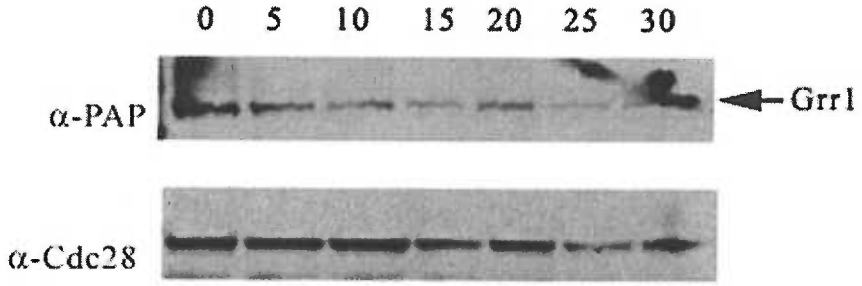
c. Gis4 is stable when treated with CHX in *grr1Δ* cells: Cycloheximide was added at a final concentration of 10 μg/mL and Gis4-TAP containing cells were harvested at indicated times. Cdc28 is included as a loading control. 5 times more lysate was loaded in this figure to compensate for the lower levels of Gis4 in *grr1Δ* cells.

Figure 10

A. wild type (Gis4-TAP)




B. wild type (TAP-Grr1)




C. *grr1Δ* (Gis4-TAP)

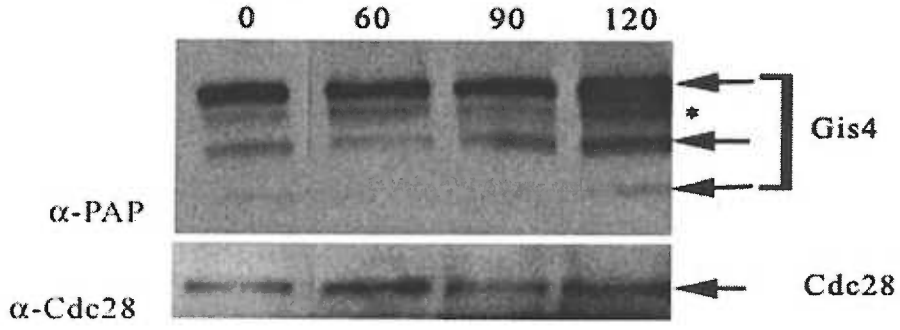


Figure 11 - The three primary bands of Gis4 are not ubiquitinated

FLAG-Gis4 was precipitated by FLAG-M2 beads and subjected to SDS-PAGE. When probed with anti-FLAG, the three primary bands of Gis4 are clearly visible (lane 1). However, when probed with anti-myc to detect ubiquitin-myc, the heavy chain (see arrow) and a non-specific, background band appear (lane 2). We know this band is non-specific because it appears in the *gis4Δ* strain as well (lane 3).

# Figure 11

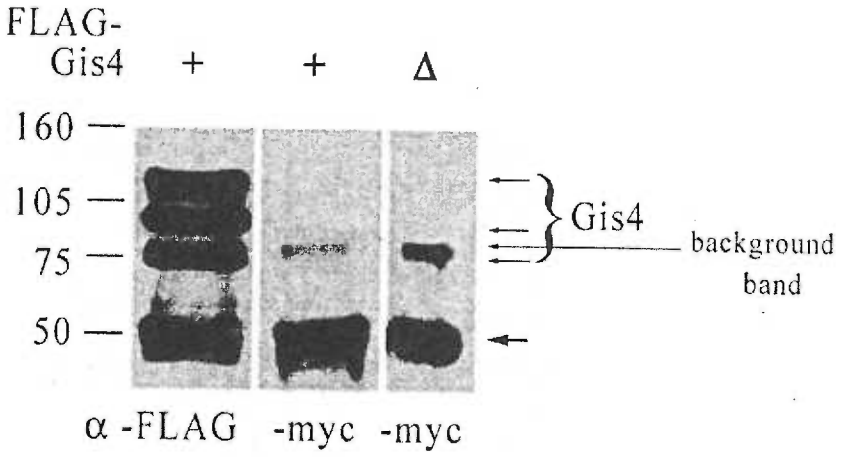


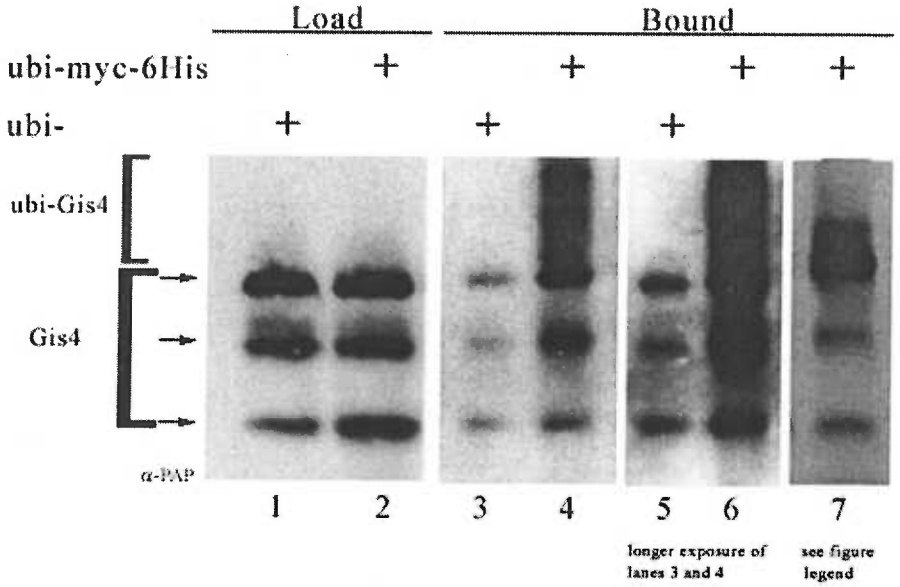
Figure 12- Gis4 is ubiquitinated in a Grr1-dependent manner

a. Gis4 is ubiquitinated: extracts were subjected to a $Ni^{2+}$NTA pull down and examined via Western Blotting to detect Gis4-TAP. Lanes 5 and 6 are a longer exposure of lanes 3 and 4 to show that there are no higher molecular weight in lane 3 bands when exposed longer. Lane 7 is from a different experiment and is indicative of the single band ubiquitin modification ("mono-ubiquitination") seen. This single band just above the highest form of Gis4's three bands can be seen in lane 3 as well.

b. Grr1 is required for Gis4 ubiquitination: Cells are treated as in A in either wild type or *grr1Δ* cells. Five times more extract was used in the *grr1Δ* pull down to make the levels of Gis4 equal (compare lanes 1 and 2). Lane 5 is a significantly longer exposure of lane 4 to demonstrate that some non-specific binding occurred as expected, yet no higher molecular weight forms were detected. The * indicates a background band seen in *grr1Δ* cells.

# Figure 12

A. wild-type (Gis4-TAP)




B. *grr1Δ* (Gis4-TAP)

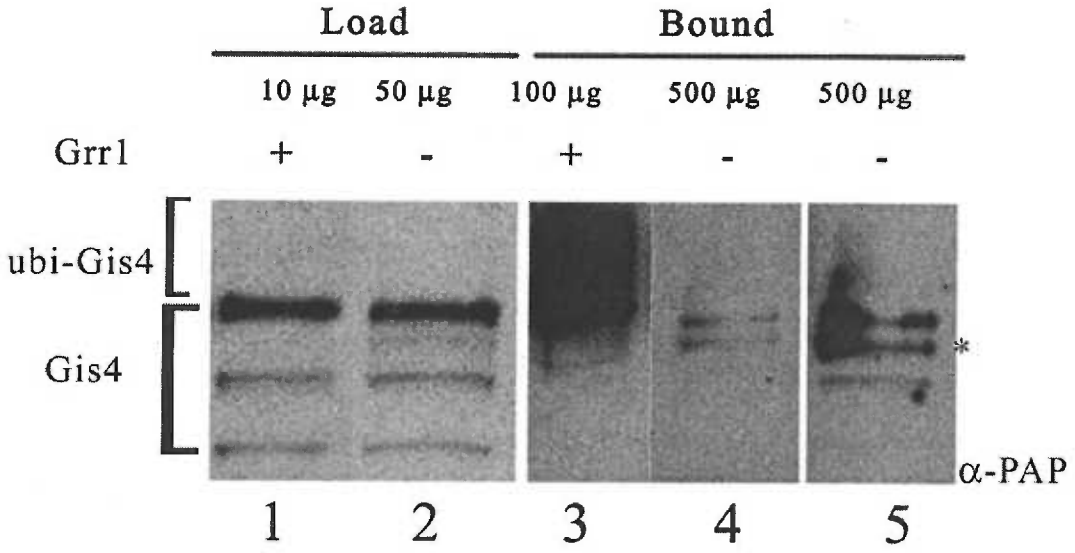


Figure 13- Over-expression of Gis4 is lethal in *grr1Δ* cells

Gis4 was expressed from a Gal1 promoter in wild-type and *grr1Δ* cells and plated on glucose or galactose media. Cells are patched as decreasing serial dilutions of cells.

This experiment was conducted with a constitutively active Translation Elongation Factor (TEF) promoter and yielded the same results. The cells were viable in glucose and inviable on galactose, as well as raffinose media (not shown)

# Figure 13

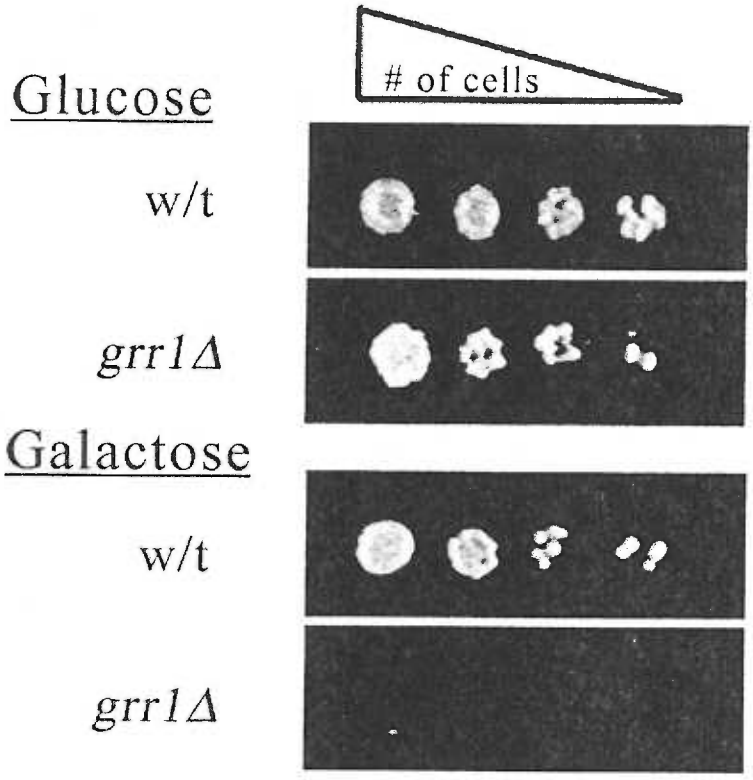


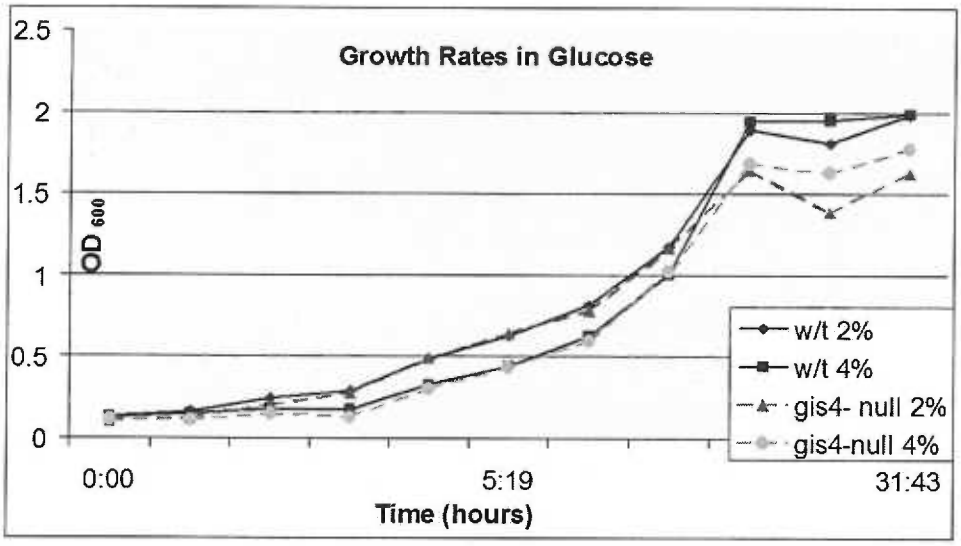
Figure 14- Growth Assays

a. Levels of Glucose does not affect growth rate in *gis4Δ* cells: The growth of wild type cells vs *gis4Δ* cells was compared in 2% and 4% glucose. Cells were grown over night in 2% glucose media. An equal number of cells (determined by absorption at $OD_{600}$) were placed into the new carbon source. This was done in triplicate and the number of cells was assessed by absorption at $OD_{600}$ and plotted against time. There appeared to be very little difference in their growth rates, although the *gis4Δ* cells had a plateau indicative of slower growth at a lower concentration of cells.

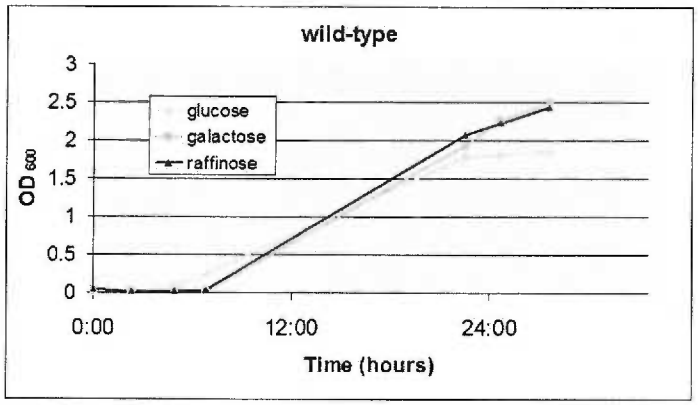
b. Other carbon sources do not affect growth rate in *gis4Δ* cells: The growth of wild type and *gis4Δ* cells was also tested in other carbon sources. Cells were grown over night in 2% glucose media. An equal number of cells (determined by absorption at $OD_{600}$) were placed into the new carbon source. This was done in triplicate and the number of cells was assessed by absorption at $OD_{600}$ and plotted against time. Again, the rates of growth were not different in 2% glucose, galactose, or raffinose.

# Figure 14

A.


Growth Rates in Glucose
2.5
2
1.5
1
0.5
0
OD 600
0:00
5:19
31:43
Time (hours)
w/t 2%
w/t 4%
gis4- null 2%
gis4-null 4%

B.


wild-type
3
2.5
2
1.5
1
0.5
0
OD 600
glucose
galactose
raffinose
0:00
12:00
24:00
Time (hours)

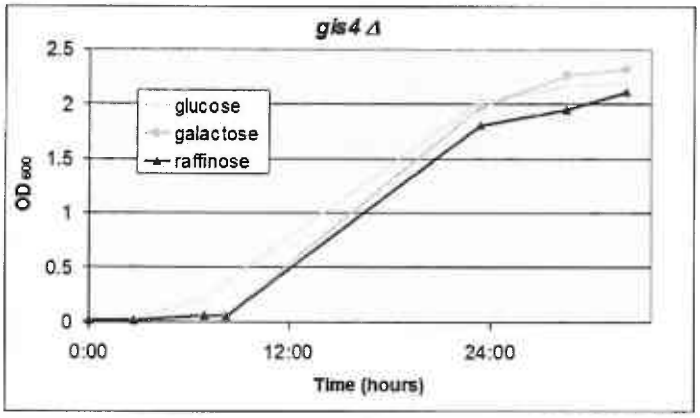

gis4 Δ
2.5
2
1.5
1
0.5
0
OD 600
glucose
galactose
raffinose
0:00
12:00
24:00
Time (hours)

Figure 15- Levels of Gis4 in various carbon sources:

The levels of Gis4 were examined in different carbon sources- including medium (2%) (lane 1) and high (4%) (lane 2) levels of glucose. Cdc28 was used as a loading control (bottom panel). Gis4 protein levels do not appear to change in either amount of glucose or galactose (lane 3). The levels appear higher in raffinose (2%) (lane 4) and glycerol (2%) (lane 5).

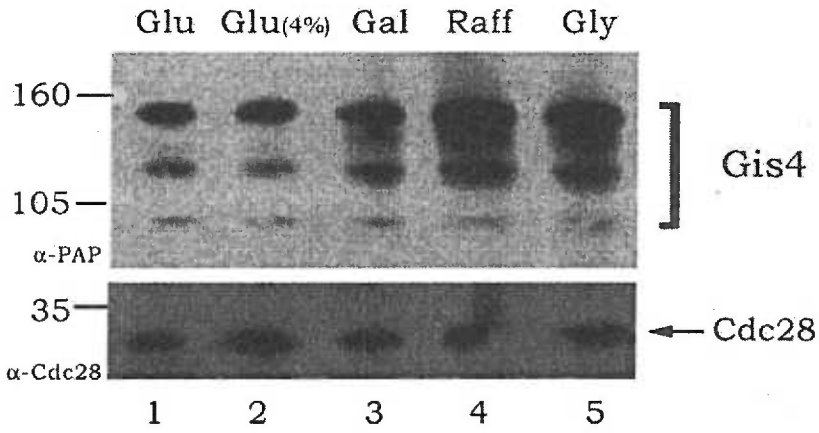

Glu
Glu(4%)
Gal
Raff
Gly
160
105
α-PAP
Gis4
35
α-Cdc28
Cdc28
1
2
3
4
5

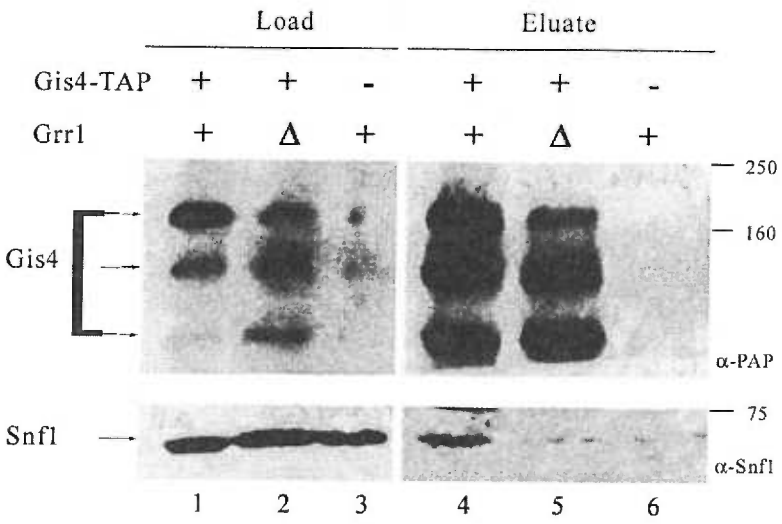
Figure 16- Gis4 binds Snf1 and requires Grr1

a. Gis4's interaction with Snf1 is dependent upon Grr1: Cells containing Grr1, Gis4-TAP, or both were subjected to precipitation using the calmodulin resin. Extracts lacking Grr1 were loaded at 5x wild type to compensate for the lower levels of Gis4, and the Snf1 band in this lane is wider, indicating greater amount of protein. Lanes 1-3 demonstrate the presence of each protein, and lanes 4-6 are the elution from the resin using Ca++. Lanes 5 and 6 have a small level of Snf1 binding, but this binding is clearly enhanced in the presence of Grr1. "L"=load, "F"=flow through, "B"=bound fraction

b. Grr1 does not interact with Snf1: Snf1 and Grr1 in yeast two-hybrid constructs were tested for interaction using β-galactosidase assays as compared to both positive and negative controls.

# Figure 16

A.


Load
Eluate
Gis4-TAP + + - + + -
Grr1 + Δ + + Δ +
250
160
Gis4
α-PAP
75
Snf1
α-Snf1
1 2 3 4 5 6

B.

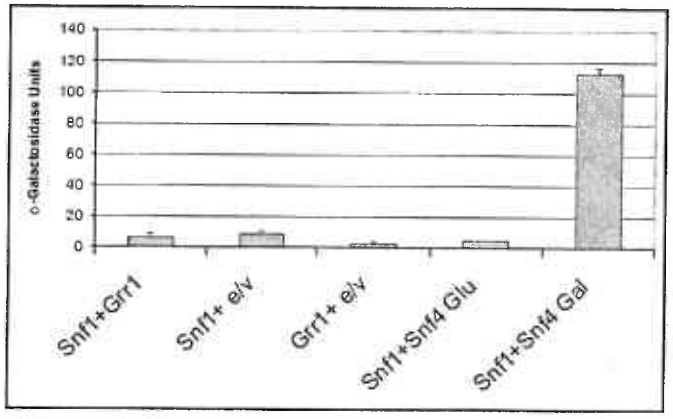

β-Galactosidase Units
140
120
100
80
60
40
20
0
Snf1+Grr1
Snf1+ e/v
Grr1+ e/v
Snf1+Snf4 Glu
Snf1+Snf4 Gal

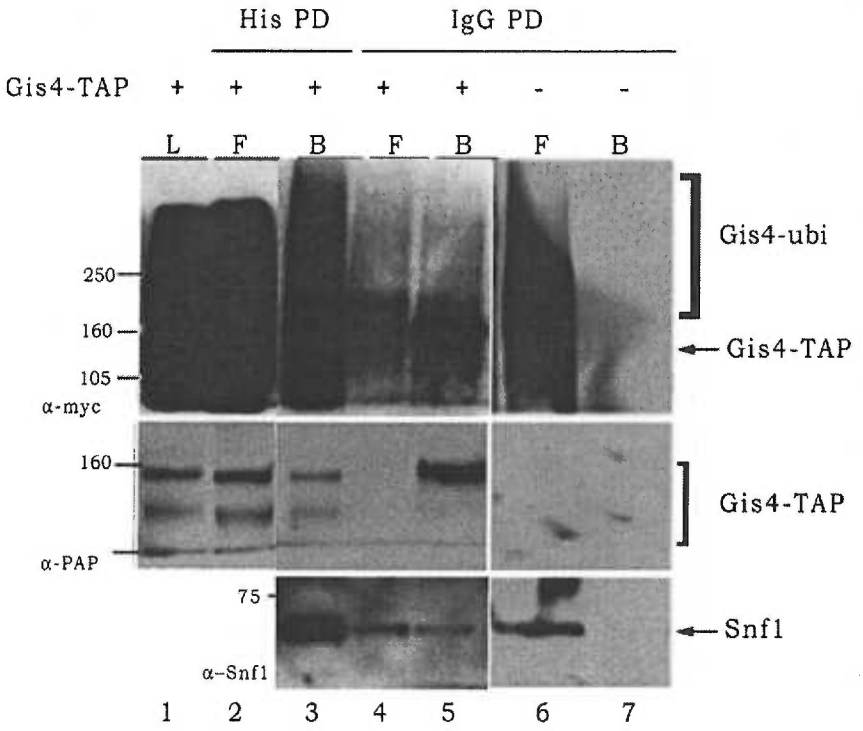
Figure 17- Ubiquitinated Gis4 binds Snf1

a. Snf1 interacts with ubiquitinated Gis4: Cells containing Gis4-TAP and Ubi4-myc expressed from a plasmid were subjected to a $Ni^{2+}$-NTA purification to precipitate Snf1 and its complexes. The beads were eluted using 500 mM imidazole and Gis4-TAP and associated proteins were pulled down using IgG sepharose via binding to the protein A tag of the TAP epitope on Gis4. Lane 3 shows the Snf1 pulled down as well as some Gis4-TAP and a large amount of ubiquitinated species. Lane 5 demonstrates that Snf1 is present in the Gis4 pull down and that the form of Gis4 that binds to Snf1 appears to be ubiquitinated. Snf1 does not come down with the IgG beads when Gis4 is absent; it is in the flow through (lane 6) and not the bound fraction (lane 7). "L"=load, "F"=flow through, "B"=bound fraction

b. The Gis4-Snf1 interaction requires an intact SCF complex: Cells from strain SLY608 (*skp1-12$^{ts}$*) containing FLAG-Gis4 (pSL 667) were grown at either 25 °C (lanes 1-2) or 37 °C (lanes 3-4). Extracts were subjected to a FLAG-M2 resin to precipitate Gis4 and its complexes. Snf1 was precipitated at 25 °C when Skp1 is active (lane 2). At 37 °C, Snf1 appears to be entirely in the flow through (lane 3).

Figure 17

A.




B.

*skp1-12* mutants

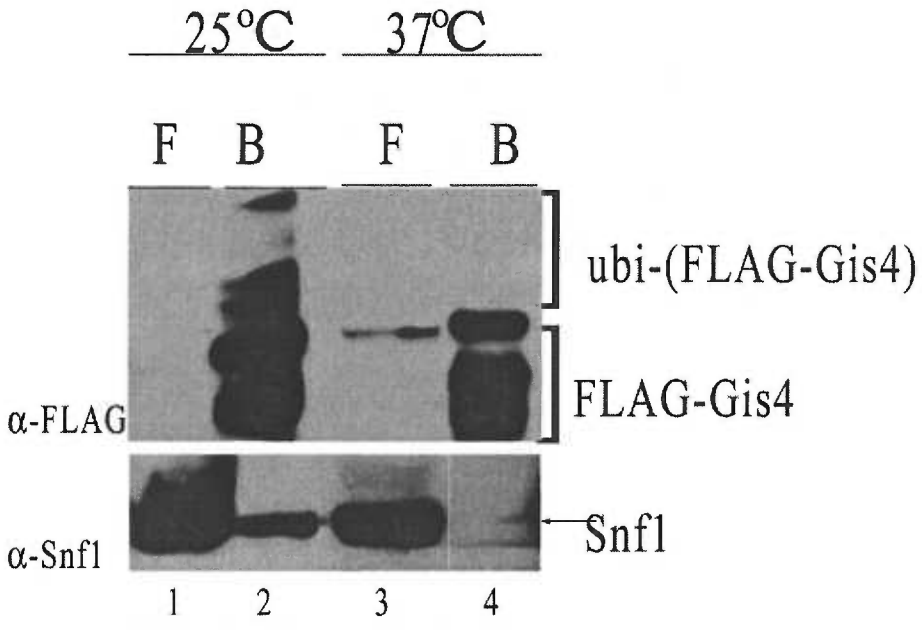


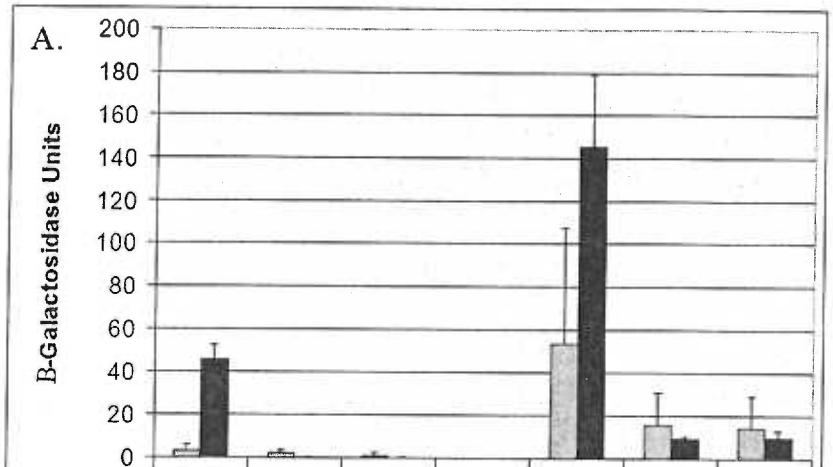
Figure 18- Gis4 positively affects Snf1 activity

a. Gis4 affects Snf1 activity: The interaction of Snf1 and Snf4 was assessed in wild type cells and cells that over-express Gis4. When Gis4 is over-expressed (right side), the interaction between Snf1 and Snf4 is increased in both glucose and galactose indicating that Snf1 is active. When Gis4 is not expressed, Snf1 only interacts with Snf4 in galactose, as expected (left side). "e/v" = empty vector

b. Gis4 interacts with the phosphorylated form of Snf1: FLAG-Gis4 was over expressed from the TEF promoter. Extracts were subjected to FLAG M2 beads for purification and assayed via Western Blot. Gis4 interacts with a lower mobility form of Snf1, perhaps indicating phosphorylation (indicated by the asterisk).

# Figure 18

A.




| Vector | Snf1+ Snf4 none | Snf1+ e/v none | Snf4+ e/v none | Snf1+ Snf4 Gis4 | Snf1+ e/v Gis4 | Snf4+ e/v Gis4 |
|---|---|---|---|---|---|---|
| Glucose | 3.0618 | 1.7685 | 1.1309 | 53.878 | 15.476 | 14.425 |
| Galactose | 45.367 | 0.1929 | 0.1885 | 145.37 | 8.7802 | 9.4519 |

B.

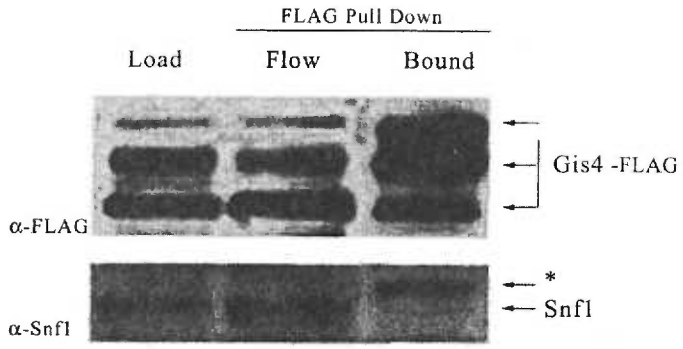
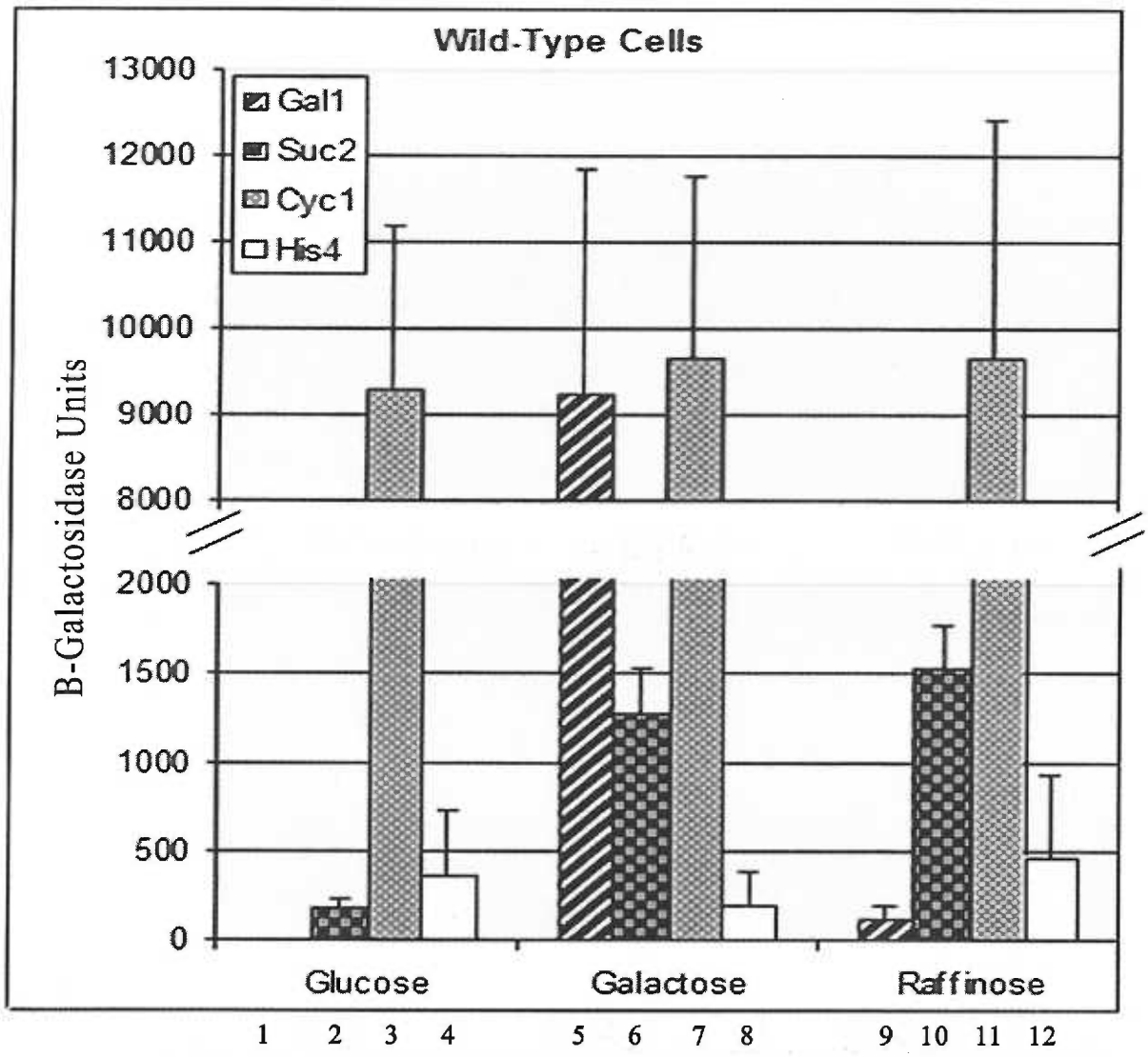


Figure 19- Deletion of Gis4 inhibits de-repression of SUC2 and CYC1

Cells were transformed with plasmids that expressed lacZ fused to the GAL1, SUC2, CYC1, or HIS4 promoter as indicated. The cells were then grown in glucose, galactose, or raffinose and reporter activity was assessed via β-galactosidase assays. A. Wild type cells B. *gis4Δ* cells

Figure 19

A


Wild-Type Cells
Gal1
Suc2
Cyc1
His4
B-Galactosidase Units
13000
12000
11000
10000
9000
8000
2000
1500
1000
500
0
Glucose
Galactose
Raffinose
1 2 3 4 5 6 7 8 9 10 11 12

B

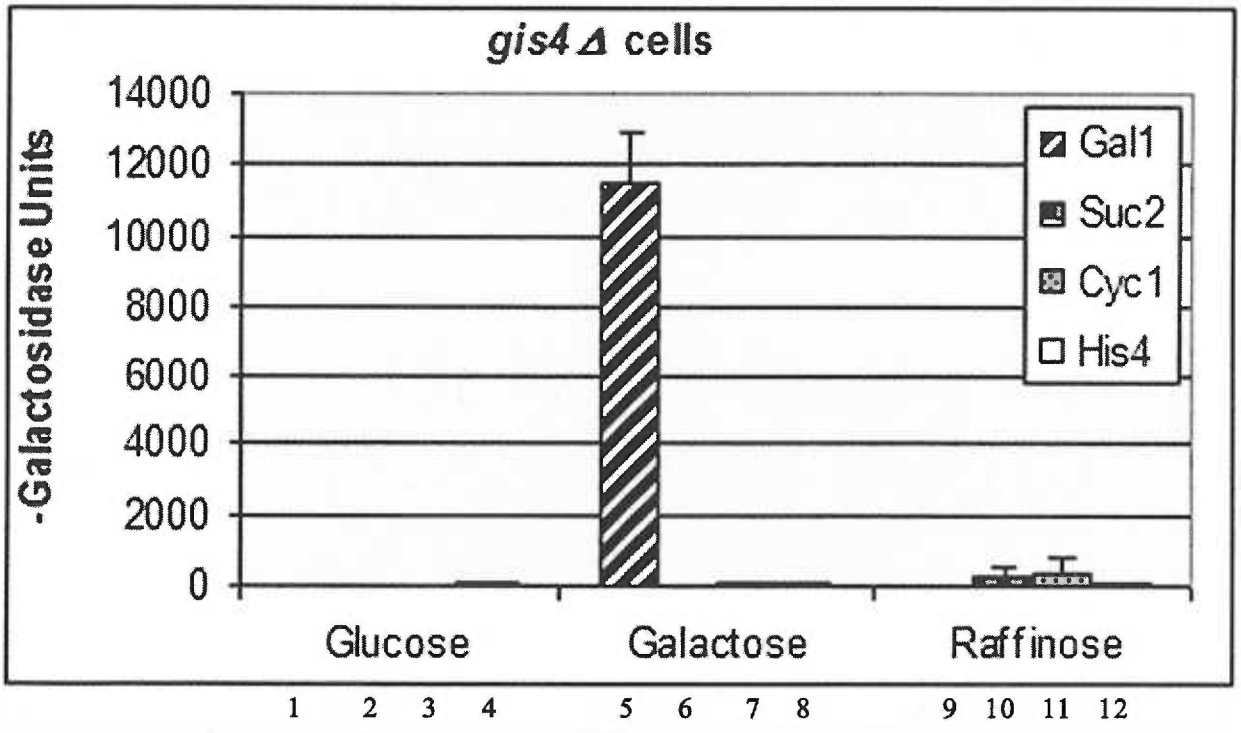

gis4Δ cells
-Galactosidase Units
14000
12000
10000
8000
6000
4000
2000
0
Gal1
Suc2
Cyc1
His4
Glucose
Galactose
Raffinose
1 2 3 4 5 6 7 8 9 10 11 12

Figure 20a- Gis4 in the scheme of glucose repression

a. High Glucose: $SCF^{Grr1}$ receives a signal through Snf3/Rgt2 and degrades Mth1, preventing Rgt1 from repressing transcription of HXT's. Snf1 is inactive and Mig1 and other repressor(s) (X) inhibit transcription of glucose-repressed genes.

b. Low Glucose: Mth1 and Std1 are able to activate Rgt1, allowing it to repress the appropriate HXT's. Snf1 is activated by upstream kinases (P) and is able to phosphorylate Mig1 and, with Gis4, inhibit other repressors, allowing expression of glucose repressed genes.

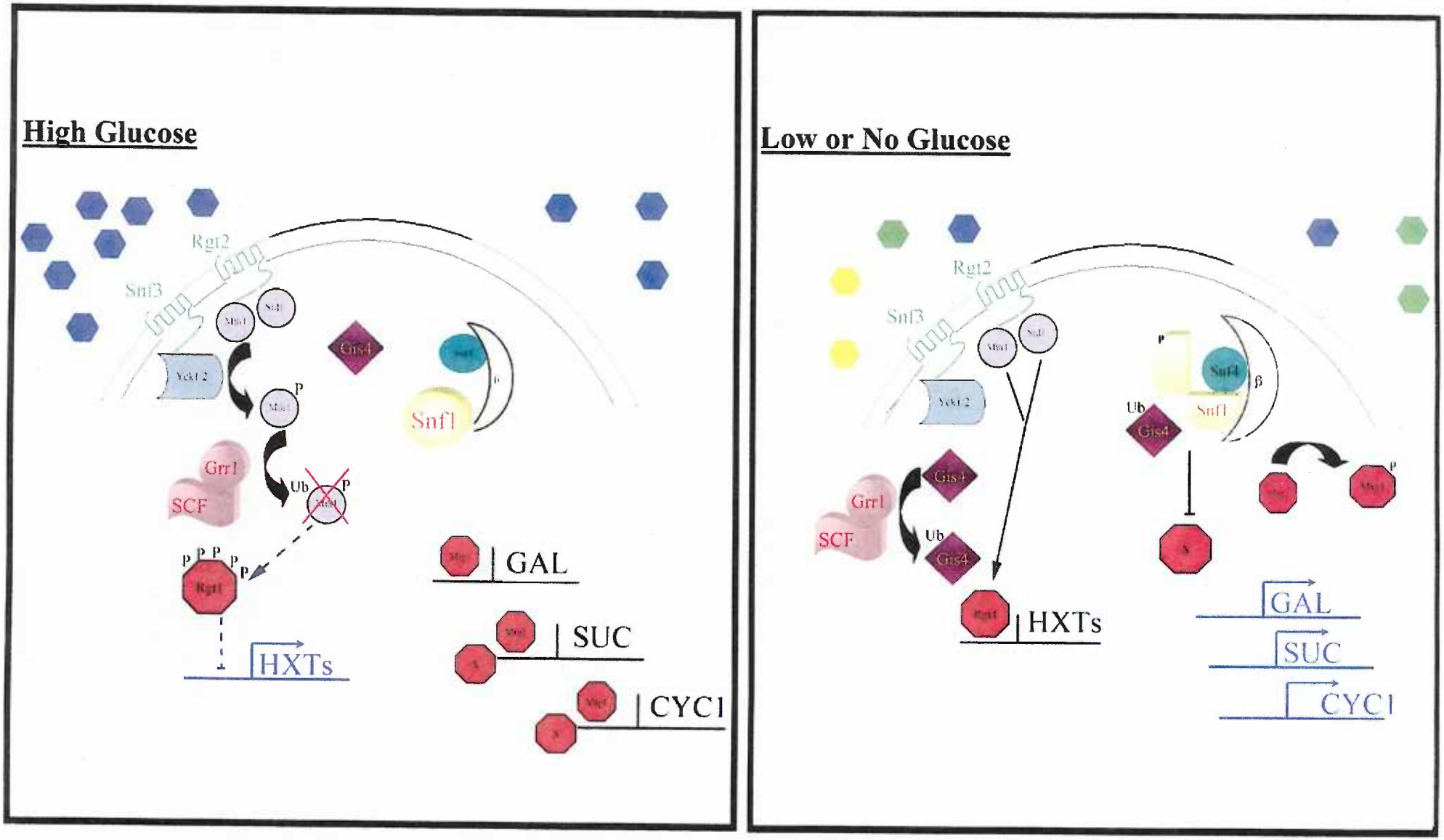

High Glucose
Rgt2
Snf3
Gis4
Snf1
Grr1
SCF
Ub
P
Rgt1
HXTs
GAL
SUC
CYC1
Low or No Glucose
Rgt2
Snf3
Gis4
Grr1
SCF
Ub
Snf1
β
HXTs
GAL
SUC
CYC1

Figure 20b- Gis4 in the scheme of glucose repression

c. *grr1Δ* mutants: these cells already have low Gis4 levels and thus are unable to activate Snf1 and remove repressors from the SUC2 and CYC1 promoters. Gal1, being repressed primarily through Mig1 can be expressed at normal levels, because Snf1 is still able to inactivate Mig1.

***grr1Δ* – Low/No Glucose**

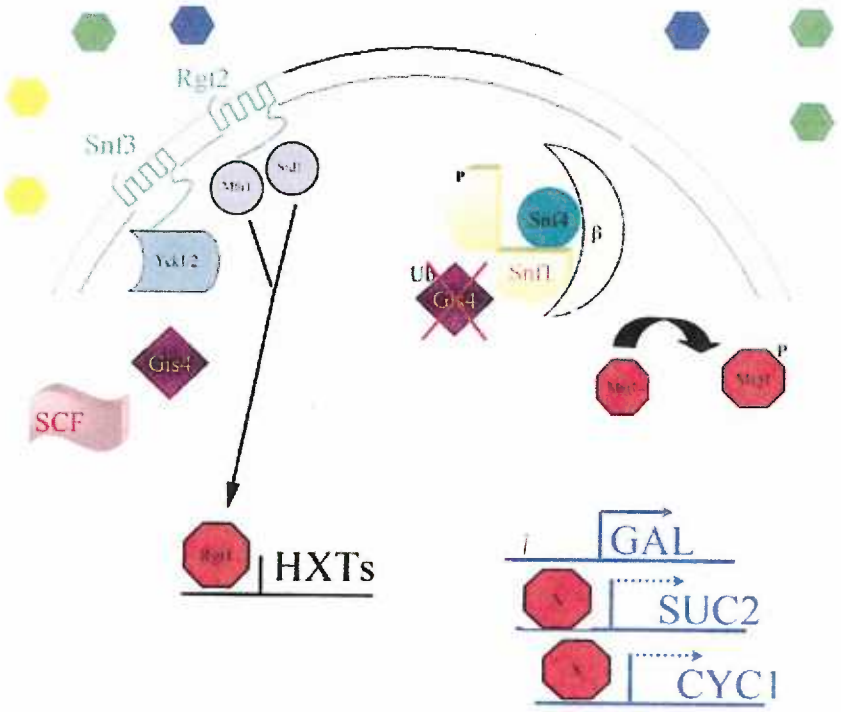
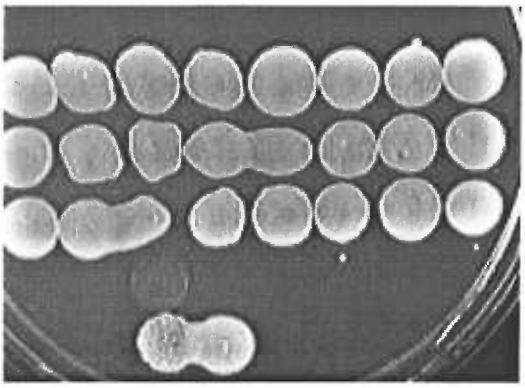
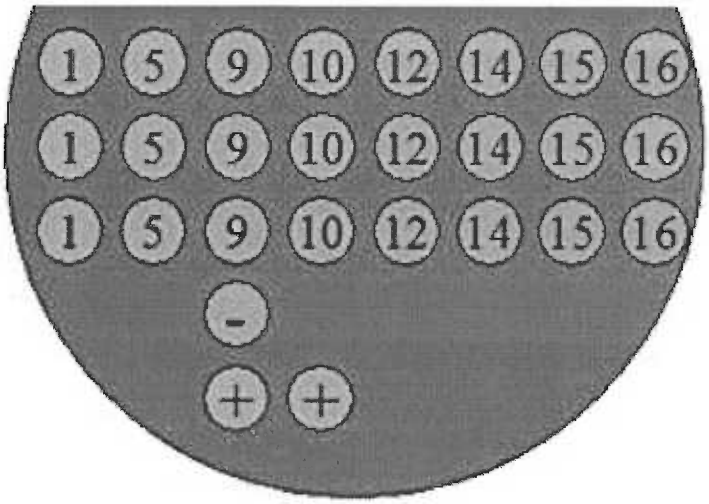


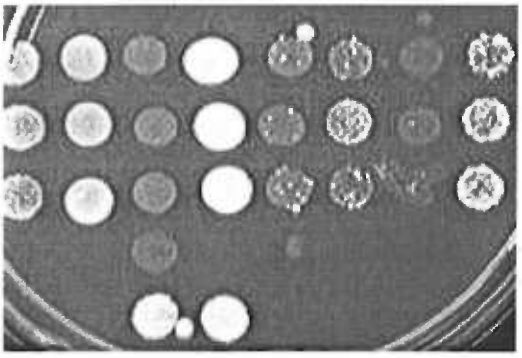
Figure 21- Yeast Two Hybrid Screen – Positive Interactors

During the screen positive interactors were isolated, and the library plasmid harvested, and retransformed to eliminate false-positives. Shown here are the first 8 clones retransformed and plated on to restrictive media. The media lacks leucine and tryptophan to maintain the plasmids, and then additionally lacks adenine or histidine to determine reporter construct activation. The plates lacking histidine have an odd surface tension when droplets are plated and the colonies do not always form perfect circles. Clones 1, 5, 10, and 16 are positive in this assay. #5 is Gis4.

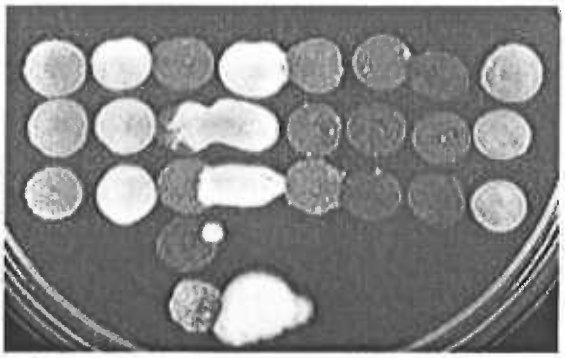


-Leu-Trp



-Leu-Trp-Ade



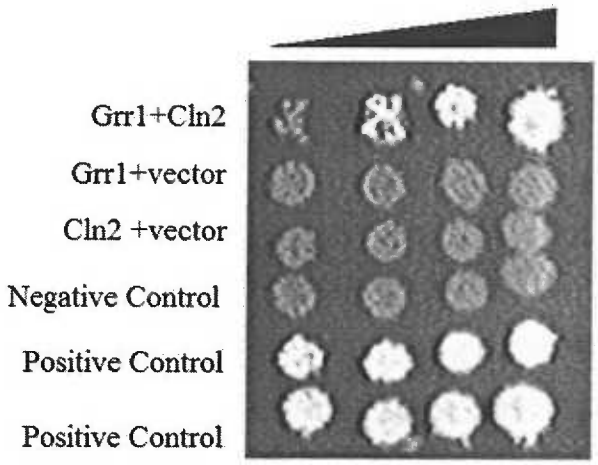
-Leu-Trp-His+3AT

Figure 22- The Grr1-Cln2 interaction in the AH109 strain

a. Grr1 and Cln2 interact in AH109 cells: The interaction between Grr1 and Cln2 was measured against positive and negative controls. Shown here are one clone and the controls on media lacking leucine, tryptophan, and adenine. The number of cells plated increases from left to right.

b. Grr1 and Cln2 interact in AH109 cells at different activities: The beta-galactosidase activity from three different clones transformed with Grr1 and Cln2, examining lacZ activity by measuring color development at $OD_{420}$. The three clones have very different activities (far right).

# AH109




# β–Galactosidase Assay- AH109

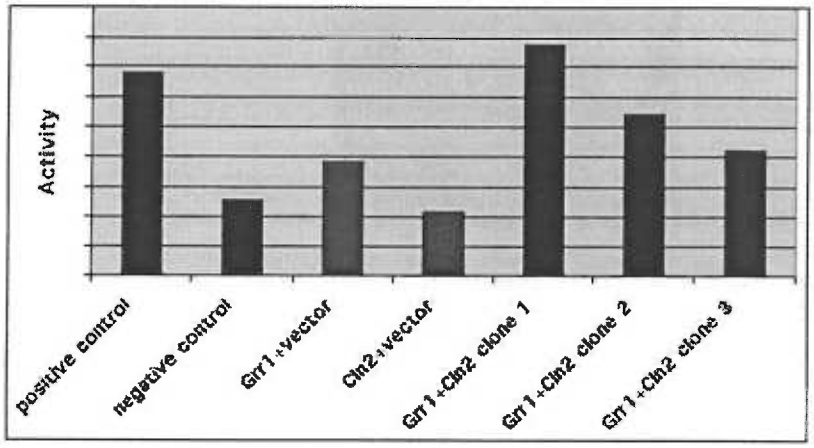
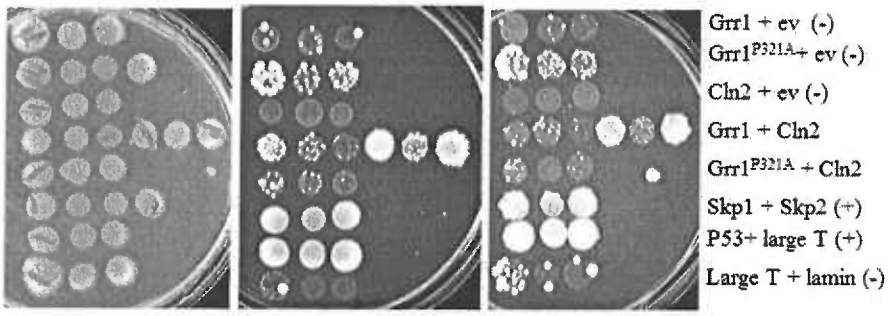


Figure 23- The Grr1-Cln2 interaction in the PJ69 strain:

a. The Grr1-Cln2 interaction is weak in PJ69 cells and Grr1P321A has trans-activation activity: The Grr1-Cln2 interaction was tested again, this time with the PJ69 strain, and included the F box mutant. Again, not all the clones transformed with Grr1 and Cln2 responded equally, only 4 of the 6 plated had robust growth. Additionally, the $Grr1^{P321A}$ mutant did not interact with Cln2 enough to induce growth in its 3 clones, but by itself, it appeared to have trans-activation ability when expressed with the empty vector alone. The other positive and negative controls worked as expected, confirming the above results.

b. The Grr1-Cln2 interaction is weak in PJ69 cells and Grr1P321A has trans-activation activity: The test for lacZ activity was equally erratic and inconclusive. Shown here is the beta-galactosidase activity from three different clones transformed with Grr1 and Cln2, examining lacZ activity by measuring color development at $OD_{420}$.

PJ69 growth test




# β–Galactosidase Assay

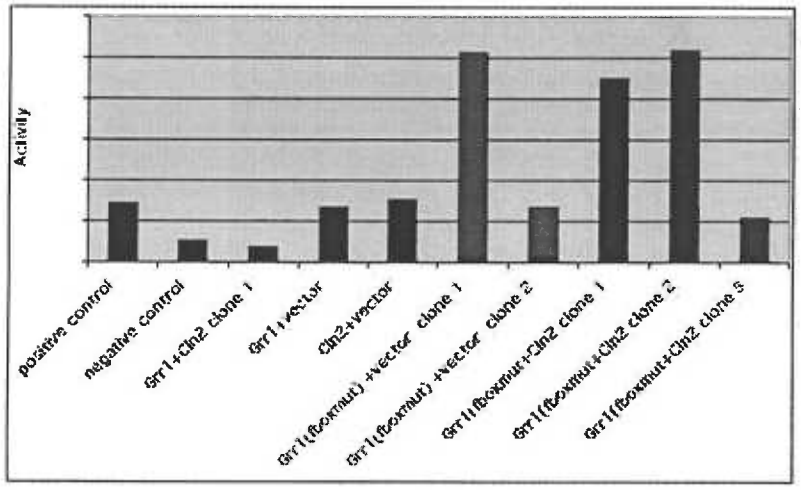
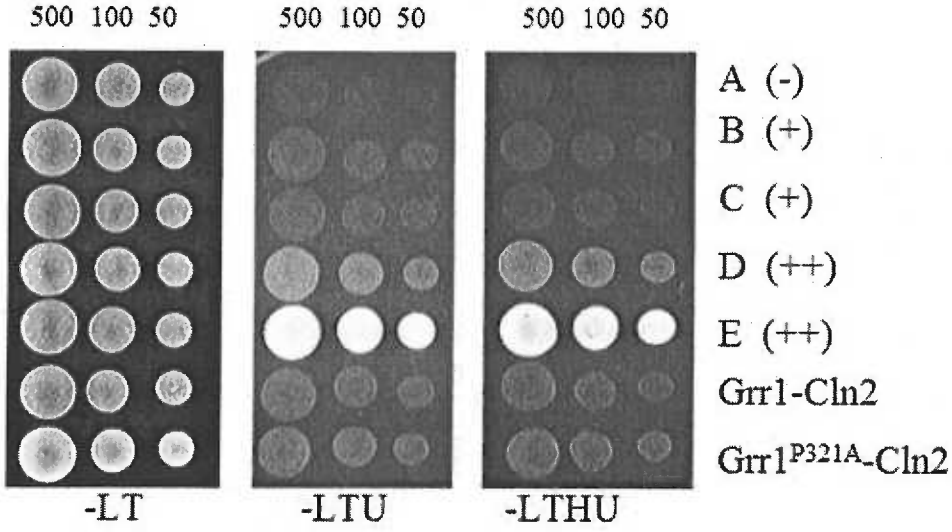


Figure 24- Reverse Two Hybrid

a. Grr1-Cln2 yeast two hybrid interaction in MaV203: The Grr1-Cln2 interaction was tested again, this time in the reverse two-hybrid strain, MAV203. Using media lacking leucine and tryptophan (for plasmid maintenance) and lacking uracil or uracil and histidine (MaV203-specific reporters), the interactions were examined. A-E represents controls from Invitrogen. A is a negative control, and B-E represent interactions of increasing strength, B being weak, and E being very strong. Recalling that the uracil promoter includes the Spo13 repressor, we concluded that the Grr1-Cln2 interaction was not strong enough to overcome the effects of the repressor. Similar to the PJ69 test, the Grr1$^{P321A}$-Cln2 interaction was non-existent as well.

b. Grr1-Cln2 reverse two hybrid interaction in MaV203: When tested via the reverse two-hybrid, Grr1-Cln2 does not appear to interact. When grown in the presence of 5FOA, strong interactions are prohibited from growing. Thus, the negative control "A" and the empty vector controls grew, as did the Grr1-Cln2 and Grr1$^{P321A}$-Cln2. The fact that the Cln2-Cdc28 clones grew indicated a problem with the Cdc28 plasmid. Only E was strong enough to overcome the repressor to grow in media lacking uracil and not on 5-FOA media.

MAV203




MAV203 growth on 0.2% 5FOA