Understanding the Role of the Vid30c in the Nutrient-Dependent Turnover of Hexose Transporters in Saccharomyces cerevisiae

by

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**ABSTRACT**

**Understanding the Role of the Vid30c in the Nutrient-Dependent Turnover of Hexose Transporters in *Saccharomyces cerevisiae***

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University of Guelph, 2012

Advisor:  
Dr. George van der Merwe

*Saccharomyces cerevisiae* is confronted with continually changing and diverse nutrient conditions. As such, it has adapted to utilize both a wide variety of nutrient sources and to preferentially use the most nutrient-rich source to obtain a competitive advantage. The transcription, intracellular trafficking and protein turnover of nutrient transporters, including the hexose transporter proteins (Hxts), are strictly regulated in response to nutrient conditions. The low affinity hexose transporter Hxt3p is highly expressed and localizes to the plasma membrane during growth in abundant glucose where it plays a major role in the transport of this sugar. However, following a shift to ethanol as a sole carbon source, Hxt3p is endocytosed and targeted to the vacuole for degradation while its expression is also repressed. In contrast, the high affinity hexose transporter Hxt7p is actively expressed and functional in the plasma membrane when glucose is limiting and nitrogen is abundant. Upon nitrogen starvation or rapamycin treatment, *HXT7* transcription decreases and the protein is targeted for degradation. The mechanisms that govern these regulatory steps are poorly understood. The Vid and Gid proteins, several of which compose the Vid30 complex (Vid30c), facilitate the nutrient-dependent degradation of the gluconeogenic enzymes FBPase and Mdh2p when glucose-
starved cells are replenished with glucose. Here we show that components of the Vid30c are needed for the ethanol-induced turnover of Hxt3p and the rapamycin or nitrogen starvation-induced degradation of Hxt7p. In addition, we demonstrate that the signals for the ethanol-induced turnover of Hxt3p and the rapamycin-induced turnover of Hxt7p converge on the Vid30c upstream of the Ras/cAMP/PKA pathway, ultimately controlling the degradation of both these hexose transporters. Finally, we provide evidence that the Vid30c controls the localization of the Ras GEF Cdc25p and may therefore directly regulate the activity of the Ras/cAMP/PKA pathway.
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List of abbreviations

ADH       aldehyde dehydrogenase

cAMP      adenosine 3’, 5’-cyclic monophosphate

CCR       carbon catabolite repression

CDB       cyclin destruction box

CP        core particle

CSM       complete synthetic medium

DIC       direct interference contrast

DIG       digoxigenin

DUB       deubiquitinating enzyme

DV        drug vehicle

EGO       escape from rapamycin-induced growth arrest

ESCRT     endosomal sorting complex required for transport

FBPase    fructose bisphosphatase

GAP       GTPase-activating protein

GEF       guanine nucleotide exchange factor

GFP       green fluorescent protein
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>GID</td>
<td>glucose induced degradation</td>
</tr>
<tr>
<td>GPC</td>
<td>GID protein complex</td>
</tr>
<tr>
<td>HA</td>
<td>hemaglutinin</td>
</tr>
<tr>
<td>HECT</td>
<td>homologous to the E6-AP carboxyl terminus</td>
</tr>
<tr>
<td>Hxt</td>
<td>hexose transporter</td>
</tr>
<tr>
<td>MFS</td>
<td>major facilitator superfamily</td>
</tr>
<tr>
<td>MVB</td>
<td>multivesicular body</td>
</tr>
<tr>
<td>NCR</td>
<td>nitrogen catabolite repression</td>
</tr>
<tr>
<td>PDS</td>
<td>post diauxic shift</td>
</tr>
<tr>
<td>PIK</td>
<td>phosphatidylinositol-kinase-related</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>RING</td>
<td>really interesting new gene</td>
</tr>
<tr>
<td>RP</td>
<td>regulatory particle</td>
</tr>
<tr>
<td>SAP</td>
<td>Sit4p activating protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble NSF attachment protein receptor</td>
</tr>
<tr>
<td>STRE</td>
<td>stress response</td>
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<tr>
<td>TAP</td>
<td>tandem affinity purification</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TOR</td>
<td>target of rapamycin</td>
</tr>
<tr>
<td>TORC</td>
<td>target of rapamycin complex</td>
</tr>
<tr>
<td>VID</td>
<td>vacuolar import and degradation</td>
</tr>
<tr>
<td>Vid30c</td>
<td>Vid30 complex</td>
</tr>
<tr>
<td>YNB</td>
<td>yeast nutrient base</td>
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<tr>
<td>YPD</td>
<td>yeast extract peptone dextrose</td>
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1.0 Introduction

1.1 Overview

Unicellular organisms are confronted with ever-changing and highly diverse nutrient conditions. As such, they have adapted to utilize both a wide variety of nutrient sources and to preferentially use the most nutrient-rich to obtain a competitive advantage. The baker’s yeast *Saccharomyces cerevisiae* preferentially utilizes glucose and fructose through the rapid, and energy-inefficient fermentation to ethanol. As an evolutionary strategy, *S. cerevisiae* rapidly consumes glucose from its environment, and upon its exhaustion adapts rapidly to consume poorer, non-fermentable carbon sources such as ethanol (its otherwise toxic by-product), galactose or maltose. A similar phenomenon occurs with the utilization of nitrogen. The yeast will consume rich nitrogen sources like ammonia or glutamine before turning to others more difficult to assimilate. The corollary to this is when yeast cells growing on a nutrient-poor source are provided with a more preferable nutrient, they quickly adapt to down-regulate the genes and inactivate the proteins involved in less preferable nutrient utilization. This adaptation is accomplished by the strict regulation of gene transcription, protein function and protein stability. To regulate this properly, yeast have adapted a series of nutrient-sensing and nutrient-signalling mechanisms that can elicit responses to repress or activate genes, inactivate or activate protein function, and have also evolved processes to target specific proteins for degradation. This introduction will outline the details of the regulation involved in these adaptive responses.
1.2 Nutrient sensing and signalling

For *S. cerevisiae* to adapt to changing environmental conditions, it must possess two basic capabilities. First, it must be able to sense the nutrient availability of its surroundings. This can be accomplished by binding of an external nutrient substrate to a plasma membrane-bound sensor or by binding an internal metabolic intermediate to a cytosolic sensor. Second, the yeast must be able to effectively transmit the sensed signal to an intracellular target and elicit a downstream response. This action is commonly accomplished by a signal cascade in which the signal is transmitted via a series of protein interactions, often involving posttranslational modifications. The signal culminates at a downstream effector, causing a change in protein function or gene expression in response to the sensed nutrient. Carbon and nitrogen represent two important nutrient sources for yeast survival that can be sensed and signalled by carbon catabolite repression, glucose activation and the target of rapamycin (TOR) pathway (Boles *et al.*, 1993; Cardenas *et al.*, 1999; Entian *et al.*, 1984). *S. cerevisiae* has adapted these mechanisms to preferentially use rich carbon sources (glucose and fructose) and nitrogen sources (ammonia and glutamine) before poorer, more difficult-to-assimilate ones (Cooper, 1982; Matern *et al.*, 1977).

1.2.1 Carbon catabolite repression

For the cell to utilize glucose efficiently, it must have a system in place to repress the genes needed for the utilization of other carbon sources when glucose is abundant. This process is called carbon catabolite repression (CCR) and is dependent on the transcriptional repressor Mig1p. When glucose is abundant in the environment, Mig1p is imported into the nucleus where it binds to the promoter region of glucose-repressible
genes. Mig1p is able to bind DNA through its zinc finger domain and recruit the general co-repressors Ssn6p and Tup1p (Treitel et al., 1995). When glucose is absent, Mig1p is exported back to the cytoplasm and CCR is effectively lifted.

When glucose is absent in the environment, the derepression of glucose-repressible genes is dependent on the Snf1-protein complex. This complex is centered on the kinase Snf1p, which is able to phosphorylate Mig1p and stimulate its transport to the cytoplasm. There are three scaffolding proteins, Sip1p, Sip2p and Gal83p which facilitate the interaction of Snf1p with the activating subunit Snf4p (Jiang and Carlson, 1997). These scaffolding proteins are also involved in the subcellular localization of the complex and therefore may offer an additional level of regulation (Vincent et al., 2001).

Snf1p contains two domains: an N-terminal catalytic domain and a C-terminal regulatory domain. The activity of Snf1p is regulated through conformational changes and is stimulated by the absence of glucose. When glucose is abundant, the regulatory domain of Snf1p interacts with the catalytic domain causing an auto-inhibitory effect. When cells are deprived of glucose, the Snf4p activating subunit binds to Snf1p, blocking auto-inhibition by the regulatory domain, thus activating Snf1p. In this active form, Snf1p is able to phosphorylate Mig1p, causing its translocation from the nucleus and relieving glucose repression (Jiang and Carlson, 1996).

The Snf1p-Snf4p interaction is regulated by phosphorylation of a threonine residue in the regulatory domain of Snf1p’s activation loop. Phosphorylation of Snf1p by the redundant kinases Sak1p, Tos3p and Elm1p stimulates Snf4p binding and abolishes the auto-inhibitory function of Snf1p, thereby activating Snf1p (Hong et al., 2003).
Dephosphorylation of Snf1p is dependent on the type-1 protein serine/threonine phosphatase Glc7p in complex with Reg1p. Recent investigation has suggested that Reg1p binds to both Glc7p and Snf1p, and, importantly, the Snf1p binding site of Reg1p is the same as the Glc7p site. When Reg1p is bound to Glc7p, Snf1p is dephosphorylated by Glc7p and the Snf1p auto-inhibitory function inactivates it. Alternatively, when Reg1p is bound to Snf1p it prevents Glc7p binding and dephosphorylating Snf1p, maintaining Snf1p in an active form. Thus the competitive binding of Reg1p to Glc7p or Snf1p regulates Snf1p activity (Tabba et al., 2010).

1.2.2 Glucose activation (Ras/cAMP/PKA)

In addition to repressing genes that are unnecessary or detrimental during growth on glucose, cells can also activate a wide array of genes needed for growth and metabolism. The major pathway for the response to glucose in *S. cerevisiae* is the Ras/adenosine 3’, 5’-cyclic monophosphate (cAMP)/ protein kinase A (PKA) pathway (Fig. 1). This pathway is involved in energy metabolism, cell cycle progression, aging, thermotolerance, glycogen accumulation, stress resistance and sporulation (Cameron et al., 1988; Longo, 2003; Mendenhall and Hodge, 1998; Smith et al., 1998).

The upstream components of glucose activation are the Ras proteins. These proteins, encoded by *RAS1* and *RAS2*, are GTPases that function as molecular switches. As GTPases, they possess the intrinsic capacity to hydrolyze guanosine triphosphate (GTP) into guanosine diphosphate (GDP) and are stimulated to do so by GTPase-activating proteins (GAPs). Ras is active when in the GTP-bound form, and becomes inactive upon GTP hydrolysis to GDP. The two GAPs that negatively regulate Ras are
Figure 1. The Ras/cAMP/PKA signalling pathway. When glucose is abundant, Ras1/2p is GTP-bound (active) via the function of the RasGEFs, Cdc25p and Sdc25p. Active Ras stimulates cAMP production via activation of the adenylate cyclase, Cyr1p. The generated cAMP binds and inactivates Bcy1p, the negative regulator of PKA. Bcy1p-cAMP then dissociates from the PKA complex and the activated TPK kinase subunits can phosphorylate downstream targets. The hydrolysis of GTP to GDP by the RasGAPs, Ira1p and Ira2p, renders Ras1/2p inactive. The phosphodiesterases, Pde1p and Pde2p, are responsible for the degradation of the cAMP signal by converting cAMP back to AMP. Adapted from Santangelo, 2006.
Ira1p and Ira2p (Tanaka et al., 1990, 1991). To reset the switch to the active form, a set of guanine nucleotide exchange factors (GEFs) are required to replace GDP with GTP. A mutational variant, Ras2Val19, exists where the GTP-bound state is maintained independently of GEFs and results in a constitutively active Ras (Crechet et al., 1990).

The two GEFs responsible for activation of Ras are Cdc25p and Sdc25p (Broek et al., 1987; Haney and Broach, 1994). **CDC25** is an essential gene and is believed to be the primary Ras-activating GEF, whereas **SDC25** is dispensable – when **SDC25** is overexpressed it can rescue a **cdc25** mutation (Boy-Marcotte et al., 1996). The result of GEF stimulation of Ras is the activation of Cyr1p, an adenylate cyclase, and the production of the intracellular secondary messenger cAMP (Matsumoto et al., 1982). This cAMP signal is degraded by the action of two phosphodiesterases, Pde1p and Pde2p (Nikawa et al., 1987; Wilson and Tatchell, 1988), that convert cAMP back to adenosine monophosphate (AMP), thereby degrading the signal molecule and blocking its downstream effects (Ma et al., 1999).

**PKA** is a heterotetrameric protein complex composed of two negative regulatory subunits (Bcy1p) and two catalytic (Tpk) subunits. The Tpk subunits possess kinase activity and are encoded by the redundant genes **TPK1**, **TPK2** and **TPK3** (Toda et al., 1987). The association of Bcy1p with the Tpk proteins maintains PKA in an inactive state. The main function of the cAMP signal is to bind and inactivate Bcy1p, thereby dissociating the regulatory subunits and activating PKA (Hixon and Krebs, 1980). Active PKA is able to phosphorylate a range of proteins involved in transcription,
metabolism and cell cycle control (Cherry et al., 1989; Dihazi et al., 2003; Gorner et al., 2002; Reinders et al., 1998).

When glucose is added to cells that are grown on glucose-limiting media, a signal is generated that converges on Ras and its GEF and GAP regulators. The exact mechanism of glucose activation of Ras remains unknown, however, it has been shown to be dependent on glucose transport via Hxts and glucose phosphorylation via Hxk1p, Hxk2p and Glk1p (Colombo et al., 2004). Upon activation of Ras there is a rapid, transient spike in cAMP levels, which increase up to 50-fold within a few minutes and then slowly decrease back to basal levels (Jaing et al., 1998). This transient spike in cAMP is enough to bind and inactivate Bcy1p (Johnson et al., 1987). With the formation of an inactive Bcy1p-cAMP complex, active PKA is available to phosphorylate its downstream effectors. Bcy1p is itself a PKA target, and upon phosphorylation its affinity for the Tpk subunits is decreased (Kuret et al., 1988). Additionally, the phosphorylation of Bcy1p by the kinases Yak1p and Mck1p causes export out of the nucleus thereby altering its regulation of the nuclear pool of PKA (Griffioen et al., 2001).

This highly regulated pathway allows the yeast cells to actively respond to high glucose availability through the consecutive activation of Ras, Cyr1p and PKA to elicit an appropriate cellular response. This cellular response includes activation of gene transcription and reorganization of metabolic proteins, all resulting in adaptation for the effective utilization of glucose.
1.2.3 The TOR pathway

In addition to responding to the availability of carbon sources, yeast cells must also be able to respond to the availability and quality of other nutrients. In a mechanism similar to CCR, cells are able to utilize any available rich nitrogen sources preferentially to poor ones and this is aptly termed Nitrogen Catabolite Repression (NCR) (Cooper, 1982). The TOR pathway responds to changes in available nitrogen and provides the mechanism for NCR; however, it also responds to changes in the availability of other nutrients, including phosphate and amino acids (Neklesa and David, 2009; Wanke et al., 2005). The discovery of the TOR pathway hinged on the observation that treatment of cells with the macrolide antibiotic rapamycin induces a similar cellular response as nutrient starvation, including a reduction in protein synthesis, induction of autophagy and entry into G0 (Barbet et al., 1996). At present, the mechanisms by which these nutrients are sensed and communicated to TOR remain unknown; however, signalling through the TOR pathway has been well studied and many components have been elucidated, as well as some mechanisms within the pathway (Fig. 2).

Two distinct TOR complexes (TORC) exist in S. cerevisiae: one rapamycin-sensitive (TORC1), the other rapamycin-insensitive (TORC2) (Zhang et al., 1995). The TORC1 signalling pathway governs temporal control of cell growth (translation, transcription, autophagy, etc.) and the TORC2 spatial control of cell growth (actin cytoskeleton and cell wall integrity) (Beck and Hall, 1999; Bickle et al., 1998; Kamada et al., 2000; Powers and Walter, 1999; Schmidt et al., 1996). For this discussion we will focus on TORC1.
Figure 2. TORC1 signalling pathway. When nutrients are abundant, TORC1 is active and represses genes required for stress, poor nutrient utilization and autophagy and stabilizes rich nutrient transporters. When one or more nutrients becomes limiting or cells are treated with rapamycin, TORC1 is inactivated, gene repression is lifted, and rich nutrient transporters are degraded. Adapted from Hall, M., 2003.
The TORC1 is composed of two partially redundant TOR proteins (Tor1p and Tor2p), Lst8p, Kog1p and Tco89p (Loewith et al., 2002; Reinke et al., 2004). Both Tor1p and Tor2p are serine/threonine kinases that are members of the phosphatidylinositol-kinase-related (PIK) kinase family (Keith and Schreiber, 1995). Lst8p contains seven WD40-repeat motifs that facilitate binding to the Tor2p kinase domain and are required for kinase function (Wullschleger et al., 2005). Kog1p possesses both HEAT-repeat and WD40-repeat motifs that facilitate interaction with both Tor1p and substrate, suggesting a role for Kog1p in presenting substrate to TORC1 (Adami et al., 2007). Tco89p shows no unique motifs and its exact role is not fully understood. Recently Tco89p has been shown to interact with the EGO (Escape from rapamycin-induced GROwth arrest) Complex, which functions in amino acid sensing, suggesting a role in signalling amino acid availability to TORC1 (Binda et al., 2009).

The function of rapamycin is elicited through its binding to the FK506-binding protein (FKBP12), encoded by FPR1. FK506 is an immunosuppressant structurally related to rapamycin. The FKBP12-rapamycin complex binds to TORC1 and inhibits kinase activity (Heitman et al., 1991). This rapamycin-induced inactivation of TORC1 mimics the inactivation of TORC1 by nutrient-starvation and therefore has been used to elucidate the function and mechanism of TOR signalling (Peng et al., 2002).

There are several downstream targets under TORC1 regulation. Rtg1p and Rtg3p are transcription factors responsible for the activation of genes involved in maintenance of intracellular glutamate supplies and signalling mitochondrial dysfunction to the nucleus, termed mitochondrial retrograde signalling (Liao and Butow, 1993). Rtg1/3p
nuclear localization is dependent on the phosphorylation state of Rtg3p and is negatively regulated by TORC1 function (Komeili et al., 2000). Active TORC1 inactivates the cytosolic regulatory protein, Rtg2p, such that the negative transcriptional regulator, Mks1p, is able to promote the phosphorylation and cytoplasmic retention of Rtg1/3p (Komeili et al., 2000).

The TORC1-dependent regulation of the transcriptional activator of NCR genes, Gln3p, and nutrient permease stability occurs via the type-2A protein serine/threonine phosphatase Sit4p, and its regulatory protein, Tap42p. TORC1 is able to promote the association of Tap42p and Sit4p by regulating the phosphorylation of the Tap42p-interacting protein Tip41p (Jacinto et al., 2001). Upon nutrient starvation, Tip41p binds to Tap42p, releasing Sit4p which is then available to dephosphorylate its downstream targets. In the form of a positive feedback loop, Sit4p is able to dephosphorylate Tip41p and rapidly amplify its phosphatase activity upon TORC1 inactivation (Jacinto et al., 2001).

One target of Sit4p is the kinase Npr1p, which functions in the regulation of nutrient permeases. Npr1p is able to affect the stability of various nutrient transporters in response to nitrogen starvation. When cells previously grown on a rich nitrogen source (ammonium) are switched to a poor nitrogen source (proline), Npr1p stimulates the internalization and degradation of the tryptophan permease Tat2p (Schmidt et al., 1998). Additionally, when cells are grown on proline, active Npr1p antagonizes the degradation of the general amino acid permease Gap1p (De Craene et al., 2001). The mechanism of Npr1p’s regulation of Gap1p is dependent on the arrestin-like Rsp5p ubiquitin ligase adaptor Aly2p. Aly2p has been shown to be a substrate of Npr1p, and its
phosphorylation directs membrane trafficking of Gap1p away from the vacuole (O’Donnell et al., 2010).

Another target of Sit4p is the transcriptional activator Gln3p, which is involved in the derepression of NCR gene transcription. When cells are grown with a rich nitrogen source, Gln3p is phosphorylated by TORC1 (Beck and Hall, 1999). Phosphorylation of Gln3p stimulates its association with the regulatory protein Ure2p, which sequesters Gln3p to the cytosol (Cox et al., 2000). Upon TORC1 inactivation, Sit4p dephosphorylates Gln3p, freeing it from Ure2p sequestration in the cytosol, and the unphosphorylated Gln3p is transported into the nucleus (Beck and Hall, 1999). Gln3p in the nucleus is then able to bind to the promoters of NCR-sensitive genes and activate transcription (Minehart and Magasanik, 1991).

TORC1 also negatively regulates the nuclear localization of the transcription factors Msn2p and Msn4p, which are responsible for activating genes involved in general stress response. This regulation is dependent on the kinase Rim15p (Cameroni et al., 2004).

As was seen with the Ras/cAMP/PKA pathway, the TOR signalling pathway enables yeast cells to adapt to changes in environmental conditions. Through the inactivation of TORC1, there is the removal of NCR, allowing for the activation of transcription of genes required for the utilization of poor nitrogen sources. Additionally, there is a reorganization of the amino acid permeases to better facilitate the acquisition of less abundant amino acids.
1.2.4 Rim15p regulation

Yeast cells grow exponentially on rich nutrient sources and they rapidly consume glucose through fermentative metabolism, while also consuming nitrogen and phosphate, leading to the eventual depletion of nutrients. This depletion of nutrients will cause a transition into a slowed growth phase called post-diauxic shift (PDS); the yeast shifts to respiratory growth with poor carbon sources, often metabolic by-products, such as ethanol generated during initial fermentative growth. Eventually, one or several nutrients will be completely consumed and the cell will transition into a quiescent or stationary phase (G₀) (Werner-Washburne et al., 1993). This transition allows yeast cells to survive during a period of nutrient starvation by the up-regulation of numerous stress response (STRE) genes, and as accumulation of the stress protectant, trehalose (Lillie and Pringle, 1980; Marchler et al., 1993; Wiemken, 1990). Other cellular responses during starvation also occur, such as down-regulation of translation, growth cessation, and widespread proteolysis (Werner-Washburne et al., 1993). As there are various nutrients that can become limiting and cause a transition to G₀, it is not surprising that several signalling pathways would converge on a single downstream effector: the Rim15p kinase that controls entry into G₀ (Fig. 3) (Pedruzzi et al., 2003).

Rim15p, a PAS (Per-Arnt-Sim) kinase, is able to integrate the signals from PKA, TORC1, the kinase Sch9p and the phosphate signalling cyclin-dependent kinase complex, Pho80p-Pho85p, to elicit the induction of stress response genes and PDS genes (Pedruzzi et al., 2003; Cameroni et al., 2004; Wanke et al., 2005). The induction of these genes is accomplished by the activation of the transcription factors Msn2p, Msn4p and Gis1p. Global transcriptional analysis has shown that these three transcription factors account
Figure 3. Rim15p regulation. When nutrients are abundant, Rim15p is phosphorylated by PKA, TORC1 or Sch9p, anchoring it in the cytoplasm via binding of 14-3-3. When nutrients become limiting, unphosphorylated Rim15p translocates into the nucleus. In the nucleus, Rim15p is responsible for the activation of Msn2/4p and Gis1p, which in turn activate STRE and PDS genes. Pho80p-Pho85p in the nucleus is able to phosphorylate Rim15p and stimulate its export from the nucleus via Msn5p. Adapted from Swinnen et al., 2006.
for almost the entire transcriptional response by Rim15p following glucose limitation at the diauxic shift (Cameroni et al., 2004).

The regulation of Rim15p is dependent on its phosphorylation and binding to the 14-3-3 protein (encoded by BMH2), which acts to anchor Rim15p in the cytoplasm (Wanke et al., 2005). Phosphorylation can occur by either PKA or TORC1 in the cytoplasm and restricts transport into the nucleus. When glucose or other nutrients become limiting, PKA and TORC1 are inactivated, resulting in the dephosphorylation of Rim15p and its dissociation from 14-3-3. In its unphosphorylated state, Rim15p is then transported into the nucleus where it can induce Msn2/4p and Gis1p function. The mechanism by which Sch9p maintains Rim15p’s cytoplasmic retention has yet to be elucidated. However, the rapamycin-induced dephosphorylation of Rim15p still occurs in a sch9 knockout strain, but Rim15p accumulates in the nucleus, indicating that Sch9p functions independently of TORC1 to control the nucleocytoplasmic distribution of Rim15p (Pedruzzi et al., 2003).

The nuclear pool of Rim15p is able to escape PKA phosphorylation because of the presence of the negative regulator Bcy1p (Griffioen et al., 2000). Studies of Rim15p-GFP localization, however, show that nuclear accumulation only occurs when the catalytic function of Rim15p is disrupted, which suggests that there is a mode of autophosphorylation that allows for a basal level of nuclear export of Rim15p (Vidan and Mitchell, 1997). Another level of regulation occurs through the Pho80p-Pho85p complex that responds to inorganic phosphate (P_1) starvation. Under conditions of P_1 abundance, the cyclin-dependent kinase Pho85p is active and phosphorylates Rim15p, stimulating its
export from the nucleus and its association with 14-3-3 (Wanke et al., 2005). The nuclear export of Rim15p is dependent on the karyopherin Msn5p (Wanke et al., 2005). The exact cellular targets of Rim15p have not been identified, though it is plausible that Msn2/4p and Gis1p are phosphorylated directly by Rim15p, a possibility that has not been shown experimentally. This regulation of Rim15p shows how the cell can integrate a variety of nutrient signals to elicit a unified response.

1.3 Protein degradation

For yeast cells to adapt to changes in environmental conditions they must modify their protein complement. These alterations involve not only the production of required proteins, but also the degradation of unnecessary proteins, ensuring that only those proteins necessary for the current conditions are present. The process of degradation usually involves posttranslational modification of specific proteins that need to be degraded and targeting of these proteins to a site for their proteolysis and degradation (Hochstrasser, 1995). The posttranslational modification process at the centre of the degradation process is called ubiquitination, which is performed by covalently linking ubiquitin to the protein to be degraded. The ubiquitin moiety targets the protein to the proteasome or the vacuole for degradation by a collection of proteases at those sites.

1.3.1 Ubiquitination

Ubiquitin is a 76 amino acid protein that is highly conserved throughout eukaryotes. Ubiquitination is the process by which the carboxyl group of the terminal glycine in ubiquitin is covalently linked to a lysine residue on a target protein. In some cases, polyubiquitination can occur, involving the formation of ubiquitin chains by the
subsequent addition of multiple ubiquitin molecules to an already target-bound ubiquitin (Chau et al., 1989). This polyubiquitin chain formation can occur at one of three lysine residues on the ubiquitin molecule, Lys29, Lys48 and Lys63, thus generating a variety of possible chain configurations (Arnason and Ellison, 1994).

The process of ubiquitination is carried out by the consecutive action of E1, E2 and E3 enzymes (Fig. 4). E1 represents the ubiquitin-activating enzyme, utilizing ATP to generate a high-energy thiolester bond between ubiquitin and its own cysteine residue. This activated ubiquitin is then transferred to a cysteine residue on an E2 ubiquitin conjugating enzyme. E2 subsequently catalyzes the transfer of ubiquitin to a lysine residue on a target protein by the bridging action of an E3 ubiquitin-protein ligase. E3 itself determines the specificity of the ubiquitin system for individual target proteins.

There are two distinct functional classes of E3s in S. cerevisiae: the ‘really interesting new gene’ (RING) or RING-like domain and the ‘homologous to the E6-AP carboxyl terminus’ (HECT) domain classes. The RING domain consists of a pair of zinc cations that interact with specific cysteine and histidine residues to form a zinc finger motif for participation in protein-protein interactions. The HECT domain, composed of a bilobal structure with a broad catalytic cleft, allows for protein-protein interactions with E2 and the subsequent formation of its own ubiquitin-thiolester bond and transfer of ubiquitin to the target protein, via conserved residues in the catalytic cleft (Huang et al., 1999).

The process of ubiquitination is reversed by the action of deubiquitinating enzymes (DUBs) that catalyze the removal of the ubiquitin moiety. This process allows
Figure 4. The mechanism of ubiquitination. The process of ubiquitination is carried out by the consecutive action of E1, E2 and E3 enzymes to covalently link ubiquitin (Ub) to a substrate. Ub-tagged substrates are targeted to the proteasome or vacuole for degradation. The Ub moiety is removed by the function of DUBs. Adapted from Ravid and Hochstrasser, 2008.
for increased regulation of the ubiquitination system, as it is necessary for the replenishment of the free ubiquitin pool; cells with decreased free ubiquitin display defects in ubiquitin-dependent proteolysis (Amerik et al., 1997).

Ubiquitination can affect a wide range of cellular functions such as nutrient transport, metabolism, cell cycle progression and transcription (Schork et al., 1995; Feldman et al., 1997; Horak and Wolf, 1997; Li and Johnson, 1997). Modification of a target protein by ubiquitination leads predominantly to the targeting of a protein for degradation, but it may also affect protein function, sorting and sub-cellular localization (Chen et al., 1996; Riezman et al., 1996; Ren et al., 2007).

1.3.2 Proteasomal degradation

The proteasome is a large, 26S protein complex with multicatalytic protease activity for the degradation of polyubiquitinated proteins (Seufert and Jentsch, 1992). It can be divided into two major subunits: the core particle (CP), or 20S particle, and the regulatory particle (RP), or 19S particle (Glickman and Coux, 2001). The barrel shape of the CP is formed by four stacked ring structures, with each ring composed of seven proteins. The CP exhibits chiastic symmetry ($\alpha\beta\beta\alpha$) such that the two outer and two inner rings are identical. The peptidolytic activity of the proteasome resides in the cavity between the two beta subunits of the CP (Groll et al., 1997) and comes in three distinct forms: a chymotryptic activity (cleaving after hydrophobic residues), a tryptic activity (cleaving after basic residues) and a post-acidic activity (Kisselev et al., 2006). Access to the catalytic core of the CP is possible only through small pores at either axial end; these
ends are therefore capped with the RP to regulate the entry of proteins into the CP (Groll et al., 2000).

When the CP becomes associated with two RP subunits it is referred to as the 26S proteasome. The RP itself can be divided into two sub-complexes: the base and the lid (Glickman et al., 1998a). The RP associates with the CP in such a way that its base is proximal to the alpha subunit of the CP and directly in line with the central pore. The base is composed of six ATPases with sequence similarity but distinct functions (Rubin et al., 1998). One well-understood ATPase, Rpt2p, functions in opening the pore to the catalytic core, thereby facilitating substrate entry (Kohler et al., 2001); another ATPase, Rpt5p, interacts with ubiquitin and is believed to function in polyubiquitinated substrate recognition (Lam et al., 2002). The exact function of the lid sub-complex is poorly understood; however, it shows sequence homology with two other cellular complexes: the COP9 signalosome and the translation initiation factor eIF3 (Glickman et al., 1998b). The lid sub-complex contains known protein domains that may help to elucidate its functional properties. One of these domains, the MPN domain (composed of Rpn8p and Rpn11p), is known to be associated with a deubiquitinating function (Maytal-Kivity et al., 2002; Verma et al., 2002; Yao and Cohen, 2002). Based on current understanding, then, the lid sub-complex is necessary for the degradation of ubiquitin-conjugated substrates, and it likely functions in recognition and removal of conjugated ubiquitin.

The proteasome is a complex and multi-functional cellular protease. It combines the catalytic activity in the center of the CP and the functional regulation of the RP to provide specific and controlled proteolytic activity. The entire process requires that the ubiquitin-conjugated substrate be first recognized by the components of the RP. The
ubiquitin moieties must then be removed for reuse and the gate to the CP opened for entry through the pore. The substrate is then unfolded to fit through the narrow pore of the CP and eventually degraded to small peptides by the catalytic activity of the three distinct proteases described above.

1.3.3 Vacuolar degradation

The vacuole contains a collection of lytic enzymes capable of degrading many cellular components (Wiemken et al., 1979). For the purpose of this review, the focus will be on the degradation of plasma membrane-bound nutrient transporters.

The plasma membrane provides a barrier between the external environment and the cytoplasm within a cell. This membrane allows a cell to maintain a hospitable and consistent internal environment even as the external environment constantly changes around it. While some small molecules can cross the plasma membrane by simple diffusion, the cell has had to develop mechanisms for the transport of requisite larger and less abundant molecules across this barrier. These mechanisms are evident in the nearly one hundred plasma membrane nutrient transporters of S. cerevisiae, which are highly adapted to transport nutrients ranging from sugars and amino acids to ions and metals into the cell. The expression of these transporters is tightly regulated to ensure that they are only transcribed when their substrate is available or needed. However, once they are present on the plasma membrane and their substrate becomes limited or is no longer required, the cell must have a mechanism to internalize and degrade them. This mechanism involves ubiquitination, endocytosis and proteolysis in the vacuole.
The only known signal for targeting a specific plasma membrane transporter for endocytosis is ubiquitination. The E3 responsible for the ubiquitination of these transporters is Rsp5p of the HECT family of ubiquitin ligases (Horak and Wolf, 1997; Krampe et al., 1998; Medintz et al., 1998; Springael and Andre, 1998). While Rsp5p does not directly interact with the target transporter, there are nevertheless a wide variety of adaptor proteins that facilitate this interaction. These adaptors, many of which are related to the mammalian arrestins, permit Rsp5p to recognize and ubiquitinate numerous unique targets under a wide range of physiological conditions (Sullivan et al., 2007; Lin et al., 2008).

Once the transporter has been ubiquitinated it is recognized by epsin/Eps15-like adaptors which contain ubiquitin-binding domains (Shih et al., 2002). Upon interaction and ubiquitin recognition, the transporter is sorted into invaginating endocytic vesicles; once these endocytic vesicles reach the membrane of the late endosome, the ubiquitinated cargo is sorted to the lumen as it develops into a multivesicular body (MVB). The sorting of cargo into the MVB pathway requires ubiquitin-bound cargo and is facilitated by the ESCRT (endosomal sorting complex required for transport) machinery (Katzmann et al., 2001).

The ESCRT system is composed of five multi-protein complexes, ESCRT-0, -I, -II, -III and Vps4p-Vta1p, all of which work in concert to sort the ubiquitinated cargo into the MVB vesicles. Three of the complexes, ESCRT-0, -I and –II have a ubiquitin binding domain that allows for recognition of ubiquitinated cargo (Shields et al., 2009). While being sorted to the lumen, the ubiquitin moieties are removed by the DUB Doa4p to be recycled back to the pool of free ubiquitin (Dupre and Haguenauer-Tsapis, 2001). This
MVB sorting is thought to be an irreversible event that is quickly followed by the fusion of the MVBs with the vacuolar membrane. Upon fusion, the membrane-bound protein cargo within the MVBs is released into the lumen of the vacuole where a number of lipases and proteinases complete the degradation (Jones et al., 1982; Teter et al., 2001).

1.4 FBPase regulation

When yeast grow on a non-fermentable carbon source, such as acetate, amino acids or ethanol, they are starved of glucose and must therefore generate it. The process of cellular glucose generation is referred to as gluconeogenesis. In this metabolic process, pyruvate is converted to glucose through the opposing reactions involved in glycolysis. Some enzymes involved in glycolysis are also involved in gluconeogenesis, but there are key regulatory steps which are unique to gluconeogenesis, and therefore require unique enzymes. Without strict regulation of these unique enzymes, glycolysis and gluconeogenesis would occur simultaneously resulting in a futile cycle that is lethal to the cell (Navas et al., 1993). For this reason, gluconeogenesis is highly regulated to ensure that it occurs only during conditions of insufficient glucose in the environment.

One widely studied metabolic enzyme regulated by carbon source is the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase). This key regulatory enzyme of gluconeogenesis catalyses the hydrolysis of a phosphate group from fructose-1,6-bisphosphate, to yield fructose-6-phosphate. In this role, FBPase is needed during glucose starvation and its function is detrimental to the cell when glucose is abundant in the environment. For this reason FBPase is highly regulated.
One form of FBPase regulation occurs at the transcription level, and involves transcriptional repression by Mig1p under glycolytic conditions (Mercado et al., 1991). The other form, catabolite inactivation, has two parts: the first part involves the rapid phosphorylation and inhibition of the enzyme in response to glucose addition; the second involves the proteolytic degradation of the enzyme in response to the same stimuli. The site of phosphorylation was determined to occur at the Ser11 residue by peptide mapping with radiolabeled $^{32}$P. The phosphorylation is performed by PKA and has been shown to reduce FBPase activity by 60% within 20 minutes of glucose addition (Rittenhouse et al., 1987). The rapid inactivation of enzyme activity by phosphorylation is independent of the targeting for proteolytic degradation (Hammerle et al., 1998). Such proteolytic degradation is an area of ongoing research centred around the site of degradation through the vacuolar pathway (Chiang and Schekman, 1991; Huang and Chiang, 1997) or the proteosomal pathway (Schork et al., 1994; Schork et al., 1995).

1.4.1 Vacuolar import and degradation pathway

The degradation of FBPase was first shown to be dependent on PEP4 (Chiang and Schekman, 1991). The PEP4 gene encodes a vacuolar aspartyl protease that has been found to activate other vacuole proteases (Ammerer et al., 1986). The deletion of this gene effectively disrupts the proteolytic function of the vacuole. The half-life of FBPase, upon switching from glucose starvation conditions to fresh glucose, was greatly increased in a pep4 strain, indicating that degradation occurs in the vacuole (Chiang and Schekman, 1991). An number of genes were discovered that arrest the vacuole-dependent degradation of FBPase: the so-called vacuolar import and degradation (VID) genes (Hoffman and Chiang, 1996). Further study into vid mutants showed that FBPase
sedimented in a high speed pellet consistent with FBPase associating with a small sub-cellular membrane structure. Analysis of this structure determined it to be a membrane-bound vesicle containing FBPase, roughly 30 – 40 nm in diameter, and distinct from the vacuole, peroxisomes, mitochondria, endoplasmic reticulum, Golgi, endosomes, and COPI and COPII vesicles (Huang and Chiang, 1997).

It has been proposed that the Vid pathway involves the following steps: the uptake and accumulation of FBPase into Vid vesicles, the trafficking of Vid vesicles from the cytosol to the vacuole, the fusion of Vid vesicles with the vacuole, the release of FBPase into the vacuole, and finally the proteolytic degradation of FBPase by vacuolar proteinases. Investigation of different \textit{VID} genes was performed to ascertain their roles in this trafficking from the cytosol to the vacuole (Chiang and Schekman, 1991; Huang and Chiang, 1997; Chiang and Chiang, 1998; Brown \textit{et al.}, 2002; Brown \textit{et al.}, 2003).

One member of the \textit{VID} family, \textit{VID22}, encodes an integral membrane protein that localizes to the plasma membrane. Deleting \textit{VID22} leads to an accumulation of FBPase in the cytosol—not in a proteinase K-protected environment—indicating that Vid22p plays a role in the transport of FBPase from the cytosol into Vid vesicles (Brown \textit{et al.} 2002). Vid22p function is indirect, though, and functions through the cytosolic protein cyclophilin A (Cpr1p). In a \textit{vid22} mutant, the reduced level of cytosolic Cpr1p blocks FBPase import into vesicles (Brown \textit{et al.}, 2001). Additional factors required for FBPase import into Vid vesicles are cytosolic ATP-binding proteins, specifically Ssa2p (Brown \textit{et al.}, 2000).
Vid vesicle trafficking requires small GTPases for effective transfer of FBPase-containing vesicles to the vacuole (Brown et al., 2003). GTPases are known to play roles in numerous cellular trafficking events, often those of vacuole fusion, by mediating tethering. Specifically, the GTPase Ypt7p is required for the proper function of Vid vesicles. The Vid vesicle trafficking events also require various SNARE proteins, including the vesicle SNAREs Nyv1p, Uti1p and Ykt6p and target SNARE protein Vam3p (Brown et al., 2003). These proteins assemble to form a SNARE complex and to promote fusion of membranes. These findings outline many similarities between Vid vesicle trafficking and heterotypic membrane fusion events (Brown et al., 2003).

Another gene, VID24, encodes a peripheral membrane protein that localizes to the outer membrane of Vid vesicles. Deletion of this gene results in accumulation of FBPase in Vid vesicles, but no delivery of these vesicles to the vacuole. It has been proposed that Vid24p plays a critical role in delivering FBPase from the Vid vesicles to the vacuole by mediating recognition, fusion or docking of Vid vesicles with the vacuole (Chiang and Chiang, 1998).

Little is known about the biogenesis of the Vid vesicles, with the exception that it requires the ubiquitin conjugating enzyme Ubc1p and is also independent of vacuole and proteosome function (Shieh et al. 2001).

1.4.2 Glucose-induced degradation pathway

The alternative site of FBPase degradation is at the 26S proteasome. Early research determined that the rate of FBPase degradation was unchanged in mutants defective in the proteolytic function of the vacuole (Schork et al., 1994). However, in
mutants defective in the proteolytic function of the proteasome, there was a highly reduced degradation of FBPase (Schork et al., 1994). This proteasomal degradation event requires polyubiquitination of FBPase and the function of the ubiquitin conjugating proteins Ubc1p, Ubc4p and Ubc5p (Schork et al., 1995).

A genome-wide screen identified a family of gene mutations that lead to defects in glucose induced-degradation (Gid) of FBPase (Hammerle et al., 1998). One such mutant, gid3, was found to stabilize FBPase under available glucose conditions by more than 30-fold (Schule et al., 2000). Gid3p is the previously described protein Ubc8p, another ubiquitin conjugating enzyme. Gid2p is required for the ubiquitination of FBPase under catabolite inactivation conditions is capable of ubiquitinating FBPase in vitro, functioning as an E3 ubiquitin ligase (Regelmann et al., 2003, Santt et al., 2008). The E3 function of Gid2p is complemented by the degenerative RING structure of Gid9p (Braun et al., 2011). Gid4p appears to function as an activator of FBPase degradation. When GID4 is artificially transcribed during gluconeogenic conditions it stimulates the degradation of FBPase (Santt et al., 2008).

1.4.3 Vid/Gid controversy

The ongoing debate over which pathway is responsible for the degradation of FBPase has seen some resolution. Recently, the genes encoding the proteins involved in both pathways have been shown to overlap in three instances. The proteins that are shared by both pathways are Vid30p/Gid1p, Vid24p/Gid4p and Vid28p/Gid5p (Regelmann et al., 2003). More importantly, Hung et al. (2004) found that after short term (1 day) glucose starvation, replenishing cells with glucose led to a non-vacuolar
degradation of FBPase, likely through the proteasome. In contrast, after long term (3 days) glucose starvation, replenishing cells with glucose led to a vacuolar degradation of FBPase.

1.4.4 Vid/Gid associated protein complexes

A systematic proteomics approach to identify protein-protein interactions led to the discovery of a large protein complex containing seven Vid and Gid proteins (Ho et al., 2002). This complex consists of Vid30p, Gid2p, Vid24p, Vid28p, Gid7p, Gid8p, Gid9p, and Moh1p (Ho et al., 2002). Of particular interest to our lab was the finding that Hxt7p, a high-affinity hexose transporter, was also recovered (Ho et al., 2002). Independently, Gid2p was identified as a member of a large cytoplasmic complex (approximately ~600 kDa), termed the Gid Protein Complex (GPC) (Regelmann et al., 2003), which was suggested to contain those Vid and Gid components identified by Ho et al. (2002). All the components of the GPC, except Moh1p, are needed for FBPase degradation in response to glucose replenishment (Chiang and Chiang, 1998; Brown et al., 2003; Regelmann et al., 2003). Consistent with these findings, a study by Pitre et al. (2006), used a Vid30-TAP tag to identify the Vid30p protein complex (Vid30c) containing all the interacting Vid and Gid proteins identified by Ho et al. (2002) except Gid7p and Moh1p, together with the uncharacterised protein Ydl176wp. Through the use of a protein-protein interaction prediction algorithm, Pitre et al. (2006) investigated the interactions between components of the Vid30c and proposed that the strongest interactions existed between Vid30p and Vid28p, which formed the core components of the proposed Vid30c.
The current view of the Vid30c is that it functions as a novel RING E3 ubiquitin ligase. The catalytic function requires the RING domains of Gid2p and Gid9p and is regulated by the presence of Gid4p (Regelmann et al., 2003; Santt et al., 2008; Braun et al., 2011).

1.5 **Hexose transporters**

Glucose is the preferred carbon source of *S. cerevisiae* and accordingly its transport into the cell is highly regulated. Transport is accomplished by a set of plasma membrane bound transport proteins called hexose transporters (Hxts) (Bisson et al., 1993; Kruckeberg, 1996). These transporters belong to the major facilitator superfamily (MFS) and transport glucose across the plasma membrane by passive, energy-independent facilitated diffusion (Marger and Saier, 1993). *S. cerevisiae* contains 20 genes encoding Hxts enabling it to adapt to a wide range of environmental glucose concentrations. How many of the 20 identified *HXT* genes actually encode functionally relevant proteins is unknown; however, it has been shown that only Hxts 1-7 are necessary for growth on glucose (Boles and Hollenberg, 1997).

1.5.1 **HXT transcriptional regulation**

Hxts are classified into two different categories: a glucose-induced, low-affinity system or a glucose-repressed, high-affinity system. The transcriptional regulation of these systems involves the combined effects of different high concentration (generally >2%) and low concentration (generally 0.1-0.5%) glucose signalling cascades (Fig. 5). Hxts 1, 2, 3 and 4 are categorized as low affinity Hxts and are expressed in response to varying glucose concentrations.
Figure 5. Regulation of hexose transporter gene transcription.  (A) *HXT3* transcription is maintained under high and low glucose concentrations by inactivation of Rgt1p.  (B) *HXT2/4* transcription is maintained under low glucose concentrations by inactivation of Rgt1p and repressed under high glucose concentrations by Mig1p.  (C) *HXT1* transcription is maintained under high glucose concentrations by inactivation of Rtg1p and further activated by an unknown activator.  See text for details.  Adapted from Ozcan and Johnson, 1999.
*HXT3* expression is controlled by the transcripational repressor Rgt1p (Fig. 5). In response to either high or low glucose conditions there is the generation of an intracellular signal via the glucose sensors Rgt2p and Snf3p, respectively (Ozcan *et al.*, 1996). Signalling through either sensor activates a component of the SCF-ubiquitin ligase complex, Grr1p, which is responsible for the ubiquitin-dependent degradation of the activators of Rgt1p, Std1p and Mth1p (Kim *et al.*, 2006). The degradation of Std1p and Mth1p results in the inactivation of Rgt1p and subsequent *HXT3* expression (Ozcan and Johnson, 1995). This combination of high and low glucose signalling maintains a high level of *HXT3* expression under a wide range of glucose concentrations, but represses expression when glucose is absent.

The expression of *HXT2* and *HXT4* is similarly controlled by the actions of Snf3p, Grr1p and Rgt1p, but also has a second level of regulation at high levels of glucose (Fig. 5). In the case of these two *HXTs*, their expression is repressed under high glucose conditions by the action of Mig1p (Ozcan and Johnson, 1996). When glucose levels are reduced, Snf1p is active and inactivates Mig1p. This combination of regulators results in expression of *HXT2* and *HXT4* under low glucose conditions and repression when glucose is high or absent.

In the case of *HXT1*, expression is controlled by the repressor Rgt1p and an as yet unknown activator (Fig. 5). In addition to the action of high glucose signalling through Rgt2p and the removal of Rgt1p repression, *HXT1* is also activated under high glucose conditions by an unknown activator. Although the activator has yet to be identified, this activation has been shown to be dependent on glucose transport and glucose
phosphorylation by the hexokinase Hxk2p, and Reg1p (Ozcan and Johnson, 1999). The combined action of these pathways maintains high $HXT1$ expression only under high glucose conditions and represses transcription when glucose is low or absent.

The expression of high-affinity glucose transporters is repressed in the presence of glucose and induced in its absence or the presence of non-fermentable carbon sources (Liang and Gaber, 1996). $HXT6$ and $HXT7$ appear to be recent gene duplications and only differ by 2 amino acids while sharing identical transcriptional regulation. Their expression is repressed by high levels of glucose via Mig1p, likely by a similar mechanism to $HXT2$ and $HXT4$ (Westholm et al., 2008). In addition, expression of $HXT6$ and $HXT7$ is induced by growth on glycerol and ethanol and induction further increases with growth on raffinose.

1.5.2 Hxt posttranslational regulation

Regulation of sugar transporters also occurs at the protein level via posttranslational modification to allow yeast cells to adapt to a sudden shift in environmental conditions. The most widely studied example is catabolite inactivation, which occurs for Hxt6p, Hxt7p, Gal2p and Mal62p. Catabolite inactivation is the process whereby the activity of these sugar transporters is depleted upon the addition of glucose to glucose-starved cells (similar to what is observable with FBPase). This process allows cells to inactivate and degrade those proteins which are no longer necessary under the prevailing environmental conditions. This event has been shown to depend on the ubiquitin ligase Rsp5p, endocytosis, and the function of vacuolar proteases (Horak and Wolf, 1997; Lucero and Lagunas, 1997; Krampe et al., 1998).
Detailed investigation has been undertaken to elucidate the mechanism of catabolite inactivation of Hxt6/7p. The inspiration for such investigation came from not only the knowledge that Gal2p and Mal62p are subjected to catabolite inactivation, but also initial findings showing a glucose-dependent turnover of Hxt6/7p. When cells grown in medium containing raffinose as the sole carbon source were switched to high concentrations of glucose, there was a reduction of 50% in Hxt6/7p levels. This degradation of Hxt6/7p requires the function of the vacuole but not the proteasome, as was found for Gal2p and Mal62p (Riballo et al., 1995; Horak and Wolf, 1997; Krampe et al., 1998).

As membrane proteins, hxts require a cellular mechanism for internalization and degradation. Therefore, turnover analysis was performed in end4ts, ren1 and act1-1ts mutants, each being defective in different steps of the endocytic pathway (Krampe et al., 1998). All mutants showed a high degree of stabilization of Hxt6/7p under catabolite inactivation conditions, indicating that the internalization of these transporters is through the endocytic pathway, as expected. To determine if ubiquitination is the signal for this internalization and degradation, rsp5 and doa4 mutants, defective in ubiquitin ligase and ubiquitin hydrolase respectively, were investigated. Both mutants show a reduced degradation of Hxt6/7p under catabolite inactivation conditions, which supported the hypothesis of ubiquitination signalling the internalization and degradation of these transporters (Krampe et al., 1998).

A proteomics-based approach used to identify ubiquitinated proteins on a global scale has found that Hxt7p was ubiquitinated at residues Lys40, Lys318 and Lys560. The same study also found Hxt6p to be ubiquitinated at Lys560 and Hxt5p at Lys61 (Peng et
Two other membrane-bound transporters show ubiquitination as a signal for degradation: Gal2p, which has been shown to be ubiquitinated in response to high glucose concentrations (Horak and Wolf, 1997), and Mal62p, whose degradation has also been shown to be dependent on Rsp5p and Doa4p (Lucero and Lagunas, 1997).

A unique turnover event occurs with the low-affinity transporter Hxt1p involving degradation in response to rapamycin treatment. Investigation has demonstrated that treatment of cells with rapamycin leads to the rapid internalization and degradation of Hxt1p. As with Hxt7p, the internalization and degradation of Hxt1p requires Rsp5p and endocytosis (Schmelzle et al., 2004).

While the underlying mechanism of the catabolite inactivation of several transporters is well understood, the signalling events that generate the response remain obscure. For example, both Grr1p and Reg1p, components of catabolite inactivation, are involved in the glucose-dependent turnover of Hxt6/7p, Gal2p and FBPase (Ozcan and Johnston, 1999; Horak et al., 2002). Still less is known about the TOR signalling components that operate in Hxt1p turnover in response to rapamycin treatment, except that neither Sit4p nor Npr1p are involved (Schmelzle et al., 2004).

The strict regulation of $HXT$ expression and protein stability allows cells to maintain only those transporters that possess the correct glucose affinity under the reigning environmental conditions, and permits them to rapidly adapt to any changes that may arise. Although great strides have been made in elucidating the mechanisms of regulation, numerous paths remain unexplored and must be investigated. Of particular importance to this study is to further what little investigation has been performed on the
conditional turnover of low-affinity Hxts and to better understand the signalling events that regulate Hxt stability in general.

1.6 Rationale for the research and thesis objectives

The Vid30c, and the individual components that comprise it, are of particular interest to our lab. More specifically, we aim to identify and define their roles in the nutrient adaptation of *S. cerevisiae*. Understanding the role of the Vid30c in yeast nutrient adaptation is of fundamental importance to cell biology, as a more concrete understanding of this process will increase our knowledge of how yeast survive such diverse environmental conditions. Additionally, this line of investigation may present possibilities for application to the wine fermentation industry, as it will further our understanding of Hxt regulation and nutrient signalling; two integral aspects of yeast adaptation during wine fermentation.

As described above, the focus of research into *VID* and *GID* genes has been to define their role in the glucose-dependent turnover of FBPase. Such research has outlined much of the signalling, mechanism and individual roles of some of these proteins. However, very little is known about some components of the Vid30c, specifically Vid28p, and no investigation has looked at a role for the Vid30c in Hxt turnover. Our lab is focused on exploring the possible involvement of the Vid30c in the aforementioned interaction of its members with Hxt7p in light of the turnover of Hxt7p occurring in a glucose-dependent manner. In addition, our investigation is broadly concerned with the involvement of the Vid30c in the nutrient-dependent turnover of Hxts in general. We hypothesize that the Vid30c is required for the degradation of Hxts,
particularly Hxt7p, during an adaptive response to changing environmental conditions. This investigation will include defining the conditions, signalling pathways involved and mechanisms of high- and low-affinity Hxt turnover. In summary, the objectives of this thesis are threefold: (i) to determine if the Vid30c is involved in Hxt turnover; (ii) to define the signalling components that regulate the Hxt turnover response; and (iii) to define the mechanism by which the Vid30c affects Hxt turnover.
2.0 Components of the Vid30c are needed for the rapamycin-induced degradation of the high-affinity hexose transporter Hxt7p in *Saccharomyces cerevisiae*

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2.1 Abstract

*Saccharomyces cerevisiae* adapts to changing nutrient conditions by regulating its genome-wide transcription profile and cell-wide protein complement in correlation with the reigning nutrient conditions. The Target Of Rapamycin (TOR) signalling pathway is one of the major control mechanisms within the cell that facilitates these changes. The transcription, intracellular trafficking and protein turnover of nutrient transporters, including the hexose transporter proteins (Hxts), are regulated in response to nutrient conditions. The Vid and Gid proteins facilitate the nutrient-dependent degradation of the gluconeogenic enzymes FBPase and Mdh2p when glucose-starved cells are replenished with glucose. Here we show that three members of the *VID* and *GID* gene families, *VID30/GID1*, *GID2*, and *VID28/GID5* are needed for the rapamycin or nitrogen starvation-induced degradation of the high-affinity hexose transporter Hxt7p. In addition, we provide evidence that the functions of several Vid and Gid proteins are in close relation to the TOR signalling pathway.
2.2 Introduction

*Saccharomyces cerevisiae* senses and adapts to its changing nutrient environment by altering its transcriptional profile and modifying the protein complement within the cell. For example, yeast cells are starved of glucose when grown with either ethanol or acetate as a carbon source. When these cells are replenished with glucose, gluconeogenic enzymes such as fructose-1,6-bisphosphatase (FBPase) and malate dehydrogenase (Mdh2p) are degraded (Horak and Wolf, 1997; Huang & Chiang, 1997; Chiang and Chiang, 1998). The degradation of FBPase can occur in the vacuole or the proteasome. Upon glucose replenishment of three day glucose-starved cells, FBPase is targeted to novel vacuolar import and degradation (Vid) vesicles, which in turn fuse with vacuoles to result in the vacuolar degradation of FBPase (Brown et al., 2003; Regelmann et al., 2003; Hung et al., 2004). A group of Vacuolar Import and Degradation (VID) genes, including *VID22*, *VID24*, *VID27*, *VID28*, and *VID30*, are needed for this function (Hoffman & Chiang, 1996; Huang & Chiang, 1997). Alternatively, upon glucose replenishment of one day glucose-starved cells, FBPase is ubiquitinated and targeted to proteasomes for degradation in a manner dependent on nine Glucose Induced Degradation (GID) genes (*GID1* to *GID9*) (Schork et al., 1995; Regelmann et al., 2003). Some *GID* and *VID* genes (*GID1/VID30*, *GID4/VID24* and *GID5/VID28*) encode the same protein product and therefore function in the proteasomal and vacuolar degradation of FBPase (Brown et al., 2003; Regelmann et al., 2003; Hung et al., 2004).

A systematic proteomics approach to identify protein-protein interactions in *S. cerevisiae* led to the identification of multiple *in vivo* protein complexes of which one complex contained seven Vid or Gid proteins (Ho et al., 2002). This complex, consisting
of Vid30p, Gid2p, Vid24p, Vid28p, Gid7p, Gid8p, Gid9p, and Moh1p, was recovered from GID7 and MOH1 FLAG-tagged strains (Ho et al., 2002). Independently, Regelmann et al. (2003) identified Gid2p as a member of a large cytoplasmic complex (approximately ~600 kDa), termed the Gid Protein Complex (GPC) and proposed that it contained those components identified in the systematic protein complex identification performed by Ho et al. (2002). All the components of the GPC, except Moh1p, are needed for FBPase degradation (Chiang & Chiang, 1998; Brown et al., 2003; Regelmann et al., 2003). Consistently, Vid30-TAP was used to identify the Vid30p protein complex (Vid30c) (Pitre et al., 2006), which contained all the interacting Vid and Gid proteins identified by Ho et al. (2002) with the exception of Gid7p and Moh1p, but included the uncharacterised protein Ydl176wp. Using a protein-protein interaction prediction algorithm to investigate interactions between components of the Vid30c, Pitre et al. (2006) proposed that the strongest interaction within the Vid30c existed between Vid30p and Vid28p to form the core components of the proposed Vid30c.

Several lines of evidence specific to individual components of the Vid30c indicate its involvement in the nutrient-related adaptation of S. cerevisiae. As stated, all the components of the Vid30c are needed for the degradation of FBPase when yeast cells adapt to glucose replenishment following glucose starvation (Chiang and Chiang, 1998; Brown et al., 2003; Regelmann et al., 2003). In addition, VID30 is needed for the effective transcription of nitrogen-regulated genes (van der Merwe et al., 2001), and Gid2p functions in the carbon-related ubiquitination and subsequent proteasomal degradation of FBPase (Regelmann et al., 2003). Also, Vid24p was identified as a peripheral membrane protein that participates in the fusion of Vid vesicles with the
vacuole to enable the degradation of FBPase (Chiang and Chiang, 1998; Brown et al., 2003). Very little is known about the specific role(s) of the remaining members of the Vid30c in nutrient-mediated adaptation of yeast.

Yeast cells use well-defined signalling pathways to respond to nutrient conditions. The Target Of Rapamycin (TOR) pathway controls a variety of cellular processes, including cell cycle progression, ribosome biogenesis and tRNA synthesis, translation, nitrogen- and carbon-regulated gene expression, and autophagy (Pedruzzi et al., 2000; Schmelzle and Hall, 2000; Raught et al., 2001; Pedruzzi et al., 2003; Lorberg and Hall, 2004; Powers et al., 2004; Roosen et al., 2005). Rich nutrient conditions activate the pivotal TOR kinases while nutrient limitation or treatment of yeast with the macrolide antibiotic rapamycin renders TOR inactive (Heitman et al., 1991). TOR regulates the activities of a series of intracellular phosphatases and kinases, such as PP2A, Sit4p and Npr1p, which in turn control a wide range of intracellular processes. For example, the nucleocytoplasmic translocation of many transcription factors, including the redundant stress response transcription factors Msn2/Msn4p, the activators Gln3p and Gat1p that are needed for nitrogen and carbon-related gene activation, and Rgt1/3p that activates the transcription of respiratory genes, are controlled by TOR (Cooper, 2002; Crespo et al., 2002; Powers et al., 2004). In addition, the ubiquitination and turnover of several nutrient-regulated permeases, including Gap1p, Tat2p, Hip1p, and Hxt1p, are controlled by TOR via Npr1p and the E3 ubiquitin ligase Rsp5p (Schmidt et al., 1998; Beck et al., 1999; De Craene et al., 2001).

Hexose transporters mediate the uptake of hexose sugars in *S. cerevisiae*. Low affinity hexose transporters, like Hxt1p and Hxt3p, are active when fermentable sugars
such as glucose and fructose are abundant, while high affinity transporters, such as Hxt6p and Hxt7p, are active when these sugars are scarce. The transcription of the HXT genes and the abundance of their protein products are regulated in response to the reigning nutrient conditions. The transcription of HXT7 is repressed by the presence of high concentrations of glucose and nitrogen starvation, but induced by non-fermentable carbon sources such as raffinose and ethanol (DeRisi et al., 1997; Gasch et al., 2000). Also, the presence of high concentrations of glucose and a limiting amount of nitrogen leads to the rapid internalization of Hxt7p from the plasma membrane and its targeting to the vacuole for degradation (Krampe et al., 1998; Krampe and Boles, 2002). Hxt7p contains three ubiquitination sites (Peng et al., 2003) and its internalization occurs in a mechanism dependent on Rsp5p, Doa4p and the endocytic pathway protein End4p (Krampe et al., 1998; Krampe and Boles, 2002). It therefore seems feasible to suggest that the endocytosis of Hxt7p occurs in an ubiquitin-dependent manner.

Hxt7p was identified as a binding partner of Gid7p (Ho et al., 2002). Also, the gid7Δ mutant has an increased ability to consume glucose in nitrogen-limited growth conditions (Gardner et al., 2005). At least one component of the Vid30c, Gid2p, is needed for the ubiquitination of a target protein, FBPase, and its subsequent degradation (Regelmann et al., 2003). We therefore hypothesized that the GPC/Vid30c or individual members of this complex are needed for the regulation of Hxt7p turnover in response to changing nutrient conditions. In this study we investigated the involvement of TOR in Hxt7p turnover and provide insight into the function of the GPC/Vid30c by analyzing the degradation of Hxt7p in cells lacking Vid28p, Vid30p, Gid2p, or Gid7p.
2.3 Material and methods

2.3.1 Strains and growth conditions

All the yeast strains used in this study are listed in Table 1. Yeast cells were grown in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose; 2% agar for solid media) for pre-culturing and transformation purposes and were always incubated at 30°C.

For growth analyses yeast cells were pre-cultured in YPD to exponential growth phase, washed with sterile water and cell densities adjusted to an initial OD$_{600nm}$ of 1.0. Ten-fold dilutions were transferred to solid YPD media containing either rapamycin (1 mg/mL in drug vehicle [90% Ethanol and 10% Tween-20]) at final concentrations of 20 or 200 ng/mL, or drug vehicle (DV) as a control. YPD + DV plates were incubated for three days and the YPD + rapamycin plates for four days.

Yeast strains used to monitor Hxt7-GFP localization and Hxt7-3HA degradation were pre-cultured in YPD to generate biomass. Cells were washed and transferred to synthetic media (0.17% (w/v) Yeast Nitrogen Base (YNB) without amino acids and ammonium sulphate) containing 3% (w/v) raffinose and 0.5% (w/v) ammonium sulphate (hereafter raffinose with ammonium). Amino acids were added to complement auxotrophic requirements. Following a four hour incubation to stimulate $HXT7$ expression, cultures were divided in two equal volumes. Samples were collected for either fluorescence microscopy or protein extraction (time zero). Rapamycin was added.

Table 1. Yeast strains used in this study.
<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>BY4742&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MAT&lt;sup&gt;a&lt;/sup&gt; his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
</tr>
<tr>
<td>BYvid30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>BY4742 VID30::KanMX4</td>
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<tr>
<td>BYgid2&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>BY4742 GLN3::KanMX4</td>
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<sup>a</sup>Open Biosystems

<sup>b</sup>This study
to one volume to a final concentration of 200 ng/mL and drug vehicle added to the other. Samples were subsequently collected at the indicated times and used for fluorescence microscopy or protein extraction.

The nutrient-dependent turnover of Hxt7-GFP was performed by shifting strains cultured in raffinose with ammonium media for four hours to YNB-based media containing either 5% glucose or 3% raffinose as carbon sources. In addition, these media either contained ammonium sulphate at 0.5% (w/v) (glucose with ammonium or raffinose with ammonium) as a nitrogen source or were devoid of ammonium sulphate (glucose without ammonium or raffinose without ammonium). Samples were collected for fluorescence microscopy prior to the shift (time zero) and at the indicated time intervals following the shift to fresh media.

The analysis of Hxt1-3HA degradation was performed by growing transformants of pTB380 (gift from Mike Hall) in glucose with ammonium media to exponential phase. Cells were washed and shifted to fresh glucose with ammonium media for four hours before rapamycin was added to a final concentration of 200 ng/mL. Samples for protein extraction were collected before (time zero) and three and six hours following rapamycin addition.

2.3.2 Strain construction

The chromosomally tagged strains used in this study were created using the PCR-based integrative transformation procedure previously described (Longtine et al., 1998). The primers used contained 100 nt at the 5’ ends homologous to the native chromosomal locus and 20 nt at the 3’ ends homologous to the specific plasmid used to amplify the
respective integration cassettes. For chromosomal tagging of HXT7, the forward and reverse primers were designed with homologous sequences upstream and downstream of the stop codon, respectively. Plasmids pFA6a-3HA-His3MX6 and pFA6a-GFP(S65T)-His3MX6 were used to generate the respective integration cassettes (Longtine et al., 1998). Following transformation, the correct integration events were verified by PCR.

The BYvid28 HXT7-GFP and BYgid2 HXT7-GFP strains were used to construct the BYvid28vid30 HXT7-GFP and BYgid2vid30 HXT7-GFP double mutants, respectively. The regions of the primers homologous to the yeast genome were 80 nt in length and were designed to replace the entire coding region of VID30 with the natMX6 cassette of pFA6a-natMX6 (Van Driessche et al., 2005). Using a similar approach, the BY4742 strain served as the parent strain to construct the BYure2 mutant. Homologous primers were designed to replace the URE2 gene with the kanMX6 cassette amplified from pFA6a-kanMX6 (Longtine et al., 1998).

2.3.3 RNA extraction and Northern analysis

Yeast strains were cultured in the indicated media prior to RNA extraction. The phenol-based RNA extraction procedure, electrophoresis and RNA transfer to nylon membrane were described previously (van der Merwe et al., 2001). A PCR DIG Probe Synthesis Kit (Roche) was used according to the manufacturer’s recommendations to generate Digoxigenin (DIG)-labelled probes with primers VID28F2 (TGGGAGACCAGTTGGCTAAG) and VID28R2 (AATGGATCAAACCACCAAGG) to detect VID28, ACT1F6 (ACCAACTGGGACGATATGGA) and ACT1R6 (TAATACGACTCAGTATAG GGCCACCAATCCACGGAGTA) to detect ACT1,
and GFP-FP (GAGAAGAACTTTTC ACTGGAG) and GFP-RP (TAGTTCATCCATGCCATGTG) to detect HXT7-GFP transcripts. Pre-hybridization was performed at 50 °C using 10 mL DIG Easy Hyb solution (Roche) for 30 minutes. Denatured probes were added to 5 mL DIG Easy Hyb solution and hybridized for 18 hours. Membranes were washed for 2×5 minutes in 2×SSC/0.1×SDS at room temperature and 2×15 minutes in 0.1×SSC/0.1×SDS at 50 °C. Membranes were blocked for 30 minutes, followed by a 30 minute incubation with anti-DIG antibodies (1:10,000 in blocking solution; Roche) and detected with CDP-Star (Roche) for 5 minutes. Membranes were exposed to autoradiography film for visualization.

2.3.4 Fluorescence microscopy

The monitoring of the subcellular localizations of Hxt7-GFP were performed by preparing slides directly from the indicated cell cultures followed by immediately analysis using the 100× objective lens of a Nikon Eclipse E600 microscope. Images were recorded using a Coolsnapfx monochrome CCD digital camera (Roper Scientific) and processed using Metamorph (Universal Imaging, Version 5.0).

FM4-64 staining was used to confirm Hxt7-GFP is internalized via the endocytic pathway. Wild type and vid28 mutant strains were incubated in raffinose with ammonium media and incubated for 210 minutes to induce HXT7 expression. Cells were concentrated (OD₆₀₀nm = 1.25) and FM4-64 (16 mM stock in DMSO) was added to a final concentration of 80 μM. Cells were incubated with FM4-64 for 15 minutes, harvested and washed, and resuspended in fresh raffinose and ammonium medium. Rapamycin was
added to a final concentration of 200 ng/mL followed by a six hour incubation after which cells were collected for analysis and imaging as described.

2.3.5 Protein extraction and Western blotting

Harvested cells were resuspended in lysis buffer (0.7 M Sorbitol, 50 mM Tris pH 7.5, 2 mM PMSF, 5 % SDS; plus protease inhibitor cocktail tablets, Roche), added to 0.3 g glass beads and vortexed for two minutes. Lysates were centrifuged at 13,000 rpm for 20 minutes to remove cell debris and the supernatants collected. Protein concentrations were determined using the DC Protein Assay (Biorad) according to the manufacturer’s recommendations. Equal amounts of protein were separated by SDS-PAGE using a 7.5% acrylamide gel and transferred to a nitrocellulose membrane for 1 hour. The ECL Detection kit (GE) was used to detect the Hxt7-3HA or Hxt1-3HA protein according to the manufacturer’s recommendations. Rabbit anti-hemagluttinin (HA) antibody (Sigma) was used as a primary antibody and donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (GE) as a secondary antibody. Equal amounts of protein in each lane were confirmed with rabbit anti-Aldehyde Dehydrogenase (ADH) (Rockland) primary antibody as previously described (Onodera and Ohsumi, 2004). Membranes were exposed to autoradiography film for visualization.

2.4 Results

2.4.1 Several gid and vid mutants have altered sensitivity to rapamycin

*VID30* is needed for the turnover of FBPase in response to a carbon shift (Hoffman and Chiang, 1996; Huang and Chiang, 1997; Regelmann *et al.*, 2003) and the
vid30Δ mutant is hypersensitive to rapamycin (van der Merwe et al., 2001). In combination, these lines of evidence suggested that the function(s) of the VID/GID genes were controlled by a nutrient-sensing pathway such as the TOR pathway. We consequently analyzed the growth of eleven mutants associated with either the GPC or Vid30c in the presence of increasing concentrations of rapamycin. Cells of the mutant (vid30Δ, gid2Δ, gid3Δ, vid24Δ, vid28Δ, gid6Δ to gid9Δ, ydl176wΔ and moh1Δ) and wild type strains were pre-cultured as described and transferred to YPD plates containing either the drug vehicle or rapamycin. We included the isogenic gln3Δ and ure2Δ mutant strains as controls as they are known to be rapamycin resistant and hypersensitive, respectively. We performed the experiment in the presence of 20, 50, 100 and 200 nM rapamycin, but only show the data of the lowest and highest rapamycin concentrations. Although the wild type and all the mutant strains showed normal growth in the presence of the drug vehicle, it was evident that seven of the mutant strains were hypersensitive to rapamycin treatment (Fig. 6). The vid30Δ, gid2Δ, gid3Δ, vid24Δ, vid28Δ, gid8Δ and gid9Δ mutants were all hypersensitive to rapamycin treatment as these strains showed a growth pattern more similar to that of the ure2Δ mutant than the wild type strain. In contrast, the gid6Δ, gid7Δ, moh1Δ and ydl176wΔ mutants showed a growth phenotype similar to the wild type strain. These growth characteristics were consistent at 20 and 200 nM rapamycin, indicating that the mutants were affected similarly by low and high concentrations of the drug. These findings suggest that all the VID/GID genes analysed, with the possible exception of GID6, GID7, MOH1 and YDL176w, have a function associated with the TOR signalling pathway.
Figure 6. Components of the Vid30c function in a TOR-dependent manner. BY4742 (WT) and the indicated deletion mutants were cultured in YPD to exponential growth phase (OD\textsubscript{600nm} ~ 0.5), harvested, washed, and ten-fold dilutions of OD\textsubscript{600nm} = 1.0 were prepared and spotted to YPD-based solid media containing either the drug vehicle (DV) or rapamycin (rap) at a final concentrations of 20 and 200 nM. Plates were incubated for three (DV) and four days (rapamycin).
It is known that the transcription of \textit{VID30} is regulated by TOR as rapamycin treatment increases its expression in YPD (van der Merwe et al., 2001). Similarly, rapamycin treatment of the wild type cells in YPD activated the transcription of \textit{GID2, GID3, GID7, GID8} and \textit{GID9} (Huang et al., 2004). Interestingly, the transcription of \textit{VID24} and \textit{GID6} were down-regulated in response to rapamycin treatment (Huang et al., 2004). No data could be found that described \textit{VID28} transcription in response to rapamycin treatment. The rapamycin hypersensitivity of the \textit{vid28\Delta} mutant (Fig. 6) along with the presence of two potential Msn2/Msn4p DNA-binding sites (STREs) in the \textit{VID28} promoter, prompted us to investigate the effect of rapamycin on \textit{VID28} expression. Wild type cells were cultured in YPD to exponential growth phase and subjected to rapamycin treatment. Samples were collected for RNA extraction directly prior to (time zero), and as indicated following rapamycin addition. Rapamycin treatment clearly induced the transcription of \textit{VID28} (Fig. 7). After 30 minutes of treatment, the expression of \textit{VID28} was induced and this level of expression remained consistent after 60 minutes and 90 minutes of rapamycin treatment. The increased expression of \textit{VID28} was therefore not transient. These lines of evidence indicate that the transcriptional regulation of \textit{VID28}, like many of the other \textit{VID/GID} genes, is controlled by the TOR pathway.
Figure 7. Transcription of *VID28* is regulated by TOR. BY4742 (wild type) was grown in YPD to exponential phase. Cells were collected for RNA extraction to represent time zero (0). Rapamycin (Rap) was added to the remaining culture to a final concentration of 200 ng/mL and incubated at 30°C with shaking. Samples were collected for RNA extractions after 30, 60 and 90 minutes as indicated. Membranes were probed for *VID28* and *ACT1* as internal control.
2.4.2 Rapamycin-induced Hxt7p turnover is dependent on Vid28p

Gid7p directly interacts with the high-affinity hexose transporter Hxt7p (Ho et al., 2002), which led to the hypothesis that \textit{GID7} is involved in Hxt7p turnover. We analysed the glucose utilization of wild type, \textit{vid28}\Delta, and \textit{gid7}\Delta strains grown in YPD media containing 20\% (w/v) glucose. Compared to the wild type, both mutant strains have increased abilities to consume glucose (data not shown). TOR is one of the major nutrient signalling pathways in yeast and \textit{vid28}\Delta is hypersensitive to rapamycin treatment. Combined, these observations suggested that Vid28p, or other members of the Vid30c, were involved in hexose transporter turnover.

We fused three copies of hemaglutinin-encoding DNA (3HA) or the \textit{GFP} gene to \textit{HXT7} in both the wild type and \textit{vid28}\Delta strains. These strains were pre-cultured in YPD to generate biomass. The expression of \textit{HXT7} is glucose-repressed and is therefore not activated in YPD. Cells were washed and shifted to fresh raffinose with ammonium media. Following a four hour incubation to induce \textit{HXT7} expression, the cells were treated with rapamycin (see Methods). Hxt7-GFP was highly expressed in raffinose with ammonium media and was clearly present in the plasma membranes of both the wild type and \textit{vid28}\Delta mutant strains (Fig. 8A, time zero). Rapamycin treatment of the wild type cells resulted in the internalization of Hxt7-GFP and ultimately its degradation with time. Loss of Hxt7-GFP from the plasma membrane, as seen in the decreased fluorescence of the plasma membrane, started after three hours and continued through six hours until its absence after 12 hours of rapamycin treatment (Fig. 8A). Hxt7-GFP localized to internal, vesicle-like structures reminiscent of multi-vesicular bodies (MVBs) (Nikko \textit{et al.}, 2003; Luhtala and Odorizzi, 2004). A stain of wild type and \textit{vid28}\Delta mutant cells with FM4-64,
Figure 8. Hxt7p internalization is rapamycin-induced and at least partially dependent on Vid28p. BY4742 *HXT7-GFP* (WT) and BYvid28 *HXT7-GFP* (*vid28Δ*) were cultured as described in the Methods. Cultures were treated with A) rapamycin at a final concentration of 200 ng/mL, or B) drug vehicle (90% ethanol + 10% Tween-20). Samples were collected at the indicated times and analysed using DIC and fluorescence microscopy. C) BY4742 *HXT7-GFP* (WT) and BYvid28 *HXT7-GFP* (*vid28Δ*) cells were incubated with FM4-64 followed by rapamycin addition and a six hour incubation period before cells were analyzed using DIC and fluorescence microscopy. The red colour indicates the FM4-64 stain and green the Hxt7-GFP. Co-localization is indicated by orange-yellow colour.
a dye used to identify the vacuole and components of the endocytic pathway, showed the co-localization of Hxt7-GFP and FM4-64 confirming these structures as components of the endocytic pathway (Fig. 8C).

In contrast to wild type cells treated with rapamycin, wild type cells treated with drug vehicle still showed fluorescence in the plasma membrane after 24 hours (Fig. 8B). Although the internalization of Hxt7p could be observed with the drug vehicle treatment alone as MVBs were formed, Hxt7-GFP was clearly present in the plasma membrane at each time point (Fig. 8B). In combination these data showed that the internalization and degradation of Hxt7-GFP is accelerated in the wild type strain in response to rapamycin treatment, indicating that TOR inactivation contributed to Hxt7p internalization and ultimately its turnover in the cell. Thus, active TOR can potentially serve to stabilize Hxt7p in the plasma membrane.

We analysed the vid28Δ HXT7-GFP strain in parallel and found Hxt7-GFP clearly remained associated with the plasma membrane for longer following rapamycin treatment. Internal structures started appearing after 6 hours of rapamycin treatment and fluorescence was still clearly present in the plasma membrane following 24 hours of rapamycin treatment (Fig. 8A). Analysis of the drug vehicle-treated strains showed similar fluorescence intensities in the plasma membranes of the wild type and vid28Δ strains after 12 hours of growth (Fig. 8B). Clear intracellular structures could be observed in the wild type strain after 18 and 24 hours of growth. These structures were largely absent from the vid28Δ strain (Fig. 8B). In combination these results show that Hxt7p is
partially stabilized in the plasma membrane in the \(vid28\Delta\) mutant in response to rapamycin treatment.

To confirm these data, we performed the same experiment with wild type and \(vid28\Delta\) strains containing \(HXT7\) chromosomally tagged with 3HA. Western analysis performed with these samples confirmed that the levels of Hxt7-3HA detected in the \(vid28\Delta\) mutant were higher than that of the wild type strain in response to rapamycin treatment (Fig. 9A). The amount of Hxt7-3HA detected in the wild type strain was abundant in the absence of rapamycin, but decreased after 6 hours and were very low after 12 hours of rapamycin treatment. In contrast, the \(vid28\Delta\) mutant still had clearly detectable levels of Hxt7-3HA present after 18 hours of treatment (Fig. 9A). The levels of Hxt7-3HA did therefore decrease in the mutant, but the turnover was markedly delayed in comparison to the wild type strain.

The persisting fluorescence of Hxt7-GFP in the plasma membrane of the \(vid28\Delta\) cells could be due to differences in the transcription of \(HXT7\)-GFP between the \(vid28\Delta\) mutant and wild type strains in response to rapamycin treatment. To investigate this possibility, wild type (\(VID28\ HXT7\)-GFP) and \(vid28\Delta\ HXT7\)-GFP strains were grown in raffinose with ammonium and RNA samples were collected prior to and 12 hours following rapamycin addition. Northern blot analysis showed that the levels of \(HXT7\)-GFP transcript were similar in wild type and \(vid28\Delta\) cells, both prior to and following rapamycin treatment (Fig. 9B). This data indicated that the transcription of \(HXT7\)-GFP was not regulated in a manner dependent on \(VID28\). Collectively, these data indicate that Vid28p plays a role in the internalization of Hxt7p in response to rapamycin treatment.
Figure 9. Degradation of Hxt7p is rapamycin-induced and at least partially dependent on Vid28p. A) BY4742 HXT7-3HA (WT) and BYvid28 HXT7-3HA (vid28Δ) strains were grown in raffinose with ammonium media followed by rapamycin addition. Samples were collected at the times indicated for protein extractions. Samples were analysed by Western blotting using anti-HA antibodies to detect Hxt7-3HA. Equal amounts of protein in each lane were with anti-ADH antibodies. B) BY4742 HXT7-GFP (WT) and BYvid28 HXT7-GFP (vid28Δ) were grown in raffinose with ammonium media followed by rapamycin addition. RNAs were extracted from samples collected prior to and 12 hours following rapamycin addition, and used for Northern analysis. A GFP probe was used to detect the transcripts of HXT7-GFP and the ACT1 probe was used as internal control.
2.4.3 Vid30p and Gid2p are involved in Hxt7p turnover

The clear involvement of Vid28p in rapamycin-induced Hxt7p internalization prompted us to investigate the roles of other Vid and Gid proteins in this process. We compared the internalization and degradation of Hxt7-GFP in the vid30Δ, gid2Δ and gid7Δ mutants to that of the wild type strain in response to rapamycin treatment. Hxt7-GFP internalization and turnover was delayed in the vid30Δ and gid2Δ strains, but not in the gid7Δ mutant. Following 12 hours of rapamycin treatment, Hxt7-GFP was still clearly associated with the plasma membranes of the vid30Δ and gid2Δ strains, but largely absent from the plasma membranes of the wild type and gid7Δ strains (Fig. 10). Western analysis of Hxt7-3HA degradation in the vid30Δ and gid2Δ strains showed the transporter was more stable in these mutant strains compared to the wild type (Fig. 11). In contrast, Hxt7-3HA was internalized and degraded similarly in both the wild type and gid7Δ strains (Fig. 11). Taken together these data indicate that in addition to Vid28p, Vid30p and Gid2p are also involved in the degradation of Hxt7p in response to rapamycin treatment.
Figure 10. Other components of the Vid30c are needed for the rapamycin-induced internalization of Hxt7p. BY4742 HXT7-GFP (WT), BYvid30 HXT7-GFP (vid30Δ), BYgid2 HXT7-GFP (gid2Δ), and BYgid7 HXT7-GFP (gid7Δ) were cultured as described in the methods. Following rapamycin addition, samples were collected at the indicated times and analysed by fluorescence microscopy.
Figure 11. Degradation of Hxt7p is at least partially dependent on Vid30p and Gid2p. A) BY4742 HXT7-3HA (WT) and BYvid30 HXT7-3HA (vid30Δ), BYgid2 HXT7-3HA (gid2Δ), and BYgid7 HXT7-3HA (gid7Δ) strains were grown in raffinose with ammonium media followed by rapamycin addition. Samples were collected at the times indicated for protein extractions and were analysed by Western blotting using anti-HA antibodies to detect Hxt7-3HA. Equal amounts of protein in each lane were confirmed with anti-ADH antibodies.
2.4.4 Core components of the Vid30c display partially overlapping function in Hxt7p internalization

We observed an increased stability of Hxt7p in both the vid28Δ and vid30Δ mutants. Our observations suggested that Vid28p had a slightly larger role in the turnover of Hxt7p than Vid30p as Hxt7p was more stable in the vid28Δ mutant than the vid30Δ mutant. Vid30p and Vid28p were proposed to be the core components of the Vid30c and could provide partial overlapping function to the complex. This potential overlapping function in the turnover of Hxt7-GFP was analysed using the vid28Δ, vid30Δ and vid28Δvid30Δ strains. Fluorescence microscopy clearly indicated the increased stability of Hxt7-GFP in the vid28Δvid30Δ double mutant compared to either of the two single mutants (Fig. 12). These observations suggest that the functions of Vid28p and Vid30p overlap in Hxt7p internalization and turnover.

Gid2p was needed for the ubiquitination of FBPase targeted for degradation (Regelmann et al., 2003). Also, Gid2p was predicted not to be part of the Vid30c core complex, but rather a member of the sub-complex due to its predicted weak interaction with the core components (Pitre et al., 2006). Analysis of Hxt7-GFP localization in the vid30Δ, gid2Δ and gid2Δvid30Δ mutants following rapamycin treatment clearly showed an increased stability of Hxt7-GFP in the double mutant versus either of the single mutants (Fig. 12). However, this increased stability was not as pronounced as that observed in the vid28Δvid30Δ double mutant. These observations indicate that at least three components of the Vid30c overlap functionally in the turnover of Hxt7p in response to TOR signalling.
Figure 12. Vid28p and Vid30p have partially overlapping function in the rapamycin-induced degradation of Hxt7p. BY4742 HXT7-GFP (WT), BYvid30 HXT7-GFP (vid30Δ), BYgid2 HXT7-GFP (gid2Δ), BYvid28 HXT7-GFP (vid28Δ), BYvid30gid2 HXT7-GFP (vid30Δgid2Δ), and BYvid28vid30 HXT7-GFP (vid28Δvid30Δ) were cultured as described in the methods. Following rapamycin addition, samples were collected at the indicated times and analysed by fluorescence microscopy.
2.4.5 Rapamycin-mediated degradation of Hxt1p is independent of the Vid30c

Hxt1p is degraded rapidly in response to rapamycin treatment (Schmelzle et al., 2004). To determine if both Hxt1p and Hxt7p were degraded in a Vid30c-dependent manner in response to rapamycin treatment, we transformed the wild type and vid30Δ, gid2Δ, vid28Δ and gid7Δ strains with pTB380 which contains HXT1 fused with 3HA at its 3’ end (Schmelzle et al., 2004). Proteins were extracted from transformants grown in glucose with ammonium media prior to and following rapamycin treatment. Similar levels of Hxt1p-3HA were detected in the wild type and respective vid/gidΔ mutant strains following three hours of rapamycin treatment (Fig. 13). These observations suggest that Hxt1p is degraded in a mechanism that is independent of Vid30c.
Figure 13. Components of the Vid30c are not needed for the rapamycin-induced degradation of Hxt1p. BY4742 (WT), BYvid30 (vid30Δ), BYgid2 (gid2Δ), BYvid28 (vid28Δ), and BYgid7 (gid7Δ) were transformed with pTB380 (HXT1-3HA). Transformants were grown in synthetic glucose with ammonium media to exponential phase and samples were collected for protein extraction prior to (time zero) and 3 hours after rapamycin treatment. Protein samples were subjected to Western analyses and probed with anti-HA antibodies to detect Hxt1-3HA. Equal amounts of protein in each lane were confirmed with anti-ADH antibodies.
2.4.6 Vid30c function in the nutrient-related turnover of Hxt7p

The rapamycin treatment of yeast cells is known to simulate nutrient starvation (Heitman et al., 1991). The presence of high concentrations of glucose combined with nitrogen starvation resulted in the rapid degradation of Hxt7p (Krampe et al., 1998; Krampe and Boles, 2002). These findings, in combination with our results showing that components of the Vid30c are needed for the rapamycin-induced internalization of Hxt7-GFP, implied a role for the Vid30c in the nutrient-dependent internalization and degradation of Hxt7p.

We cultured wild type \(HXT7\)-GFP and \(vid28\Delta vid30\Delta\) \(HXT7\)-GFP strains in raffinose with ammonium media to induce Hxt7-GFP expression and shifted these cells to abundant glucose with or without ammonium sulphate and abundant raffinose with or without ammonium sulphate media. Samples were collected at six hour intervals following the respective media shifts and the internalization of Hxt7-GFP was monitored by fluorescence microscopy. When cells were shifted to glucose-based media, with or without ammonium sulphate, Hxt7-GFP was rapidly internalized from the plasma membranes and degraded at similar rates in both the \(vid28\Delta vid30\Delta\) and wild type strains (data not shown).

We next investigated the link between nitrogen availability and the stability of Hxt7-GFP in the plasma membrane by using raffinose as a carbon source to prevent glucose-mediated regulation of Hxt7p. When wild type \(HXT7\)-GFP and \(vid28\Delta vid30\Delta\) \(HXT7\)-GFP cells were grown in raffinose with ammonium media (time zero), we found a comparable presence of Hxt7-GFP in the plasma membranes of both wild type and
vid28Δvid30Δ double mutant strains (Fig. 14A). Following a shift of these cells into fresh raffinose with ammonium media, the fluorescence intensities remained similar for the first 12 hours followed by a slow but gradually decrease in intensity as internal structures became visible. This observation is similar to that seen in the drug-vehicle treated cells (Fig. 8B) and was thought to be a result of nitrogen depletion. We investigated this possibility by analysing the presence of ammonia in the growth media at times zero, 6, 12 and 24 hours and found significant levels (1.14±0.03 g/L) of ammonia were still present in the media after 24 hours of growth. The reason for this gradual loss Hxt7p from the plasma membrane is therefore not depletion of ammonia.

In contrast, Hxt7-GFP was less stable in the plasma membrane of the wild type strain when cells were shifted to raffinose without ammonium media. Internalization of Hxt7-GFP started occurring within 6 hours post shift with a concurrent decrease in fluorescence from the plasma membrane (Fig. 14B). The vid28Δvid30Δ double mutant, however, showed a delayed turnover of Hxt7-GFP in these conditions. Hxt7-GFP was still clearly present at high levels in the plasma membrane of the vid28Δvid30Δ double mutant after 12 hours in the raffinose without ammonium media. In addition, 24 hours post shift Hxt7-GFP was still present in the plasma membrane of the vid28Δvid30Δ cells while it was not detectable in the wild type strain. These results mimic the rapamycin-induced internalization of Hxt7p (compare Fig. 12 and 14B). Rapamycin treatment might therefore mimic nitrogen starvation-induced Hxt7p turnover. In combination, these findings suggest that the nitrogen-mediated internalization and ultimate degradation of Hxt7p is dependent on Vid30c.
Figure 14. Nutrient-dependent internalization of Hxt7p is at least partially dependent on **VID28**. BY4742 *HXT7-GFP* (WT) and BY*vid28vid30* *HXT7-GFP* (*vid28Δvid30Δ*) were grown in raffinose with ammonium to exponential phase, harvested, washed and shifted to A) raffinose with ammonium, and B) raffinose without ammonium media. Following the nutrient shift, samples were collected at the times indicated and analysed by fluorescence microscopy.
2.5 Discussion

The roles of individual components of the GPC/Vid30c in carbon-related protein turnover is known (Brown et al., 2003; Regelmann et al., 2003; Hung et al., 2004). Nonetheless, the nutrient-related roles of the \( VID \) and \( GID \) genes are not necessarily restricted to adaptation to preferred carbon sources. To this end, \( VID30 \) is needed for nitrogen-regulated activation of gene expression, is transcriptionally regulated by TOR and the \( vid30\Delta \) mutant is hypersensitive to rapamycin treatment (van der Merwe et al., 2001). Here we expand our understanding of the nutrient response in yeast by providing evidence that the functions of the \( VID/GID \) genes are related to the TOR signalling pathway. We investigated the involvement of the TOR signalling pathway in the regulation of Hxt7p presence in the plasma membrane and found rapamycin treatment induced the internalization and degradation of this high-affinity hexose transporter. Linking the TOR signalling pathway to the regulation of Hxt7p is consistent with the known role of TOR in the turnover of other nutrient permeases, including Gap1p, Tat2p and Hip1p (Schmidt et al., 1998; Beck et al., 1999; De Craene et al., 2001; Schmelzle et al., 2004).

We show that at least three components of the Vid30c, Vid30p, Gid2p, and Vid28p, were needed for the efficient internalization of Hxt7p in response to rapamycin treatment. To our knowledge, this is the first report in which components of the Vid30c have been linked to the internalization and degradation of a plasma membrane protein. The colocalization of Hxt7-GFP and FM4-64 indicates that the Vid and Gid proteins not only function in the turnover of proteins via the Vid or proteasomal pathways as previously described (Brown et al., 2003; Regelmann et al., 2003; Hung et al., 2004), but
also have a role in protein turnover via the endocytic pathway. In combination, these observations predict a role for the Vid30c that is pivotal to a variety of protein turnover mechanisms within the cell.

Vid28p and Vid30p were proposed to be the core components of the Vid30c, while Gid2p was suggested to form part of a subcomplex of the Vid30c that interacts weakly with the core complex (Pitre et al., 2006). The loss of Vid28p function delayed Hxt7p turnover more severely than the loss of either Vid30p or Gid2p. A double mutant lacking both VID28 and VID30 showed significant delay in Hxt7p internalization, indicating that these two proteins have some degree of functional overlap. These data support the proposed model in which all the components of the Vid30c interact with both Vid28p and Vid30p (Pitre et al., 2006). Thus, deleting either VID28 or VID30 could still support the existence of a complex containing most of the components of the Vid30c. However, the vid28Δvid30Δ double mutant should prevent the formation of the Vid30c. It is important to note that turnover of Hxt7p still occurred in the vid28Δvid30Δ double mutant, albeit at a significantly delayed rate. This indicates that either the remaining components of the Vid30c can still support the degradation observed in the vid28Δvid30Δ double mutant, or another pathway that functions in parallel to the Vid30c still enables the internalization and degradation of Hxt7p.

Gid2p has previously been shown to be involved in the ubiquitination of an enzyme targeted for degradation following a nutrient shift (Regelmann et al., 2003). Hxt7p contains multiple ubiquitination sites and is degraded in response to either glucose replenishment or nitrogen starvation or both. Our findings show a prolonged presence of Hxt7p in the plasma membrane following rapamycin treatment in cells lacking Gid2p,
but internalization and degradation of Hx7p did occur. The delayed turnover was prolonged in cells lacking both Gid2p and Vid30p, further supporting the model that individual components of the Vid30c function in concert to mediate the turnover of Hx7p.

The internalization of Hxt7-GFP in the **vid28Δ** (Fig. 3A and B) and **vid28Δvid30Δ** mutants (Fig. 12) revealed that the intracellular trafficking of Hxt7-GFP to the vacuole might be affected by the Vid30c. Similar to the wild type strain, these mutant strains have clear intracellular structures, possibly MVBs, visible after 12 hours. However, these structures are not as obvious in the mutant strains, which also show a concomitant increase in fluorescence in the plasma membranes. This effect is more pronounced in the **vid28Δvid30Δ** double mutant than the **vid28Δ** single mutant. These observations suggest that the targeting of Hxt7-GFP from the MVB to the vacuole might be dependent on the Vid30c. The increase in Hxt7-GFP fluorescence on the plasma membrane at later time points in the mutants, particularly in the **vid28Δvid30Δ** double mutant, may be a result of recycling of endocytosed Hxt7-GFP back to the plasma membrane. Alternatively it is possible that there is a small amount of Hxt7-GFP being translated at later time points and its trafficking differs between the wild type and mutant strains. In the **vid28Δvid30Δ** double mutant newly translated Hxt7-GFP may be trafficked to the plasma membrane; whereas newly translated Hxt7-GFP is trafficked to the vacuole for degradation in the wild type. The potential for a low level of translation of Hxt7-GFP at later time points is supported by the low, but detectable levels of **HXT7** RNA present at 12 hours after rapamycin treatment (Fig. 9B). Understanding this phenomenon will require further investigation.
Gardner et al. (2005) showed that a gid7Δ mutant ferments glucose and fructose more effectively than a wild type strain in nitrogen-limited broths. The Gid7p-Hxt7p interaction provided a potential explanation for the increased sugar utilization and suggested Gid7p could be involved in the nutrient-dependent degradation of Hxt7p. Our findings are not consistent with this model, showing that TOR-regulated Hxt7p turnover was not dependent on Gid7p. The specific function of Gid7p remains unclear.

In contrast to HXT7, the low affinity transporter HXT1 is highly expressed in the presence of glucose (DeRisi et al., 1997; Gasch et al., 2000). Upon rapamycin treatment, Hxt1p is degraded in a mechanism dependent on End4p (a component of the endocytic pathway) and Rsp5p (an E3 ubiquitin ligase) (Schmelzle et al., 2004), both of which have been shown to be involved in Hxt7p degradation (Krampe et al., 1998; Krampe & Boles, 2002). We tested the stability of Hxt1p in the vid/gid mutants after 3 hours of rapamycin treatment (Fig. 13) and found Hxt1p degradation occurred similarly in the mutant and wild type strains. Although Hxt7p and Hxt1p are needed in the plasma membranes of yeast cells in different carbon conditions, the internalization and degradation of both these permeases are regulated by the TOR pathway and require the proper functioning of similar components of cellular ubiquitination and endocytosis machineries. It is anticipated that the signalling mechanisms downstream of TOR resulting in these two endocytic events would be different. Here we show that components of the Vid30c are needed for the TOR-mediated degradation of Hxt7p, but not for Hxt1p degradation.

Our investigation of the nitrogen starvation-dependent internalization and degradation of Hxt7p clearly showed that the Vid30c is needed for this process to occur in the absence of glucose. Hxt7p remained present at similar levels in the plasma
membranes of both wild type and vid28Δvid30Δ cells grown in raffinose with ammonia media, indicating that the signalling cascade needed for the activation of Hxt7p internalization was not activated in these growth conditions. However, Hxt7p endocytosis was activated in raffinose media lacking nitrogen and this event was dependent on Vid28p and Vid30p. Interestingly, we could not find any impact of Vid28p and Vid30p on the internalization of Hxt7p in abundant glucose conditions, irrespective of nitrogen availability. The combination of these two nutrient conditions, high glucose and nitrogen starvation, leads to the rapid degradation of Hxt7p and appears to occur independent of the Vid30c. These findings suggest that Hxt7p degradation occurs in both Vid30c-dependent and Vid30c-independent mechanisms. The precise signalling mechanism that governs the nitrogen-induced Vid30c-dependent internalization of Hxt7p is currently unclear.

The precise molecular mechanism by which Vid28p, and ultimately the Vid30c, function in the TOR-mediated internalization of Hxt7p is currently unclear. There are multiple stages in the process of Hxt7p internalization and degradation in response to nutrient conditions where Vid28p and other components of the Vid30c could potentially exert its function. These include i) regulating the activities of specific phosphatase and kinases, potentially Sit4p or Npr1p, in response to nutrient signals; ii) activating components of the ubiquitination machinery, potentially Rsp5p, to enable the ubiquitination of Hxt7p; iii) initiating the endocytosis of Hxt7p; iv) trafficking Hxt7p to the vacuole. Elucidating the precise molecular function of the Vid30c will increase the understanding of nutrient adaptation of *S. cerevisiae.*
2.6 Acknowledgements

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3.0 The regulation of hexose transporter turnover converges on the Vid30c and requires inactivation of the Ras/cAMP/PKA pathway in *Saccharomyces cerevisiae*

3.1 Abstract

The expression and turnover of hexose transporters are controlled by a variety of nutrient-related signals in yeast. The low-affinity hexose transporter Hxt3p is highly expressed and localizes to the plasma membrane during growth in abundant glucose where it plays a major role in the transport of this sugar. However, following a shift to ethanol as a sole carbon source, Hxt3p is endocytosed and targeted to the vacuole for degradation while its expression is also repressed. In contrast, the high-affinity hexose transporter Hxt7p is actively expressed and functional in the plasma membrane when glucose is limiting and nitrogen is abundant. Upon nitrogen starvation or rapamycin treatment, *HXT7* transcription decreases and the protein is targeted for degradation. The mechanisms that govern these regulatory steps are poorly understood. Here we show that the signals for the ethanol-induced turnover of Hxt3p and the rapamycin-induced turnover of Hxt7p converge on the Vid30 complex which, via the Ras/cAMP/PKA pathway, ultimately controls the degradation of both these hexose transporters.

3.2 Introduction

The Target Of Rapamycin (TOR) and Ras/cAMP/Protein Kinase A (PKA) signalling pathways enable *Saccharomyces cerevisiae* to respond to nutrient availability and stress (Cameron *et al.*, 1988; Barbet *et al.*, 1996; Smith *et al.* 1998; Longo *et al.*, 1998).
Two Tor kinases, Tor1p and Tor2p, are pivotal proteins in the TORC1 signalling cascade that has wide-ranging effects in the cell. Rich nutrient conditions activate the TORC1 pathway to promote cell cycle progression and protein synthesis, while preventing autophagy and the expression of stress response genes (Barbet et al., 1996; Powers and Walter, 1999; Kamada et al., 2000). By contrast, the TORC1 is inactivated by nutrient starvation or rapamycin treatment resulting in cell cycle arrest, a decrease in protein synthesis, and the increased expression of stress response and nitrogen-regulated genes (Beck and Hall, 1999). Similarly, the Ras/cAMP/PKA pathway also antagonizes the stress response and promotes cell proliferation in the absence of stress and the presence of abundant glucose (Mendenhall et al., 1998; Longo et al., 2003). Glucose limitation or cell stress inactivate this pathway leading to cell cycle arrest, the synthesis of complex carbohydrates, the activation of stress response genes, and the derepression of glucose-repressed genes (Cameroni et al., 2004). Interestingly, these two distinct pathways show a level of cross communication as TOR signalling has been shown to converge on the cAMP/PKA pathway (Pedruzzi et al., 2003; Schmelzle et al., 2004).

The activity of PKA is controlled by intracellular cAMP (Hixson and Krebs, 1980). In the presence of glucose, the two redundant small G proteins Ras1p and Ras2p are activated via the guanine exchange factors Cdc25p and Sdc25p (Broek et al., 1987; Haney 1994). Cdc25p is an essential gene, whereas Sdc25p is dispensable (Boy-Marcotte et al., 1996; Lolch-Mallol et al., 2004). It would appear that Cdc25p is the determinant guanine exchange factor in Ras activation; however, overexpression of SDC25 rescues growth of a cdc25Δ deletion strain, indicating that Sdc25p has adequate functionality (Boy-Marcotte et al., 1989). Active Ras1/2p in turn activates the adenylyl cyclase,
Cyr1p, to produce cAMP (Matsumoto et al., 1982). The production of cAMP activates PKA by releasing it from its inhibitory interaction with the regulatory subunit Bcy1p (Hixson and Krebs, 1980). The activity of Ras1/2p is negatively modulated by the GTPase activating proteins Ira1p and Ira2p (Tanaka et al., 1990, 1991), while the intracellular level of cAMP is decreased by the phosphodiesterases Pde1p and Pde2p (Nikawa et al., 1987; Wilson and Tatchell, 1988). Active PKA prevents cell cycle arrest, post-diauxic shift gene expression and glycogen accumulation by phosphorylating and inactivating Rim15p, a kinase essential for the activation of these processes (Pedruzzi et al., 2003; Cameroni et al., 2004). Conversely, in the absence of glucose or in response to stress, the decrease in cAMP allows for Bcy1p to bind and inactivate PKA, resulting in the activation of Rim15p (Schmelzle et al., 2004).

Hexose transporters (Hxts) are regulated at the transcriptional and posttranslational levels to allow yeast to adapt to varying sugar concentrations in the environment. If conditions become unfavorable for the expression of a specific HXT gene, the cell must repress its transcription and degrade the remaining protein. This degradation occurs via endocytosis and proteolysis in the vacuole. For example, HXT7 encodes a high-affinity hxt, whose transcription is induced by low levels of glucose or a non-fermentable carbon source, and Hxt7p localizes to the plasma membrane (Liang and Gaber, 1996; Snowdon et al., 2008). However, in response to glucose abundance, nitrogen starvation or rapamycin treatment, HXT7 transcription is repressed and Hxt7p is degraded (Krampe et al., 2002; Snowdon et al., 2008). By contrast, HXT3 encodes a low-affinity hxt that is actively expressed in glucose abundance, but repressed (Roberts et al., 2006) and the gene product degraded when only a non-fermentable carbon source like
ethanol is supplied (Snowdon et al., 2009). Despite much research into the turnover of Hxts, the mechanisms that govern these processes are not fully understood.

The Vid/Gid proteins play an important role in yeast adaptation to different nutrient conditions. These proteins assemble into a multi-component complex termed the Vid30 complex (Vid30c) (Pitre et al., 2006) and have a role in the degradation of FBPase and Mdh2p following the transition from gluconeogenic to glycolytic growth conditions (Regelmann et al., 2003; Hung et al., 2004). At least three of these proteins, Vid30p, Gid2p and Vid28p, are needed for the turnover of Hxt7p upon rapamycin treatment or nitrogen starvation. Additionally, the growth of several vid/gid mutants is sensitive to the presence of rapamycin in the medium (Snowdon et al., 2008) and the transcription of the VID/GID genes increases in the presence of non-fermentable carbon sources (Roberts et al., 2006). The function(s) of the Vid30c therefore seems to correlate with growth on poor carbon and nitrogen sources.

In this study we further investigate the link between the Vid30c and the regulatory mechanisms that govern Hxt turnover. Here we show that Vid28p functions upstream of Ras2p and mediates the Rim15p-dependent, ethanol- and rapamycin-induced degradation of Hxt3p and Hxt7p, respectively.

3.3 Materials and methods

3.3.1 Strains and growth conditions

All the yeast strains used in this study are listed in Table 2. Yeast cells were grown in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose) or synthetic
Table 2. Yeast strains used in this study.

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<sup>a</sup>Open Biosystems

<sup>b</sup>This study
drop out media [0.17% Yeast Nutrient Base without amino acids and ammonium sulphate, 2% (w/v) glucose, 0.5% (w/v) ammonium sulphate and CSM (-URA 0.77g/L, -LEU 0.69g/L or -MET 0.75g/L, MP Biomedicals)] for pre-culturing and transformation purposes and were incubated at 30°C, unless otherwise stated.

Yeast strains used to monitor Hxt3p-GFP and Cdc25p-GFP localization and degradation were pre-cultured in YPD to generate biomass. Cells were washed and transferred to glucose media [0.17% (w/v) Yeast Nitrogen Base without amino acids and ammonium sulphate, 2.5% (w/v) glucose, 0.5% (w/v) ammonium sulphate and CSM (-URA 0.77g/L, -LEU 0.69g/L or -MET 0.75g/L, MP Biomedicals)]. Amino acids were added to complement auxotrophic requirements and 100 µM CuSO4 was added to stimulate $CDC25$ expression from the $CUP1$ promoter. Following a three hour incubation to stimulate $HXT3$ or $CDC25$ expression, cells were imaged using fluorescence microscopy or harvested for protein extraction (time zero). The remaining culture was washed and transferred to ethanol media [0.17% (w/v) Yeast Nitrogen Base without amino acids and ammonium sulphate, 2% (v/v) ethanol, 0.5% (w/v) ammonium sulphate and CSM (-URA 0.77g/L, -LEU 0.69g/L, MP Biomedicals)]. Amino acids were added to complement auxotrophic requirements and/or suppress expression from the $MET25$ promoter. Samples were subsequently collected at the indicated times and used for fluorescence microscopy or protein extraction. In the case of Cdc25-GFP localization studies, 50 ml of culture was concentrated down to 500 µl and treated with 1 µl of DAPI (1 mg/ml) for ten minutes before imaging.
Yeast strains used to monitor GFP-Hxt7 localization and degradation were cultured as described in Snowdon et al., (2008) with the following exceptions. 100 µM CuSO₄ was added during the four hour incubation to stimulate HXT7 expression from the CUP1 promoter. After cells were extracted at time zero, the remaining cells were washed twice with sterile water and re-suspended in fresh raffinose with ammonium media.

### 3.3.2 Strain construction

The chromosomally tagged strains used in this study were created using the PCR-based integrative transformation procedure previously described (Longtine et al., 1998). The primers used contained 75-100 nt at the 5’ ends homologous to the native chromosomal locus and 20 nt at the 3’ ends homologous to the specific plasmid used to amplify the respective integration cassettes. For chromosomal tagging of HXT3 and CDC25, the forward and reverse primers were designed with homologous sequences upstream and downstream of the respective stop codons. Plasmid pFA6a-GFP(S65T)-His3MX6 was used to generate the respective integration cassettes (Longtine et al., 1998). For HXT3 promoter replacement, the forward and reverse primers were designed with homologous sequences upstream and downstream of the start codon, respectively, and pYM-N35 (natNT2::MET25p) was used as the template to generate the integration cassette (Janke et al., 2004). A similar approach was used for CDC25 promoter replacement, except pYM-N2 (natNT2::CUP1p) was used as the template to generate the integration cassette (Janke et al., 2004). For chromosomal tagging and promoter replacement of HXT7, the forward and reverse primers were designed with homologous sequences upstream and downstream of the start codon, respectively. pYM-N4
(natNT2::CUP1p-GFP) was used to generate the respective integration cassette (Janke et al., 2004).

Disruption of the VID30 gene was performed by using 80 nt primers with regions homologous to the yeast genome and were designed to replace the entire coding region of VID30 with the bleMX6 cassette of pFA6a-bleMX6 (Van Driessche et al., 2005).

Overexpression of VID28 was accomplished by the replacement of the native promoter with the PGK1 promoter. For VID28 promoter replacement, the forward and reverse primers were designed with homologous sequences upstream and downstream of the start codon, respectively. Plasmid pCW1 (Christopher Walkey; UBC) was used to generate the respective integration cassette. Following transformation, the correct integration events were verified by PCR.

Hexose transporter analysis in response to RIM15 overexpression was performed using the indicated strains transformed with pVW904 (2µ LEU2 TDH3p-RIM15-myc13; (Wanke et al., 2005)) and YEplac181 (2µ LEU2) as the control. For the Ras2p work, plasmids YCp50 (Control; CEN URA3), YCp50-RAS2 and YCp50-RAS2Val19 were transformed into the indicated strains to observe the effects of expression of a constitutively active RASVal19 allele.

The generation of BYtor1-l was performed as described by Cruz et al., 2001. Transformants were selected based on their resistance to rapamycin and the proper point mutation was confirmed by sequencing.
3.3.3 RNA extraction and Northern analysis

The extraction of RNA and analysis by Northern blotting was performed as outlined in 2.3.3.

3.3.4 Fluorescence microscopy

The monitoring of the subcellular localizations of GFP-tagged proteins was performed as outlined in 2.3.4.

3.3.4 Protein extraction and Western blotting

Harvested cells were resuspended in lysis buffer (1% NP40, 0.25% deoxycholate, 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM PMSF; plus protease inhibitor cocktail tablets, Roche), added to 0.3 g glass beads and vortexed for two minutes. Lysates were centrifuged at 5,000 rpm for 3 minutes to remove cell debris and the supernatants collected. Protein concentrations were determined using the DC Protein Assay (Biorad) according to the manufacturer’s recommendations. Equal amounts of protein were separated by SDS-PAGE using a 7.5% or 10% acrylamide gel for anti-GFP and anti-ADH, respectively. Proteins were transferred to a nitrocellulose membrane for 1 hour at 100 V. A mouse anti-GFP antibody (Roche) was used as a primary antibody and donkey anti-mouse immunoglobulin G conjugated to horseradish peroxidase (GE) as a secondary antibody. The ECL Detection kit (GE) was used to detect the secondary antibody according to the manufacturer’s recommendations. Equal amounts of protein in each lane were confirmed with rabbit anti-Aldehyde Dehydrogenase (ADH) (Rockland) primary antibody as previously described (Onodera and Ohsumi, 2004) and donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (GE) as a secondary antibody. Membranes were exposed to autoradiography film for visualization.
3.4 Results

3.4.1 Vid30c is needed for the ethanol-induced regulation of Hxt3

HXT3 encodes a low-affinity hexose transporter that is actively transcribed when glucose is abundant (Ozcan et al., 1999; Roberts et al., 2006). In the absence of glucose, the transcription of this gene is repressed (Roberts et al., 2006) and Hxt3p is targeted for degradation (Snowdon et al., 2009). Since the Vid30c is needed for the nitrogen starvation-induced internalization and degradation of Hxt7p (Snowdon et al., 2008), we reasoned that this complex might also participate in the turnover of Hxt3p when glucose is replaced with ethanol as the sole carbon source. Wild type and individual Vid30c mutant cells carrying HXT3-GFP were grown in glucose media to activate the expression of HXT3, and then Hxt3-GFP was monitored upon a switch to ethanol as the sole carbon source. In glucose (time zero), HXT3 transcription was activated and Hxt3-GFP was visible on the plasma membrane in all the strains tested (Figs. 15A and 15C). In the wild type strain, Hxt3-GFP was internalized with Hxt3-GFP faintly visible on the plasma membrane three hours after the switch to ethanol media and almost completely degraded after six hours. In contrast, when monitoring the mutant strains we observed delayed internalization and degradation of Hxt3-GFP in the vid30Δ, gid2Δ, vid24Δ, vid28Δ, gid8Δ and gid9Δ mutants as Hxt3-GFP was clearly more abundant in the plasma membrane after 3 hours in ethanol media (Fig. 15A). Furthermore, our previous findings suggested that Vid28p and Vid30p, the proposed core components of the Vid30c (Pitre et al., 2006), had at least a partially overlapping function since the vid28Δvid30Δ double mutant delayed Hxt7p turnover more efficiently than either of the respective single mutants (Snowdon et al., 2008). Consistently, the ethanol-induced internalization and degradation
Figure 15. Components of the Vid30c are required for Hxt3p turnover. BY4742 (WT), vid30Δ, gid2Δ, vid24Δ, vid28Δ, gid7Δ, gid8Δ, gid9Δ and ydl176wΔ expressing HXT3-GFP were cultured in glucose media as described in the methods, time 0. Following a switch to ethanol media, samples were collected at the indicated times and analyzed by (A) fluorescence microscopy, (B) Western analysis with an anti-GFP antibody and (C) Northern analysis with a GFP probe. Identical Western blots and Northern blots were probed with an anti-ADH antibody and an anti-ACT1 probe as loading controls, respectively.
of Hxt3-GFP in the vid28Δvid30Δ strain was severely delayed in comparison to the respective single mutants (Figs. 15A and 16A). These results were confirmed by Western analyses (Figs. 15B and 16B). In combination, these data implicate the Vid30c in the turnover of two different Hxts, Hxt3p and Hxt7p (Snowdon et al., 2008), in response to different nutrient stimuli.

We next analyzed the transcription of HXT3-GFP in response to a glucose to ethanol shift and confirmed previous findings that the gene was highly induced in glucose media, but repressed with ethanol as a sole carbon source (Fig. 15C) (Ozcan and Johnson, 1999, Roberts and Hudson, 2006). Slight but reproducibly higher levels of HXT3-GFP transcription were detected in the vid30Δ, gid2Δ, vid24Δ, vid28Δ and vid28Δvid30Δ strains compared to the wild type after 3 hours of ethanol treatment (Fig. 15C and 16E). These findings suggested that the Vid30c also controlled the efficient repression of HXT3 transcription in the absence of glucose.

To separate the ethanol-mediated transcriptional regulation of HXT3 from Hxt3p turnover, the same experiments were performed with HXT3-GFP under the control of the methionine repressible MET25 promoter (Janke et al., 2004) in the wild type and vid28Δvid30Δ strains. Cells were grown to exponential phase in the glucose media devoid of methionine to induce HXT3-GFP expression, washed to remove glucose, and transferred to ethanol media containing methionine to repress HXT3-GFP transcription. We confirmed the severely delayed ethanol-induced internalization and degradation of Hxt3p in the vid28Δvid30Δ double mutant using fluorescence microscopy and Western blotting (Figs. 16C and 16D). Note that the phenotypes of Hxt3-GFP were similar regardless of HXT3-GFP being expressed from the native HXT3 or MET25 promoter.
Figure 16. The combined deletion of VID28 and VID30 has an increased effect on Hxt3p turnover, which is maintained when the native promoter is exchanged for the MET25 promoter. BY4742 (WT) and vid28Δvid30Δ expressing HXT3-GFP from either the native promoter or the MET25 promoter were cultured in glucose media (A, B and E) or glucose media minus methionine (C and D) as described in the methods, time 0. Following a switch to ethanol media (A, B and E) or ethanol media plus methionine (C and D), samples were collected at the indicated times after the shift and analyzed by fluorescence microscopy (A and C), Western analysis with an anti-GFP antibody (B and D) and Northern analysis with a GFP probe (E). Identical Western blots and Northern blots were probed with anti-ADH antibody and an ACT1 probe as loading controls, respectively.
Together, these results confirmed the participation of the Vid30c in the regulation of gene expression and protein turnover of Hxt3p as the cell adapts to ethanol as the sole carbon source.

### 3.4.2 Active Ras/cAMP/PKA prevents ethanol-induced Hxt3p turnover

The Ras/cAMP/PKA signalling pathway plays a major role in controlling the response of yeast to glucose in the environment (Mbonyi and Thevelein, 1988). The activation of Ras1/2p occurs in response to abundant glucose in the environment via the stimulating action of the RasGEF Cdc25p (Haney, 1994). Active Ras1/2p stimulates the adenylate cyclase Cyr1p to produce cAMP, which in turn activates PKA and stimulates cell proliferation (Matsumoto et al., 1982). The RAS2Val19 allele renders PKA constitutively active (Crechet et al., 1990). We hypothesized that inactivation of PKA is needed for the turnover of Hxt3-GFP in ethanol and tested if constitutively active Ras2pVal19 would impact the turnover of Hxt3-GFP in a shift to ethanol. Using the MET25p-HXT3-GFP strain, fluorescence microscopy and Western analysis showed that the native RAS2 allele supported the internalization of Hxt3-GFP at 3 hours and almost complete degradation 6 hours after an ethanol shift, while Ras2pVal19 stabilized Hxt3p in the plasma membrane even after 6 hours in ethanol media with no internalization and very little degradation visible throughout the entire time course (Fig. 17A and 17B). Active Ras2p therefore prevents the turnover of Hxt3p.
Figure 17. The turnover of Hxt3p requires inactivation of the Ras/cAMP/PKA pathway. (A and B) BY4742 expressing MET25p-HXT3-GFP was transformed with YCp50, YCp50-RAS2 and YCp50-RAS2<sup>VAL19</sup>. Cells were cultured in glucose media minus methionine as described in the methods, time 0. Following a switch to ethanol media plus methionine, samples were collected at the indicated times and analyzed by (A) fluorescence microscopy and (B) Western analysis with an anti-GFP antibody. (C and D) BY4743 (WT) and BY<sup>bcy1Δ/Δ</sup> expressing HXT3-GFP were cultured in glucose media as described in the methods, time 0. Following a switch to ethanol media, samples were collected at the indicated times and analyzed by (A) fluorescence microscopy and (B) Western analysis with an anti-GFP antibody. Identical blots were also probed with an anti-ADH antibody as a loading control.
Bcy1p binds and inactivates PKA in the absence of glucose (Hixson and Krebs, 1980). Consequently, PKA is constitutively active in the absence of BCY1 (Toda et al., 1987). We analyzed the turnover of Hxt3p in the bcylΔ mutant and, similar to the observations for the constitutively active Ras2pVal19, no internalization or degradation of Hxt3p was observed in the bcylΔ mutant (Fig. 17C and 17D). Collectively, these results demonstrate that inactivation of Ras2p and PKA is integral to the signalling of Hxt3p turnover.

### 3.4.3 Tor1p and Ras2p, but not Npr1p, impacts rapamycin-induced Hxt7p turnover

The nitrogen-regulated turnover of amino acid permeases is known to be regulated by Npr1p in the TORC1 pathway (Schmidt et al., 1998; MacGurn et al., 2011). We used the CUP1p-HXT7-GFP construct to analyze the rapamycin-induced degradation of Hxt7p (Snowdon et al., 2008) in the constitutively active tor1-1 strain. In this construct, the native promoter was exchanged for a copper-inducible one to eliminate the impact of TORC1 signalling on HXT7 transcription. Our results indicate that the tor1-1 allele prevents Hxt7p internalization and turnover (Fig. 18A and 18B). We next tested if Hxt7p turnover was dependent on Npr1p and found internalization and degradation profiles in response to rapamycin treatment were similar in the wild type and npr1Δ mutant (Fig. 18C and 18D). Additionally, there was no impact of NPR1 overexpression in either wild type or vid28Δvid30Δ strains (Fig. 18E). Collectively, these results indicate that the rapamycin-induced degradation of Hxt7p is TORC1-regulated, but in a manner independent of Npr1p.
Figure 18. The rapamycin-induced turnover of Hxt7p signals through Tor1p, but is Npr1p-independent. (A and B) BY4742 (WT) and tor1-1 expressing CUP1p-GFP-HXT7 were cultured in raffinose media plus CuSO₄ as described in the methods, time 0. After washing, the cells were resuspended in fresh raffinose media and treated with rapamycin. Samples were collected at the indicated times and analyzed by (A) fluorescence microscopy and (B) Western analysis with an anti-GFP antibody. Identical blots were also probed with an anti-ADH antibody as a loading control. (C and D) BY4742 (WT) and npr1Δ expressing CUP1p-GFP-HXT7 were cultured in raffinose media plus CuSO₄ as described in the methods, time 0. After washing the cells, they were resuspended in fresh raffinose media and treated with rapamycin. Samples were collected at the indicated times and analyzed by (C) fluorescence microscopy and (D) Western analysis with an anti-GFP antibody. Identical blots were also probed with anti-ADH antibody as a loading control. (E) BY4742 (WT) and vid28Δvid30Δ expressing HXT7-GFP were transformed with pRS425 (control; 2μ LEU2) and pEJ23 (2μ LEU2 HA-NPR1). Cells were cultured in raffinose media as described in the methods, time 0. The cells were then treated with rapamycin and samples were collected at the indicated times and analyzed by fluorescence microscopy.
Several studies have shown close interactions between the TOR and Ras/cAMP/PKA pathways ranging from TOR and Ras/cAMP/PKA converging as separate pathways on the same molecular target (Pedruzzi et al., 2003), TOR functioning to control PKA as a downstream effector (Martin et al., 2004) to TOR and PKA having antagonistic effects in the cell (Ramachandran and Herman, 2011). Since Hxt3p turnover is controlled by Ras/cAMP/PKA (Fig. 17) and Tor1p controls rapamycin-induced Hxt7p turnover in a mechanism independent of Npr1p (Fig. 18), we tested the involvement of Ras/cAMP/PKA in rapamycin-induced Hxt7p turnover. While cells expressing native RAS2 displayed normal rapamycin-induced turnover of Hxt7-GFP, it was clear that Hxt7-GFP remained in the plasma membrane with limited, if any, internalization following rapamycin treatment of cells expressing constitutively active RAS2\textsuperscript{VAL19} (Fig. 19A). Higher levels of Hxt7-GFP were also detected by Western analysis in RAS2\textsuperscript{VAL19} strains after rapamycin treatment (Fig. 19B). These observations indicate that like ethanol-induced Hxt3p turnover, the rapamycin-induced internalization and degradation of Hxt7p is dependent on the inactivation of Ras2p.
Figure 19. The turnover of Hxt7p requires inactivation of Ras2p. BY4742 expressing *HXT7-GFP* were transformed with YCp50, YCp50-*RAS2* and YCp50-*RAS2*<sup>VAL19</sup>. Cells were cultured in raffinose as described in the methods, time 0. The cells were then treated with rapamycin and samples were collected at the indicated times and analyzed by (A) fluorescence microscopy and (B) Western analysis with an anti-GFP antibody. Identical blots were also probed with an anti-ADH antibody as a loading control.
3.4.4 Ras2p functions downstream of Vid28p in the turnover of Hxt3p

The absence of *VID28* had the most severe effect of all the single mutants on Hxt3p internalization and degradation (Fig. 15A and 15B). This prompted us to test the impact of the overexpression of *VID28* on Hxt3-GFP turnover. The native promoter of *VID28* was replaced with the constitutively active *PGK1* promoter in the MET25_HXT3-GFP strain. The overexpression of *VID28* showed a clear acceleration of the internalization and degradation of Hxt3-GFP in a glucose to ethanol shift, when compared to the parent strain (Fig. 20A and 20B). The localization of Hxt3-GFP to compartments of the endocytic pathway appears earlier and the Hxt3-GFP levels decrease more quickly in the *PGK1p-VID28* strain than in the wild type strain. Thus, the increased expression of *VID28* antagonizes the stability of Hxt3p in the plasma membrane.

To determine the relationship of Vid28p function to that of Ras2p, we monitored the ethanol-induced turnover of Hxt3-GFP in a *PGK1p-VID28* strain expressing *RAS2*<sup>VAL19</sup>. It was clear that the Ras2p<sup>Val19</sup> phenotype was dominant over that of *VID28* overexpression (Fig. 20C and 20D). These observations suggest that Vid28p functions upstream of Ras2p to control the internalization and degradation of Hxt3p in response to a shift from glucose to ethanol.
Figure 20. Overexpression of \( VID28 \) increases the rate of Hxt3p turnover, which is rescued by constitutively active Ras2p. (A and B) BY4742 (WT) and \( PGK1p-VID28 \) transformed with \( PGK1p-VID28 \) expressing \( MET25p-HXT3-GFP \) were cultured in glucose media minus methionine as described in the methods, time 0. Following a switch to ethanol media plus methionine, samples were collected at the indicated times and analyzed by (A and C) fluorescence microscopy and (B and D) Western analysis with an anti-GFP antibody. Identical blots were also probed with an anti-ADH antibody as a loading control.
3.4.5 Cdc25p localization in ethanol is controlled by components of the Vid30c

Cdc25p is the major RasGEF needed for the GDP/GTP exchange on Ras2p to activate the protein (Jones et al., 1991). Active Ras2p-GTP leads to the activation of PKA (Santangelo, 2006). As a negative feedback mechanism, Cdc25p is phosphorylated by PKA leading to the inactivation of Cdc25p’s RasGEF activity and exclusion from the nucleus (Belotti et al., 2010; Jian et al., 2010).

We tested the localization of Cdc25-GFP in response to a switch from glucose to ethanol. Since expression of CDC25-GFP from its native promoter is weak and poorly visualized by fluorescence microscopy, we expressed the fusion gene using the CUP1 promoter. Interestingly, Cdc25-GFP localized to the nucleus when cells were grown in glucose in the presence of copper (Fig. 21A; time zero). When wild type cells were shifted to ethanol media devoid of copper, the Cdc25-GFP signal in the nucleus decreased with time until only a weak signal was present after three hours (Fig. 21A). By contrast, the nuclear signal remained strong in the vid28Δvid30Δ double mutant during the time course after a shift to ethanol and appeared to localize to specific punctate loci within the nucleus (Fig. 21A). These observations suggest that Vid28p and Vid30p, and potentially other components of the Vid30c, are needed to regulate the nucleo-cytoplasmic localization of Cdc25p.

Investigation into Cdc25p posttranslational modification has demonstrated that its nucleo-cytoplasmic localization is dependent on its phosphorylation state (Belotti et al., 2010; Jian et al., 2010). Cdc25p also contains a cyclin destruction box (CDB) motif at its
Figure 21. The nucleocytoplasmic localization of Cdc25p is affected by \textit{vid28\textDelta vid30\textDelta}. BY4742 (WT) and \textit{vid28\textDelta vid30\textDelta} expressing \textit{CUP1p-CDC25-GFP} were cultured in glucose media plus CuSO$_4$ as described in the methods, time 0. Cells were then washed, and following a switch to ethanol media, samples were collected at the indicated times and analyzed by (A) fluorescence microscopy and (B) Western analysis with an anti-GFP antibody. DAPI staining was performed to localize the nucleus. Identical blots were probed with an anti-ADH antibody as a loading control. Punctate loci are denoted with an arrow.
amino-terminal end (Kaplon and Jacquet, 1995). These CDBs are required for the ubiquitin-dependent degradation of cyclins (Nugent et al., 1991). Indeed, the CDB present in Cdc25p is necessary for its glucose-induced degradation (Kaplon and Jacquet, 1995). Additionally, Cdc25p is degraded in response to heat shock, oxidative stress and ethanol shock, but its phosphorylation and localization are not affected (Wang et al., 2004). These data, along with the stable Cdc25-GFP signal in vid28Δvid30Δ (Fig 21A), motivated us to investigate the possible Vid30c-dependent degradation of Cdc25p. Surprisingly, we did not detect any degradation of Cdc25p when cells were switched from glucose to ethanol media (Fig 21B). However, the amount of Cdc25p in the vid28Δvid30Δ strain was clearly higher than in the wild type, suggesting a role for the Vid30c in the stability of Cdc25p. Additionally, we observed a discrepancy between the migration rate of Cdc25p among the two strains, with Cdc25p species from the wild type migrating more slowly than the Cdc25p species from the vid28Δvid30Δ strain (Fig. 21B). This difference in migration suggests a possible posttranslational modification of Cdc25p in the wild type, potentially phosphorylation, which does not occur in the vid28Δvid30Δ strain. Taken together, these data suggest that the Vid30c is required for both the translocation of Cdc25p from the nucleus to the cytoplasm and the stability of Cdc25p when cells are shifted from glucose to ethanol, and this may be a result of a posttranslational modification.

3.4.6 Rim15p is the downstream effector of PKA in the Vid30c-dependent turnover of Hxt3p and Hxt7p

PKA controls the activity of several downstream effectors in the presence of abundant glucose, including the Rim15p kinase (Reinders et al., 1998). We investigated
the potential role of Rim15p in the turnover of Hxt3p and Hxt7p. While the levels of Hxt3-GFP and its localization to the plasma membrane were comparable in the wild type and \textit{rim15}\textsuperscript{Δ} mutant grown in glucose, the internalization of Hxt3-GFP in \textit{rim15}\textsuperscript{Δ} cells was markedly delayed when the cells were shifted to ethanol, as the protein was still visible on the plasma membrane and detectable by Western after 6 hours (Fig. 22A and 22B). Similarly, Hxt7-GFP was expressed at similar levels and localized to the plasma membrane in the wild type and \textit{rim15}\textsuperscript{Δ} mutant strains grown in raffinose, but the \textit{rim15}\textsuperscript{Δ} mutants showed a significant delay in internalization and degradation when the cells were treated with rapamycin (Fig. 22C and 22D). In combination, these results demonstrate that active Rim15p is needed for ethanol and rapamycin-induced internalization and degradation of Hxt3p and Hxt7p, respectively.

To investigate the relationship between the Vid30c and Rim15p, we tested whether the overexpression of \textit{RIM15} (pVW904; (Wanke et al., 2005) would rescue the delayed Hxt3p turnover phenotype of the \textit{vid28}\textsuperscript{Δ}\textit{vid30}\textsuperscript{Δ} double mutant. When grown in glucose, the levels of Hxt3-GFP were similar in the wild type strain whether \textit{RIM15} was overexpressed or not (Fig. 23A; time zero). \textit{RIM15} overexpression therefore has no impact on Hxt3p stability during growth in glucose. However, following a shift to ethanol media, \textit{RIM15} overexpression accelerated the turnover of Hxt3-GFP in the wild type as the protein levels were lower after 3 hours and barely detectable after 6 hours in ethanol media (Fig. 23A). Interestingly, the reduced Hxt3p turnover in the \textit{vid28}\textsuperscript{Δ}\textit{vid30}\textsuperscript{Δ} double mutant was rescued when \textit{RIM15} was overexpressed (Fig. 23A). Overexpression of \textit{RIM15} did not impact the transcriptional regulation of \textit{HXT3} in either glucose or ethanol (Fig. 23B).
Figure 22. The turnover of both Hxt3p and Hxt7p is dependent on the function of Rim15p. (A and B) BY4742 (WT) and rim15Δ expressing MET25p-HXT3-GFP were cultured in glucose media lacking methionine as described in the methods, time 0. Following a switch to ethanol media plus methionine, samples were collected at the indicated times and analyzed by (A) fluorescence microscopy and (B) Western analysis with an anti-GFP antibody. (C and D) BY4742 (WT) and rim15Δ expressing CUP1p-GFP-HXT7 were cultured in raffinose media plus CuSO₄ as described in the methods, time 0. After washing the cells, they were resuspended in raffinose media and treated with rapamycin. Samples were collected at the indicated times and analyzed by (C) fluorescence microscopy and (D) Western analysis with an anti-GFP antibody. Identical blots were also probed with an anti-ADH antibody as a loading control.
Figure 23. Rim15p functions downstream of the Vid30c in Hxt3p turnover. (A and B) BY4742 (WT) and vid28Δvid30Δ expressing MET25p-HXT3-GFP (A) or HXT3-GFP (B) were transformed with YEplac181 (2μ LEU2) and pVW904 (2μ LEU2 TĐH3p-RIM15-myc13). Cells were cultured in glucose media lacking methionine as described in the methods, time 0. Following a switch to ethanol media plus methionine, samples were collected at the indicated times and analyzed by (A) Western analysis with an anti-GFP antibody and (B) Northern analysis with a GFP probe. Identical Western blots and Northern blots were probed with an anti-ADH antibody and ACT1 probe as loading controls, respectively. (C and D) BY4742 (WT), PGK1p-VID28, rim15Δ and PGK1p-VID28 rim15Δ expressing HXT3-GFP were cultured in glucose media as described in the methods, time 0. Following a switch to ethanol media, samples were collected at the indicated times and analyzed by (C) fluorescence microscopy and (D) Western analysis. Identical blots were also probed with an anti-ADH antibody as a loading control.
We further tested the relationship of Vid28p to Rim15p by analyzing the turnover of Hxt3-GFP in the rim15Δ mutant when VID28 is overexpressed. If Rim15p functions downstream of Vid28p, the accelerated turnover of Hxt3p observed when VID28 is overexpressed should be delayed by the deletion of RIM15. Our findings confirm this epistasis as the overexpression of VID28 could not rescue the delayed Hxt3p turnover phenotype of the rim15Δ mutant (Fig. 23C and 23D). Taken together, these data indicate that Rim15p, as an effector of PKA, functions downstream of the Vid30c to facilitate Hxt3p turnover with ethanol as the sole carbon source.

3.4.7 Turnover of Hxt3p and Hxt7p is dependent on Rsp5p and Art8p

The endocytosis of several amino acid permeases and hexose transporters is dependent on ubiquitination by the E3 ubiquitin ligase Rsp5p (Galan et al., 1996; Springael et al., 1998; Krampe et al., 2002; Hatakeyama et al., 2010). Members of the Art family of arrestin-like proteins recruit Rsp5p to nutrient transporters targeted for endocytosis by functioning as adaptors and giving Rsp5p substrate specificity (Lin et al., 2008; Nikko et al., 2008). It is not known if Rsp5p, or which of the Arts, are needed for the internalization and subsequent degradation of Hxt3p and Hxt7p. We analyzed the ethanol-induced internalization and degradation of Hxt3-GFP in the rsp5-1 mutant. As this strain dies with prolonged exposure to ethanol at 37°C, we used 30°C as the non-permissive temperature to analyze the temperature-sensitive mutation. It was clear that Hxt3p-GFP remained on the plasma membrane with no discernible internalization occurring even after 6 hours in ethanol (Fig. 24A). The lack of protein degradation was also confirmed by Western (Fig. 24B). These results confirm that Rsp5p is the E3 ligase needed for the turnover of Hxt3p. The glucose-induced turnover of Hxt7p is known to be
dependent on Rsp5p (Krampe et al., 1998; Krampe et al., 2002). Similarly, we found that the rapamycin-induced turnover of Hxt7p is also dependent on Rsp5p (Fig. 25A and 25B).

We screened four arrestin-like proteins (Art3p, Art4p, Art6p, and Art8p) for any potential involvement in Hxt3p and Hxt7p turnover, and found that only the art8Δ mutant significantly delayed Hxt3p turnover (Fig. 24C and 24D). The endocytosis of Hxt3-GFP was still observed in the absence of ART8, but to a much lesser extent than in the parent strain, suggesting that other Rsp5p adaptor proteins, in addition to Art8, might be needed for the endocytosis of Hxt3p. In contrast, none of these art mutants had a major impact on Hxt7p turnover; however, art8Δ did appear to have a slight effect on Hxt7-GFP internalization (Fig. 25C).
Figure 24. The turnover of Hxt3p is dependent on Rsp5p and Art8p. (A and B) BY4742 (WT) and rsp5-1 and (C and D) BY4742 (WT), art3Δ, art4Δ, art6Δ and art8Δ expressing HXT3-GFP were cultured in glucose media as described in the methods at 23 °C (A and B) and 30 °C (C and D), time 0. Following a switch to ethanol media and 30 °C (A and B), samples were collected at the indicated times and analyzed by (A and C) fluorescence microscopy and (B and D) Western analysis with an anti-GFP antibody. Identical blots were also probed with an anti-ADH antibody as a loading control.
Figure 25. The turnover of Hxt7p is dependent on Rsp5p and Art8p. (A and B) BY4742 (WT) and rsp5-3 and (C and D) BY4742 (WT), art3Δ, art4Δ, art6Δ and art8Δ expressing HXT7-GFP were cultured in raffinose media as described in the methods at 23 °C (A and B) and 30 °C (C and D), time 0. After treatment with rapamycin and a switch to 30 °C (A and B), samples were collected at the indicated times and analyzed by (A and C) fluorescence microscopy and (B and D) Western analysis with an anti-GFP antibody. Identical blots were also probed with an anti-ADH antibody as a loading control.
3.5 Discussion

The Vid30c (Pitre et al., 2006), also known as the GID complex (Santt et al., 2008), has been studied extensively in yeast adaptation to glucose after growth with non-fermentable carbon sources (such as ethanol or acetate), as the Vid30c is needed for the ubiquitination and degradation of FBPase and Mdh2p (Hoffman et al., 1996; Regelmann et al., 2003; Santt et al., 2008). Here we show components of this complex also play an important role in the adaptation to gluconeogenic growth conditions following growth with glucose as the sole carbon source. HXT3 is actively expressed and Hxt3p localizes to the plasma membrane when glucose is abundant, but HXT3 transcription is repressed (Roberts et al., 2006) and the protein is endocytosed and degraded when ethanol is the sole carbon source (Snowdon et al., 2009). This latter adaptation depends on the Vid30c, specifically the proposed core components Vid28p and Vid30p (Pitre et al. 2006), the proposed regulatory protein of the complex in glucose, Vid24p (Santt et al., 2008), and the two catalytic components Gid2p and Gid9p (Santt et al., 2008; Braun et al. 2011), as the transcriptional repression and more noticeably the turnover of Hxt3p are delayed in the absence of these genes (Figs. 15 and 16). It has also been shown that components of the Vid30c are needed for the nitrogen starvation-induced degradation of Hxt7p (Snowdon et al., 2008) and nitrogen-regulated gene expression (van der Merwe et al., 2001). These results show that several components of the Vid30c do not function solely in the adaptation of yeast to glucose replenishment, but are also needed for the adaptation to a range of nutrient conditions.

Several pathways are involved in signalling the presence or absence of nutrient abundance in the environment. Importantly, the Ras/cAMP/PKA pathway promotes cell
proliferation in response to abundant glucose in the environment (Mbonyi et al., 1988). PKA prevents entry into stationary phase by inactivating Rim15p, which promotes entry into G₀ (Pedruzzi et al., 2003). Our subsequent hypothesis that an active Ras/cAMP/PKA pathway will prevent turnover of Hxt3p was supported by the predominant retention of Hxt3p-GFP in the plasma membrane in ethanol when cells were either expressing \( RAS2^{VAL19} \) or lacking \( BCY1 \) (Fig. 17). Also, PKA is inactive in gluconeogenic carbon sources (Thevelein et al., 1999), resulting in the activation of Rim15p (Pedruzzi et al., 2003). Our data show that active Rim15p is needed for the turnover of Hxt3p in ethanol (Figs. 22A and 22B). Similar to the turnover of Hxt3p, the rapamycin-induced internalization of Hxt7p is prevented in cells expressing \( RAS2^{VAL19} \) (Fig. 19) and by the absence of \( RIM15 \) (Figs. 22A and 22B). Overall, our observations indicate that the Ras/cAMP/PKA pathway needs to be inactivated to enable the turnover of Hxt3p and Hxt7p in response to ethanol and rapamycin treatment. These observations are supported by previous studies in which TORC1 inhibits the activity of the Ras/cAMP/PKA pathway in response to rapamycin treatment (Soulard et al., 2010).

TORC1 and Npr1p are involved in the endocytosis and subsequent degradation of amino acid permeases in a nitrogen-dependent manner (Schmidt et al., 1998; De Craene et al., 2001). Here, we confirm that the previously reported rapamycin-induced degradation of Hxt7p (Snowdon et al., 2008) is dependent on Tor1p, but surprisingly Npr1p is not involved in this process (Fig. 18).

It was previously shown that the rapamycin-induced degradation of Hxt1p is also prevented by the expression of \( RAS2^{VAL19} \) (Schmelzle et al., 2004). Unlike Hxt1p, the degradation of Hxt3p and Hxt7p is dependent on components of the Vid30c (Figs. 15, 16
and Snowdon et al., 2008). The \textit{vid28\Delta vid30\Delta} double mutant has a more pronounced impact on \textit{HXT3} repression and Hxt3p (Fig. 16) or Hxt7p (Snowdon et al., 2008) turnover than either of the respective single mutants, indicating that some functional overlap exists within the complex. There exists a strikingly similar retention of these Hxts on the plasma membranes of the \textit{vid28\Delta vid30\Delta} double mutant cells and the cells expressing \textit{RAS2^{VAL19}} (Figs. 16, 17, 19, and Snowdon et al., 2008). In addition, the overexpression of either \textit{VID28} or \textit{RIM15} resulted in the accelerated turnover of Hxt3p (Figs. 20A, 20B and 23A). These results suggest that the function of the Vid30c is closely linked to the activity of the Ras/cAMP/PKA pathway. To this end, while the overexpression of \textit{VID28} accelerates Hxt3p endocytosis and degradation, constitutively active \textit{RAS2^{VAL19}} rescues this phenotype (Figs. 20C and 20D). Therefore, the Vid30c most certainly functions upstream of Ras2p to regulate the ethanol and rapamycin-induced internalization of Hxt3p and Hxt7p, respectively.

Our data suggest that the Ras/cAMP/PKA pathway must be inactive to facilitate the Hxt turnover process. Our demonstration that Cdc25p localization and stability is regulated by the Vid30c (Fig. 21), suggests that in the \textit{vid28\Delta vid30\Delta} double mutant, Ras/cAMP/PKA remains active in ethanol, with Cdc25p accumulating in the nucleus rather than being exported to the cytosol. By contrast, in the wild type strain Cdc25p is exported to the cytosol in response to an ethanol shift and Ras/cAMP/PKA is inactivated to facilitate the turnover of the Hxts. In support of this observation, active, GTP-bound Ras2p has been localized to the nucleus of logarithmically growing cells. In addition, when these cells reach the diauxic shift, GTP-bound Ras2p localizes to the cytosol (Leadsham et al., 2009). These observations suggest that Ras activation does not only
occur at the plasma membrane as previously thought (Bhattacharya et al., 1995). The nuclear accumulation of Cdc25p in $vid28\Delta vid30\Delta$ cells growing in ethanol (Fig. 21A) suggests that Ras2p is activated in the nucleus when the Vid30c is absent. This hypothesis is currently under further investigation. Interestingly, we observe a difference in the relative molecular mass of Cdc25p on Western blots between the wild type and $vid28\Delta vid30\Delta$ strains (Fig. 21B), suggesting that the Vid30c is responsible for a posttranslational modification of Cdc25p. This modification could be a phosphorylation event, which would be in agreement with the observation that phosphorylated Cdc25p is excluded from the nucleus (Belotti et al., 2010).

The current model for the function of the Vid30c is built upon its known function as a ubiquitin ligase (Regelmann et al., 2003; Santt et al., 2008; Braun et al., 2011). Therefore, Cdc25p might represent a direct target of the Vid30c, as the stability of Cdc25p is moderately affected in a $vid28\Delta vid30\Delta$ strain. Alternatively, the Vid30c may affect the stability of a yet unidentified phosphatase or regulatory subunit of a phosphatase that possibly acts on Cdc25p, thereby regulating Cdc25p phosphorylation. The increased levels of an active phosphatase in the $vid28\Delta vid30\Delta$ double mutant would account for the altered molecular mass of Cdc25p observed in this strain (Fig. 21B). In either case, this collection of data suggests the possibility of a unique, nuclear branch of Ras regulation that may play an important role in yeast adaptation to nutrient conditions.

Nutrient transporters, including hexose transporters and amino acid permeases, are targeted for endocytosis and subsequent vacuolar degradation by ubiquitination (Galan et al., 1996; Springael et al., 1998; Springael et al., 1999; Dunn et al., 2001; Krampe et al., 2002; Hatakeyama et al., 2010). While the Vid30c functions as an E3
ubiquitin ligase (Santti et al., 2008), Rsp5p is an essential E3 ligase needed for the ubiquitination of nutrient transporters (Galan et al., 1996; Springael et al., 1999; Krampe et al., 2002; Hatakeyama et al., 2010). Also, an assortment of arrestin-like proteins function as adaptors for recruiting Rsp5p to nutrient transporters targeted for degradation (Lin et al., 2008; Nikko et al., 2008). Here we show that Hxt3p endocytosis in response to ethanol treatment is dependent on Rsp5p and Art8p (Fig. 24C and 24D), confirming that Rsp5p and the arrestin-like adaptor Art8p function in the endocytosis and degradation of Hxt3p. The glucose-induced degradation of Hxt7p is dependent on Rsp5p (Krampe et al., 1998), and here we confirm that the rapamycin-induced degradation of Hxt7p is as well (Fig. 25A and 25B). In the case of Hxt7p, the implication of a particular arrestin-like Rsp5p adaptor is not as clear. Our results did not demonstrate a major role for any individual Art protein, but this may be due to the overlapping function of Arts or the involvement of an Art protein that was not tested. Although the Vid30c itself functions as an E3 ubiquitin ligase, its direct target(s) to facilitate Hxt3 and Hxt7 endocytosis and degradation remain unknown.

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4.0 General discussion and future directions

Previous research into the function of the Vid30c, or its individual components, has been exclusively focused on understanding its role in FBPase turnover in response to glucose replenishment (Brown et al., 2003; Regelmann et al., 2003; Hung et al., 2004). Here, we expand on the understanding of the Vid30c and show that it is also involved in the turnover of two membrane-bound proteins in response to unique nutrient changes. We show that the high-affinity Hxt7p is internalized and degraded in response to nitrogen starvation and rapamycin treatment in a Vid30c-dependent manner. We also show that the low-affinity Hxt3p is internalized and degraded in response to a shift from glucose to ethanol in a Vid30c-dependent manner. These findings present a novel function for the Vid30c in the turnover of integral membrane proteins, whereas previous research had only identified a role for them in the turnover of the cytosolic enzymes FBPase and Mdh2p (Hung et al., 2004). Additionally, the turnover of FBPase and Mdh2p occurs in response to the replenishment of glucose to glucose-starved cells (Hung et al., 2004). We have thus established a greater role for the Vid30c in the adaptation to environmental changes, with a function during glucose and nitrogen starvation.

Our investigation here sought not only to define the roles of individual components of the Vid30c, but also to further our understanding of the holo-complex (Vid30c). Previous studies had used single VID/GID mutants to understand how individual components affected phenotypes such as FBPase degradation (Brown et al., 2002; Regelmann et al., 2003; Hung et al., 2004). Here we utilized double mutants to investigate functional overlap within the Vid30c. We found that a combination of gid2Δvid30Δ and vid28Δvid30Δ increased the stability of Hxt7p, as compared to the
individual mutants, when cells were treated with rapamycin. Additionally, we found that
the \textit{vid28\textsuperscript{Δ}vid30\textsuperscript{Δ}} strain showed very little internalization or degradation of Hxt3p and
Hxt7p in response to a switch from glucose to ethanol and rapamycin treatment,
respectively. These results show that there is a significant level of functional overlap
among the individual components of the Vid30c and that deletion of both \textit{VID28} and
\textit{VID30} appears to almost completely destroy the function of the Vid30c. This work has
led to a greater understanding of how the function of individual components affects the
entire Vid30c.

During the completion of the work presented in this thesis, there was a growing
amount of data published on the function of the Vid30c as a ubiquitin ligase (Regelmann
\textit{et al.}, 2003; Santt \textit{et al.}, 2008; Braun \textit{et al.}, 2011). Our results show that the Vid30c
plays a role in Hxt turnover. Previous studies have implicated ubiquitination, via Rsp5p,
as being responsible for targeting nutrient permeases to the vacuole for turnover (Galan
\textit{et al.}, 1996; Springael \textit{et al.}, 1998; Springael \textit{et al.}, 1999; Dunn \textit{et al.}, 2001; Krampe
\textit{et al.}, 2002; Hatakeyama \textit{et al.}, 2010). Despite the potential for the Vid30c to function directly
in the ubiquitination of Hxt3p and Hxt7p, we show that the ubiquitin ligase Rsp5p is
responsible for targeting these Hxts for degradation. Our data does not dispute the role of
the Vid30c as a ubiquitin ligase, but it is not involved in the direct ubiquitination of Hxts
as it is for FBPase. As such, we aimed to define more accurately where the Vid30c
functions during these turnover events.

Through the manipulation of genes required for glucose and nitrogen signalling,
we were able to outline the signalling events required for the degradation of both Hxt3p
and Hxt7p. We showed that for Hxt3p degradation to occur in response to ethanol there
must be inactivation of Ras2p and PKA followed by activation of Rim15p. In the case of Hxt7p degradation in response to rapamycin treatment, there must be inactivation of both Tor1p and Ras2p followed by activation of Rim15p. These data suggest that the major signalling event of both Hxt3p and Hxt7p turnover is the activation of Rim15p. The fact that Rim15p activation is dependent on the inactivation of both Ras2p and Tor1p has been previously shown (Pedruzzi et al., 2003); however, a role for Rim15p in protein turnover has not been shown previously. To our knowledge this is the first demonstration that Rim15p functions in nutrient permease turnover.

Once the key signalling events of Hxt3p turnover had been outlined, we were able to investigate the potential of Vid30c function within the Ras/cAMP/PKA signalling pathway. This line of investigation was driven by the knowledge that Rsp5p, not the Vid30c, was likely responsible for Hxt3p ubiquitination, and therefore the Vid30c might function upstream, in signalling the turnover. Through a series of epistasis experiments we demonstrated that the Vid30c functions upstream of Rim15p, and more surprisingly, upstream of Ras2p. To our knowledge, these data represent the first evidence of the Vid30c functioning in a potential signalling role.

The activation of Ras2p is controlled by the RasGEFs, Cdc25p and Sdc25p, which stimulate the exchange of GDP with GTP on Ras2p (Haney and Broach, 1994). The two RasGEFs possess functional redundancy, but Cdc25p was found to be essential, whereas Sdc25p is dispensable (Boy-Marcotte et al., 1996). Additionally, the stability and localization of Cdc25p is regulated by carbon source (Wang et al., 2004; Belotti et al., 2010; Jian et al., 2010). When cells are shocked with high concentrations of ethanol there is a rapid degradation of Cdc25p (Wang et al., 2004) and when cells are starved of
glucose, Cdc25p is phosphorylated and transported out of the nucleus (Belotti et al., 2010; Jian et al., 2010). We therefore investigated a potential role for the Vid30c in Cdc25p stability. This investigation revealed no turnover of Cdc25p in response to a shift from glucose to ethanol, but there were higher levels of total Cdc25p protein in the vid28Δvid30Δ strain, indicating a potential role for the Vid30c in Cdc25p stability. More interestingly, we demonstrated that the Vid30c regulates the nucleocytoplasmic localization and possibly the posttranslational modification of Cdc25p. Cdc25p has been shown previously to be phosphorylated and this phosphorylation directly affects its nuclear localization (Belotti et al., 2010; Jian et al., 2010); it is possible then, that phosphorylation represents the posttranslational modification event that is dependent on the Vid30c. With the known role of the Vid30c as a ubiquitin ligase, it is unlikely that the Vid30c is involved in directly phosphorylating Cdc25p. Rather, a more likely scenario is that the Vid30c is affecting the stability of a phosphatase or regulatory unit of a phosphatase. An alternative scenario is that the regulation of Cdc25p seen in the vid28Δvid30Δ strain is simply a secondary effect of a feedback loop. More specifically, the vid28Δvid30Δ strain may maintain the Ras/cAMP/PKA pathway active by a different mechanism, and as a result a downstream kinase, potentially Rim15p, is unable to phosphorylate Cdc25p. Either way, whether the effect of vid28Δvid30Δ deletion on Cdc25p is direct or indirect, it represents a significant finding that the Vid30c regulates the central Ras/cAMP/PKA signalling pathway. It is therefore also of great interest to identify the direct target of the Vid30c in response to a shift to ethanol or rapamycin treatment.
Currently, the phosphatase(s) responsible for the dephosphorylation of Cdc25p has not been identified. However, there is significant evidence that points to Sit4p as a candidate: (i) the dephosphorylation of Cdc25p occurs in the nucleus and Sit4p is a nuclear phosphatase (Sutton et al., 1991; Huh et al., 2003); (ii) Cdc25p was found to physically interact with Sap185p, a protein that complexes with Sit4p and is required for its function (Gavin et al., 2006; Collins et al., 2007); (iii) VID28 displays a negative genetic interaction with SAP190, which encodes the Sit4p activating protein, Sap190p (Costanzo et al., 2010). We therefore hypothesize that the Vid30c functions in Cdc25p regulation by directing either Sit4p or one of its Sit4p Activating Proteins (SAPs) for ubiquitin-dependent degradation. We aim to investigate this by: (i) determining the impact of overexpression of SIT4, SAP4, SAP155, SAP185 and SAP195 on Cdc25p localization and phosphorylation; (ii) determining the stability of Sit4p and the SAPs in a vid28∆vid30∆ mutant strain, compared to wild type; (iii) using immunoprecipitation to determine whether there is any physical interaction between any of the Vid30c components and Sit4p or any SAPs; (iv) determine if the Vid30c is directly involved in the ubiquitination of Sit4p or any of the SAPs. This analysis will help to determine the exact function of the Vid30c in Hxt turnover and more firmly define a role for the Vid30c in nutrient signalling. Additionally, it will further define the mechanistic regulation of the Ras/cAMP/PKA signalling pathway.

Based on the data presented in this thesis we propose a model for the role of the Vid30c in Hxt turnover. Our proposed model is centered on the observed function of the Vid30c in the nucleocytoplasmic distribution of Cdc25p (Fig. 26). We believe that this regulation of Cdc25p and the resulting impact on the Ras/cAMP/PKA pathway is the
Figure 26. Proposed model for the role of the Vid30c in Hxt turnover. The Vid30c controls the nuclear export of Cdc25p in response to nutrient deprivation, likely through modulation of its phosphorylation state. As a result of the nuclear exclusion of Cdc25p, there is down regulation of the Ras/cAMP/PKA signalling pathway, which results in the import of Rim15p into the nucleus. The nuclear import of Rim15p is responsible for stimulating the internalization and degradation of both Hxt3p and Hxt7p. Dotted lines signify unknown mechanisms or indirect function.
basis for the observed impact of Vid30c component mutants on Hxt3p and Hxt7p turnover. With respect to Hxt3p turnover, when glucose is abundant we see localization of Cdc25p to the nucleus, independent of Vid30c function, as well as an active Ras/cAMP/PKA pathway. The active Ras/cAMP/PKA signalling maintains Rim15p in the phosphorylated state and restricted to the cytoplasm. The inactivation, and thus the nuclear exclusion of Rim15p is crucial for the maintained stability of Hxt3p on the plasma membrane when abundant glucose is available. Alternatively, when cells are starved of glucose the Vid30c is activated by an as yet unknown mechanism. Activation of the Vid30c results in the export of Cdc25p from the nucleus. We speculate that the impact of the Vid30c on Cdc25p export is likely due to the Vid30c-dependent degradation of a particular SAP protein. In this case, the depletion of a specific SAP protein would no longer enable the Sit4p-dependent dephosphorylation of Cdc25p and thus phosphorylated Cdc25p would be transported out of the nucleus and Ras activation would no longer occur. The resulting inactivation of Ras/cAMP/PKA signalling no longer maintains Rim15p in a phosphorylated form and it is able to enter the nucleus and signal for Hxt3p degradation to proceed. The mechanism by which Rim15p promotes Hxt3p degradation is not known, however it may be a result of Rim15p-dependent transcriptional activation of genes required for the turnover process. A gene such as ART8 is an attractive candidate, as induction of ART8 transcription would allow for Art8p to direct the E3 ligase Rsp5p to Hxt3p in a Rim15p-dependent manner.

The Hxt7p turnover event has not been investigated as thoroughly, but we speculate that the underlying mechanisms remain the same. When nitrogen is abundant the active TORC1 pathway prevents Rim15p entry into the nucleus. If cells are then...
switched to a medium devoid of nitrogen or are treated with rapamycin, then the TORC1-dependent phosphorylation of Rim15p no longer occurs and Rim15p is transported into the nucleus where it may signal the turnover of Hxt7p. Additionally, under nitrogen depletion or rapamycin treatment we speculate that the Vid30c inactivates the Cdc25p-dependent activation of Ras signalling by a mechanism similar to that described above for glucose starvation. Therefore, in both turnover events the dependency on the Vid30c is based on its role in the inactivation of the Ras/cAMP/PKA pathway.

To further clarify our model it is useful to apply this model to our observed results of Vid30c component mutants. When we look at a \( \text{vid28}\Delta\text{vid30}\Delta \) mutant strain grown in 2.5% glucose medium there is stability of Hxt3p on the plasma membrane due to active Ras/cAMP/PKA and the resulting exclusion of Rim15p from the nucleus. When these cells are then switched to 2% ethanol medium the lack of a functional Vid30c does not allow for the proper down regulation of the Ras/cAMP/PKA. The increased Ras/cAMP/PKA signalling in a \( \text{vid28}\Delta\text{vid30}\Delta \) mutant strain does not allow Rim15p entry into the nucleus and Hxt3p degradation is impaired. In the case of Hxt7p, when \( \text{vid28}\Delta\text{vid30}\Delta \) mutant cells are grown in 3% raffinose there is stability of Hxt7p due to active TORC1’s exclusion of Rim15p from the nucleus. When these cells are then deprived of nitrogen or treated with rapamycin the inactivation of TORC1 is insufficient to allow Rim15p transport into the nucleus because of the increased Ras/cAMP/PKA activation in a \( \text{vid28}\Delta\text{vid30}\Delta \) mutant strain and Hxt7p degradation is impaired. We are suggesting that the major impact of disrupting the function of Vid30c components is the increased Ras/cAMP/PKA activation. There are key aspects of this model that need to be further investigated. Firstly, we need to define the mechanism by which the Vid30c
responds to nutrient availability. It has been suggested that the composition of the complex varies depending on nutrient conditions. Specifically, that Vid24p is only present upon a switch to glycolytic conditions and may function to activate the complex (Santt et al., 2008); this form of regulation may be similar for glucose starvation and rapamycin treatment. Secondly, as discussed above, we need to determine how the Vid30c affects Cdc25p localization. Finally, we need to investigate the function of Rim15p in this model to determine how it is able to signal the turnover of Hxts.

Although our understanding of the role of the Vid30c during nutrient adaptation in yeast has been furthered by the work presented in this thesis, there is still much that remains unknown. We have shown that the Vid30c plays a role in the turnover of the low-affinity hexose transporter, Hxt3p, and the high-affinity hexose transporter, Hxt7p; however, there was no function for the Vid30c in the rapamycin-dependent turnover of Hxt1p. As there are at least four other functional Hxts encoded in the yeast genome, it is of interest to determine if the Vid30c is required for their turnover in response to nutrient conditions. Such investigation would provide a more global view of the Vid30c’s regulation of Hxt stability.

The major interest of our lab is the nutrient adaptation of yeast; more specifically, we are interested in yeast adaptation to fermentation stresses. We have shown here that Hxt3p is internalized and degraded when cells are shifted from glucose-containing media to ethanol as the sole carbon source. These environmental conditions bear similarity to those that yeast will encounter during wine fermentation when abundant glucose is rapidly converted to ethanol. Additionally, we showed that Hxt7p is internalized and degraded when cells are starved for nitrogen. Again, these environmental conditions are
similar to those of wine fermentation; depletion of nitrogen as fermentation nears completion. Our data therefore suggest that these Hxts might be down-regulated near the end of fermentation. In agreement with this, Perez et al. (2005) showed that both Hxt3p and Hxt7p protein levels decrease towards the end of fermentation. The loss of hexose transport capacity is the major cause of ‘stuck fermentations’ and causes great financial loss to the wine industry (Bisson, 1999). It would therefore be of great interest to determine what impact deletion of Vid30c components would have on Hxt stability during wine fermentation, with the aim of increasing the rate and fidelity of fermentation. Such investigation could provide direction in the development of new yeast strains better adapted to nitrogen-poor grape musts, restarting stuck fermentations or overall faster wine production, with significant financial implications.
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