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Hygromycin B Hypersensitive (*hhy*) Mutants Implicate an Intact Trans-Golgi and Late Endosome Interface in Efficient Tor1 Vacuolar Localization and TORC1 Function

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Abstract

S. cerevisiae vacuoles are functionally analogous to mammalian lysosomes. Both also serve as physical platforms for Tor Complex 1 (TORC1) signal transduction, the master regulator of cellular growth and proliferation. Hygromycin B is a eukaryotic translation inhibitor. We recently reported on hygromycin B hypersensitive (*hhy*) mutants that fail to grow at sub-translation inhibitory concentrations of the drug and exhibit vacuolar defects (Banuelos et al., 2010). Here, we show that *hhy* phenotype is not due to increased sensitivity to translation inhibition and establish a super *HHY* (*s-HHY*) subgroup of genes comprised of *ARF1*, *CHC1*, *DRS2*, *SAC1*, *VPS1*, *VPS34*, *VPS45*, *VPS52*, *VPS54* that function exclusively or inclusively at trans-Golgi and late endosome interface. Live cell imaging of *s-hhy* mutants revealed that hygromycin B treatment disrupts vacuolar morphology and the localization of late endosome marker Pep12, but not that of late-endosome independent vacuolar SNARE Vam3. This, along with normal post-late endosome trafficking of the vital dye FM4-64, establishes that severe hypersensitivity to hygromycin B correlates specifically with compromised trans-Golgi and late endosome interface. We also show that Tor1p vacuolar localization and TORC1 anabolic functions, including growth promotion and phosphorylation of its direct substrate Sch9, are compromised in *s-hhy* mutants. Thus an intact trans-Golgi and late endosome interface is a requisite for efficient Tor1 vacuolar localization and TORC1 function.

Keywords

Yeast; hygromycin hypersensitive mutants; *hhy* mutants; trans-Golgi and late endosome interface; TORC1 regulation

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INTRODUCTION

Hygromycin B is an aminoglycoside antibiotic isolated from *Streptomyces hygroscopicus* that inhibits translation and is cytotoxic to budding yeast at concentrations >100 µg/ml (Eustice and Wilhelm 1984; Cabanas 1978). At lesser concentrations, hygromycin B has been shown to be detrimental to certain yeast mutants. The growth of yeast mutants defective in glycosylation functions is inhibited at 50 µg/ml hygromycin B (Ali et al. 2004). Most interestingly, a small group from over 200 different vacuolar trafficking mutants have been reported to exhibit growth sensitivities at hygromycin B concentrations below 50 µg/ml including a handful of vacuolar protein sorting (*vps*) mutants and endosome and vacuole interface 1 (*env1*) mutant (Conboy and Cyert 2000; Ali et al. 2004; Mukherjee et al. 2006; Takahashi et al. 2008). The vacuole of *Saccharomyces cerevisiae* is analogous to the mammalian lysosome. Both organelles carry out vital cellular functions including macromolecule degradation, receptor down-regulation, autophagy, and stress survival responses (Bowers and Stevens, 2005; Ostrowicz et al. 2008; Veses et al. 2008; Li and Kane 2009; Armstrong 2010; Richards et al. 2012; Hecht et al. 2014; Klionsky and Eskelinen 2014; Feyder et al. 2015). We recently performed a genomic screen of the yeast haploid deletion strain collection of non-essential genes for hypersensitivity to hygromycin B (*hhy*) mutants; the small collection of *hhy* mutants are presented in Table 1 and all were shown to have defects in vacuolar trafficking, morphology and/or stress survival function (Banuelos et al. 2010). Furthermore, *HHY* gene collection is enriched for established gene functions at the Golgi and late endosome trafficking interface.

Many functional proteins of the vacuole enter the endomembrane system in the ER and transit through Golgi. Such membrane and luminal vacuolar proteins are then delivered from trans Golgi to the lysosomal vacuole by one of two conserved vesicular mechanisms. The late endosome-dependent pathway delivers vacuolar cargo in a clathrin dependent manner through the late endosomal compartment (Vida et al. 1993; Payne and Scheckman 1985; Seeger and Payne 1992). An alternative late-endosome independent pathway delivers vacuolar cargo in a clathrin-independent manner and utilizes the adaptor protein AP3 (Armstrong 2010; Gautreau et al. 2014; Saftig & Klumperman 2009; Li and Kane 2009; Kummel and Ungermann 2014; Ostrowicz et al. 2008; Richards et al. 2010; Viotti 2014; Wickner 2010). The vacuole membrane also houses conserved proteins and protein complexes that do not enter the endomembrane system, but associate with the vacuole membrane on its cytoplasmic side. These include protein complexes involved in regulation of vacuolar fusion/fission including Yck3, Env7 and Vac8 (LaGrassa et al. 2005; Manandhar et al. 2013; Manandhar et al. 2014; Subramanian et al. 2006). They also include the conserved master regulatory signaling complex involved in regulation of cell proliferation and growth, Tor Complex 1 (TORC1) (reviewed in Loewith and Hall 2011). TORC1 positively regulates several anabolic processes including ribosome biogenesis, protein synthesis and nutrient uptake; it also negatively regulates catabolic processes such as autophagy and ubiquitin-mediated proteolysis (Hall 2008; Cybulski and Hall 2009; reviewed in (Reggiori et al. 2013; Delorme-Axford et al. 2015)). mTOR complex 1 (mTORC1) is known to activate stress-responsive transcription factors (reviewed in Aramburu et al. 2014; Ho and Gasch, 2015). Most recently, TORC1 pathway has been linked to neutral lipid

homeostasis in yeast (Madeira et al. 2015) and vacuolar fragmentation (Stauffer and Powers, 2016). TORC1 function, in turn, is regulated through nutrient sensing and by the RAGulator or its yeast counterpart, the EGO complex -- both of which are also localized to the vacuolar/lysosomal membrane. TORC1 upregulation is associated with disease states including cancer and diabetes (Chakrabarti, et al. 2010). Tor is also involved in regulation of the aging process (McCormic et al. 2011). The TORC1 resident kinase, Target of Rapamycin (Tor) is a conserved phosphoinositide 3-kinase (PI3K)-related serine/threonine kinase whose direct substrate includes ribosomal kinase S6k in mammals and its homolog Sch9 in yeast (Rohde et al. 2008; Soulard et al. 2009; Neufeld 2010; Noda and Ohsumi 1998; Urban et al. 2007). While higher eukaryotes only have one *TOR* gene, yeast encodes Tor1 and Tor2 proteins, which can substitute for each other in TORC1. Additional proteins in TORC1 are Lst8, Kog1 and Tco89 (Wullschleger et al. 2006; Sturgill et al. 2008). Recently, it has been discovered that ubiquitin is involved in TORC1 function and regulation through binding with Kog1, a regulatory subunit of TORC1 (Jiang, 2016). While mTORC1 is recruited to lysosomes and late endosomes in a nutrient sensitive manner, yeast TORC1 and Tor1 appear to constitutively reside on vacuole membranes (Betz and Hall 2013; Binda et al. 2009). All *hhy* mutants are also hypersensitive to rapamycin and caffeine (Banuelos et al. 2010) – two drug sensitivities suggestive of compromised TORC1 function (Kuranda et al. 2006; Reinke et al. 2006; reviewed in Aronova et al. 2007). This led us to hypothesize that TORC1 function may be compromised in *hhy* mutants and to further explore the state of vacuolar trafficking and TORC1 in these mutants.

Here we report further studies of *hhy* mutants that establish a super hygromycin B hypersensitive subgroup (*s-hhy* mutants) and genetically, as well as phenotypically, point to a compromised trans-Golgi and late endosome interface in those mutants. We also show that Tor1p vacuolar localization and several parameters of TORC1 function are defective in *s-hhy* mutants. Taken together, our results implicate intact Golgi and late endosome interface in efficient Tor1p vacuolar localization and TORC1 anabolic functions. Our studies also support a therapeutic potential for sub-translation inhibitory levels of hygromycin B in regulation of TORC1 activity, as well as TORC1 hyperactivity in disease.

MATERIALS AND METHODS

Yeast strains and plasmids

S. cerevisiae deletion strains used in these studies were from the MAT- α haploid deletion strain library of non-essential genes derived from the parental strain *BY4742* (Genotype: *MAT α his3 1 leu2 0 ura3 0 lys2 0*) and were gifts from Dr. Gregory Payne, University of California, Los Angeles (UCLA). All yeast strains used in this study are listed in Table 2. Each mutant contains a PCR based disruption of its open reading frame by integration of a KanMX4 module through homologous recombination (Wach et al., 1994). *TOR1-3XGFP* expressing strain MP52-2A (Genotype: [YL516] MAT α ; TOR1-D330-3xGFP) was a gift from Dr. Claudio De Virgilio and has been described previously (Binda et al., 2009). A PCR based approach was used to obtain *hhy* and *vps16* mutants in a *TOR1-3xGFP* background (*TOR1-GFP*). Primers were designed approximately 50–100 bases upstream and downstream of each target gene (see Table 3 for primers used in this study). PCR

amplification was performed with Phusion polymerase (NEB) using genomic DNA extracted from each respective deletion strain. Amplicons containing the kanamycin resistance cassette (KanMX4) flanked by gene-specific upstream and downstream sequences were subjected to agarose gel electrophoresis and purification using Zymoclean Gel DNA Recovery Kit (Zymo Research; Orange, CA) prior to separate transformations into *TOR1-3XGFP* background. Transformants were selected on YPD plates supplemented with 200 µg/ml geneticin (G418), a functional analog of kanamycin; correct kanamycin cassette insertion and gene deletion were confirmed by PCR using KanMX4 and locus-specific primer pairs. Plasmid p1462 encoding Sch9-HA was a gift from Dr. Claudio De Virgilio and has been described before in Urban et al, 2007. *PEP12-RFP* and *VAM3-RFP* expressing plasmids were gifts from Dr. Christian Ungermann (University of Osnabrück) and have been described before (Markgraf, et al., 2009). Their auxotrophic markers were changed from Trp to Leu in order to be utilized in our strains. Yeast transformations were performed by the Lithium Acetate (PLATE) method.

Media and growth analyses

Cells were routinely grown (Sherman, 2002) in YPD (2% peptone, 1% yeast extract and 2% dextrose) or supplemented selective (SM) medium with or without agar at 30 °C unless stated otherwise. Deletion strains with a KanMX4 cassette were selected for as described above. For liquid growth kinetics studies, overnight cultures were diluted to an $OD_{600} = 0.2$ in YPD and transferred to a 96-well microtiter plate. Growth of each strain was assessed in presence of hygromycin B or cycloheximide. Sub-inhibitory concentrations for each drug were experimentally determined by growth assays of the parental wild type (BY4742 α). Final concentrations for growth assessment in hygromycin B were 0, 10, 20 and 40 µg/ml. Final concentrations for growth assessment in cycloheximide were 0, 0.025, 0.05, and 0.1 µg/ml. PBS and DMSO served as vehicles for hygromycin B and cycloheximide treatments, respectively. The microtiter plates were incubated at 30°C with aeration (220 rpm), and depending on the instrument used, OD_{600} or OD_{595} was determined at specified intervals as stated in legends of Figures 1 and 2. Growths for each treatment condition were carried out in triplicates and the average absorbance was used to make each graph. Each strain was assessed in at least three separate experiments with reproducible results. Growth assays were also performed for *hhy* mutants in the *TOR1-GFP* background and showed similar profiles to those in the parental background (data not presented).

Microscopic localization and vacuole morphology studies

To preserve fluorescence of fusion proteins, cells were grown and manipulated in amber conical tubes and microtubes. $OD_{600} = 0.8$ of cells were divided into hygromycin B-treated (10 µg/ml) and mock-treated samples. Total hygromycin B treatment time for all samples was 4 hours in YPD at 30°C after which cells were washed three times in PBS. 1 µl oxryase was added for each 25 µl of cell suspension to preserve fluorescence, and samples were kept on ice until addition to concanavalin A-treated glass slides to improve cell adherence. In experiments where cells were also labeled with the endocytic and vacuolar marker dye FM4-64 [N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl)hexatrienyl] pyridium dibromide] (Molecular Probes Inc., Eugene, OR), staining was carried out as described by Vida and Emr (1995) after the first hour of hygromycin B treatment or mock treatment. All

strains were viewed with an Olympus Fluoview 1000 confocal laser scanning system mounted on an inverted microscope (Olympus IX-81) and a 60x oil immersion UPLSAPO (NA 1.35, WD 0.15 mm) objective. The Argon ion (488 nm) and blue/red diode (405 nm/635 nm) lasers were used for image capturing. Fluorescent and nomarski optics images were captured from at least ten random fields for each strain and from three separate experiments; shown images represent the overall consensus of localization patterns visualized. Images were captured using Olympus's FV-10-ASW 3.0 software, were transferred as JPG files to Photoshop CS2 for cropping and were then merged to visualize colocalization. Vacuolar morphology for each strain was assessed by scoring at least 200 cells into four categories: Wild-type, fragmented, enlarged or absent. TOR1-GFP vacuolar localization was assessed in at least 200 cells and the p-value was calculated using a chi-squared test.

Cell viability and recovery assays

Strains were grown overnight, diluted to $OD_{600} = 0.2$ in YPD and divided into two tubes of untreated and treated with hygromycin B to a final concentration of 10 $\mu\text{g/ml}$. Cultures were then incubated at 30°C with aeration for 4 hours. At 4 hours, 1.1 ml of cell culture was removed; one ml was stained with propidium iodide (Molecular Probes Inc., Eugene, OR) and 0.1 ml was used to measure OD_{600} . Hygromycin B was removed from the remaining treated culture by washing 3 times in 3 ml YPD. Washed cells were then incubated for an additional 24 hours at 30°C with aeration, after which OD_{600} was assessed and an additional propidium iodide staining was performed. Viability was assessed by scoring at least 300 cells from three separate experiments. For plate assays of recovery, *s-hhy* cultures were treated with 10 $\mu\text{g/ml}$ hygromycin B for 4 hours, then washed and quantitatively spotted as 10-fold serial dilutions on YPD plates using a multichannel micropipetter as performed by Binda et al (2009). For viability staining, one μl of 1 $\mu\text{g/ml}$ propidium iodide was added to 1 ml of fresh cell culture in amber tubes, and cells were incubated at 30°C for 30 minutes with aeration. Cells were collected by centrifugation, resuspended in PBS after three washes, and prepared for microscopy as above. Nomarski optics and fluorescence images were captured from random fields and used to count dead (fluorescing) and total cells. Heat killed cells served as positive staining control. For rapamycin recovery assays, experiments were performed as detailed for hygromycin B; cells were assessed immediately and 4 hours after treatment, and then 24 and 48 hours after removal of drug. All growth recovery assays were performed in triplicates and for at least two separate experiments, and standard error was calculated.

Sch9 phosphorylation analyses

p1462 plasmid expressing Sch9-HA has been previously described (Urban et al. 2007). Wild type and *s-hhy* mutants were transformed with p1462, and Sch9-HA phosphorylation was analyzed in westerns with anti-HA monoclonal antibody (Cell Signaling Technology, Beverly, MA) and chemiluminescent approach (Thermo Scientific, Pittsburg, PA) using the Sch9 chemical fragmentation analysis described by Urban et al. (2007) with the following modifications. In addition to the previously described treatments, cells were treated with hygromycin B (10 $\mu\text{g/ml}$, 4 hours) or in combination with cycloheximide (25 $\mu\text{g/ml}$, 30 minutes) where cycloheximide was added during the last 30 minutes of the hygromycin B

treatment. Each mutant was analyzed at least 2 times and from two independent cell preparations. Lower exposure bands were quantified densitometrically using image J. To compare extent of Sch9 phosphorylation, mid region of the fragmentation profile was used as it was consistently visualized in all mutant samples. Extent of Sch9 phosphorylation was calculated by expressing the densitometry value of the slowest migrating band (most highly phosphorylated) divided by that of the total values of the slowest and fastest migrating (non-phosphorylated) bands for each mutant and all treatments of the mutants. For comparison, this ratio was set to 100% for wild-type (WT) cells without treatment, and Sch9 phosphorylation of untreated mutants was expressed as percent of WT in parallel experiments.

RESULTS

Deletion of *HHY* genes that function at trans-Golgi and late endosome interface leads to super-hypersensitivity to Hygromycin B

hhy mutants are unable to grow in presence of 25 µg/ml of hygromycin B (Banuelos, 2010). In order to determine the minimum inhibitory concentration of hygromycin B and study the growth kinetics of *hhy* mutants in a range of hygromycin B concentrations, we conducted 24-hour growth assays in liquid media in the presence of 0, 10, 20 and 40 µg/ml hygromycin B (Figure 1). As expected, hygromycin B did not affect growth of the parental wild type strain at any of the tested concentrations. *hhy* mutants, however, exhibited a dichotomy of growth phenotypes in the presence of hygromycin B. *arf1*, *chc1*, *drs2*, *sac1*, *vps1*, *vps34*, *vps45*, *vps52* and *vps54* lacked significant growth under all three tested hygromycin B concentrations; this subset of *hhy* mutants will be referred to as super-hypersensitive to hygromycin B (*s-hhy*). *bud32*, *dhh1*, *paf1*, and *tpd3* exhibited increased growth inhibition with increasing hygromycin B concentration and will be referred to as dose-dependent hypersensitivity to hygromycin B (*d-hhy*) mutants. *s-hhy* mutant *arf1* repeatedly exhibited a slight dose dependent growth at the lowest tested hygromycin B concentration. Consistent with previous reports, *bud32*, *chc1*, *dhh1*, *drs2*, *paf1*, *tpd3* and *vps34* strains showed compromised fitness relative to wild type in absence of hygromycin B. In the course of these studies, two of the original *hhy* mutants were dismissed as their phenotypes could not be confirmed-- *yelo59w* and *sbh2*. Additionally, several deletion mutants of genes involved in vesicular trafficking were re-evaluated at the original screen conditions due to their reported genetic and/or physical interactions with *HHY* genes. Of these, only *vps1* lacked significant growth, was assessed to be a *s-hhy* mutant, and was included as such in subsequent studies. The *s-HHY* and *d-HHY* genes and summaries of their known molecular functions (as gleaned from yeastgenome.org) are presented in Table 1.

The dichotomy of hygromycin B sensitivities of *s-hhy* and *d-hhy* deletion mutants corresponds to the functional dichotomy of the deleted genes. *s-HHY* genes are directly involved in vesicular trafficking functions, while *d-HHY* genes are not. Furthermore, the common denominator in the vesicular trafficking function of *s-HHY* gene products is their role, either exclusively or inclusively, at the trans-Golgi and late endosome interface. Thus,

super-hypersensitivity to hygromycin B in *hhy* mutants correlates with compromised trans-Golgi and late endosome interface.

Alternatively, the observed *s-hhy* phenotype may have been due to increased sensitivity to the translation inhibitory function of hygromycin B. This possibility was probed via growth assays of wild type and *hhy* mutant strains using another potent translation inhibitor, cycloheximide. Cycloheximide concentrations sub- to minimally-inhibitory to growth of the wild type parental strain were experimentally determined to range from 0.025 µg/ml to 0.10 µg/ml (data not presented). 24-hour growth assays were performed with wild type parental and *hhy* mutant strains in presence of 0, 0.025, 0.05, and 0.1 µg/ml cycloheximide. Most *hhy* mutants did not show significantly increased growth sensitivities to cycloheximide relative to that observed with wild type, nor was there a dichotomy of sensitivities based on gene function as seen with hygromycin B treatments (Figure 2). Thus, the unique hygromycin B hypersensitivity of *hhy* mutants does not appear to be due to increased vulnerabilities to translation inhibition. Taken together, these results suggest that the compromised trans-Golgi and late endosome interface of *s-hhy* mutants correlates with their super-hypersensitivity to hygromycin B.

Hygromycin B treatment of *s-hhy* mutants disrupts late endosome-dependent but not late endosome-independent post-Golgi trafficking

The vacuolar defects of *hhy* mutants, the absence of increased growth sensitivity to another potent translation inhibitor, and the common function of *s-hhy* mutants at the trans-Golgi and late endosome interface led us to hypothesize that a compromised trans-Golgi and late endosome interface is highly susceptible to hygromycin B in *s-hhy* mutants. To test this, we investigated the state of both late endosome-dependent and late endosome-independent trafficking in the *s-hhy* mutants. *s-hhy* mutants have been shown biochemically to have severe defects in correct trafficking and proteolytic processing of the common endosome-dependent vacuolar marker carboxypeptidase Y, resulting in secretion of significant levels of the enzyme (Bankaitis et al. 1986; Banta et al. 1988; Robinson et al. 1988; Rothman et al. 1989; Bonangelino et al. 2002). Therefore, we instead explored localization of Vam3 and Pep12 as respective markers of the two trafficking pathways by live cell imaging. Pep12-RFP and Vam3-RFP are high copy plasmids with two copies of DsRed attached to the N terminus as described in Markgraf et al. (2009). Vam3 is a Q-SNARE that resides on the vacuole membrane and is transported there by the endosome-independent pathway (reviewed in Ostrowicz, et al. 2008). Pep12 is a late-endosome Q-SNARE that is delivered to that compartment in a clathrin-dependent manner and serves as a late endosome marker (Becherer 1996). WT and *s-hhy* mutant strains were transformed with plasmids constitutively expressing either Vam3-RFP or Pep12-RFP, and localization patterns of the two proteins were assessed in live cells mock-treated or treated for four hours with 10 µg/ml hygromycin B, which is the lowest tested inhibitory dose for *s-hhy* mutants (Figure 3). The four-hour treatment time was experimentally determined to be the shortest incubation that resulted in visible changes in slow-growing *s-hhy* mutants. As expected, mock-treated wild type strain exhibited localization of Vam3-RFP to vacuolar membranes, which are marked as the crater boundaries in nomarski optics imaging, and localization of Pep12-RFP to 1–3 punctate structures characteristic of the late endosome. In contrast, while the punctate

staining pattern of Pep12-RFP was slightly compromised in mock-treated *s-hhy* mutants, the vacuolar staining of Vam3-RFP was not. Upon hygromycin B treatment, no changes were observed in Vam3-RFP nor Pep12-RFP localizations in wild type strain. In contrast, within 4 hours of hygromycin B treatment, Pep12-RFP punctate staining patterns were abrogated in the *s-hhy* mutants while Vam3-RFP vacuolar localization remained undisturbed. These results indicate that in *s-hhy* mutants, Golgi and late-endosome interface is compromised and further disrupted at sub-translation inhibitory levels of hygromycin B, while late endosome-independent post-Golgi trafficking is not.

Vacuolar morphology and Tor1-GFP localization are disrupted in Hygromycin B treated *s-hhy* mutants

We have previously shown that *hhy* mutants are sensitive to caffeine and rapamycin suggestive of defects in TORC-1 signaling pathway (Banuelos et al., 2010). Caffeine is a phosphodiesterase inhibitor that has been reported to target yeast TOR Complex1 (TORC1) and TOR Complex2 (TORC2) (Guan et al. 2009; Reinke et al. 2006; Wanke et al. 2008). Rapamycin is an anti-fungal agent that directly targets TORC1 (Park et al. 2005; Kuranda et al. 2006; reviewed in Levin 2005; Aronova et al., 2007). Since TORC1 localizes to the vacuole (Betz and Hall 2013; Binda et al. 2009), and all *HHY* genes have been implicated in vacuolar function and/or trafficking, a defective TORC1 signaling due to vacuolar trafficking defects is plausible. We hypothesized that TORC1 vacuolar localization is defective and hypersensitive to hygromycin B in *hhy* mutants and investigated the localization of the TORC1 kinase Tor1. Tor1 and Tor2 are the interchangeable kinase subunits of TORC1 and, unlike *TOR2*, *TOR1* is not an essential gene. Thus, we confirmed that *tor1* mutants are not hypersensitive to hygromycin B in the wild type background (Figure 1). Additionally, deletion of *TCO89*, another non-essential component of TORC1, did not lead to hygromycin B hypersensitivity (Figure 1). Together, these controls indicate that absence of Tor1 (and its presumable replacement by Tor2) or a structurally compromised TORC1 is not sufficient for hygromycin B hypersensitivity. For the localization studies, we utilized a strain in which *TOR1* gene is endogenously tagged with 3x-GFP (Tor1-GFP) in a central region, and the fusion protein is active and localizes to the vacuole membrane (Binda et al. 2009). *HHY* genes were individually deleted in that background, and Tor1-GFP localization was assessed by confocal microscopic live cell imaging after 4 hours of either mock-treatment or treatment with 10µg/ml hygromycin B. Cells were also stained with the vital endocytic vacuolar membrane marker FM4-64 to assess the endocytic pathway, mark the vacuolar membrane and quantitatively score for vacuolar morphology for each mutant. FM4-64 is a lipophilic styryl vital dye that localizes to the vacuolar membrane through the endocytic pathway and moves from plasma membrane through early endosomes and late endosomes to the vacuole (Vida and Emr 1995). Representative images from treated and mock-treated strains are presented in Figure 4 and quantified results of scored cells for Tor1-GFP localization and vacuolar morphology are summarized in Table 4.

In wild type cells, in presence or absence of hygromycin B, vacuole morphology is unchanged and both Tor1-GFP and FM4-64 localize to vacuole membranes. *hhy* mutants, however, exhibited two distinct Tor1-GFP localization and vacuolar morphology phenotypes upon hygromycin B treatment; this dichotomy again corresponds to their *s-hhy* and *d-hhy*

classifications. In most *s-hhy* mutants, Tor1-GFP vacuolar localization was slightly compromised in untreated cells and further perturbed in presence of hygromycin B. *s-hhy* mutants also exhibited their previously reported aberrant vacuolar morphologies in the absence of hygromycin B, which were then exaggerated upon treatment. As in *s-hhy* mutants, Tor1-GFP vacuolar localization was slightly compromised in untreated *d-hhy* cells. However, in contrast to *s-hhy* mutants, both Tor1-GFP localization and vacuolar morphology were not further compromised in hygromycin B-treated *d-hhy* strains. Studies of Vam3-RFP or Pep12-RFP localization in *TOR1-GFP* background confirmed that hygromycin B treatment (10µg/ml for 4 hours) disrupts Tor1-GFP and Pep12-RFP localizations but not that of Vam3-RFP (data not presented).

The onset of severe vacuolar morphology defects upon hygromycin B treatment supports a general block in endosome-dependent vacuolar trafficking that includes, but may not be limited to, localization of Pep12 and Tor1. The undisturbed pattern of FM4-64 localization to the vacuole in hygromycin B treated *hhy* strains is indicative of undisturbed bulk endocytosis which includes late endosome to vacuole stage of trafficking. Taken together, these results further pinpoint the trans-Golgi and late endosome interface specifically as the compromised stage of vacuolar trafficking in *s-hhy* mutants and as correlative with hygromycin B super-hypersensitivity. Furthermore, as Tor1 trafficking in *s-hhy* mutants is defective and hypersensitive to hygromycin B, our results also implicate intact trans-Golgi and late endosome interface as a requisite for efficient Tor1 vacuolar localization.

Hygromycin B treated *s-hhy* mutants are viable but exhibit growth arrest and defects in recovery from growth arrest

Since our results established compromised Tor1 vacuolar localization, we next probed whether TORC1 function was compromised and/or hypersensitive to hygromycin B in *hhy* mutants. First we assessed whether the observed lack of growth of mutants in 10 µg/ml hygromycin B is due to growth arrest indicative of defective TORC1 signaling or whether it is due to cell death. To assess cell death, a viability assay was performed using propidium iodide, which only penetrates dead cells due to their leaky membranes. Cells were stained with propidium iodide after the routinely used 4 hour hygromycin B treatment and were subsequently analyzed by epifluorescence and stained cells were quantified by scoring (Figure 5a). Hygromycin B-treated *hhy* mutants showed slight or no drop in cell viability relative to their mock-treated counterparts, implicating growth arrest in presence of hygromycin B.

Deletion of individual components of TORC1, or those of the EGO complex which regulates TORC1, results in inability to recover from rapamycin induced growth arrest (Dubouloz et al., 2005). More recently, EGO complex-TORC1 interactions were shown to be perturbed in vacuolar trafficking Vps-C mutants as assessed by their defect in recovery from rapamycin induced growth arrest (Kingsbury et al. 2014). To evaluate the state of EGO complex-TORC1 interactions in *hhy* mutants, we assessed the ability of *hhy* mutants to recover from both hygromycin B-induced and rapamycin-induced growth arrest. Wild type and mutant strains were either mock-treated or treated with 10µg/ml hygromycin B for 4 hours, the drug was removed by multiple washes, and cells were resuspended in YPD without hygromycin

B and incubated with aeration for another 24 hours. The OD₆₀₀ of each individual culture was read immediately following 4-hour incubation and again after 24 hours post treatment (Figure 5b). All strains remained viable 24 hours post-hygromycin B treatment as assessed by propidium iodide staining (data not presented). In wild-type cells, both treated and mock-treated cells showed equivalent growth 24 hours post-hygromycin B treatment. Interestingly, *hhy* mutants once again exhibited a dichotomy in cell cycle arrest recovery that corresponded to that seen for hygromycin B hypersensitivity and Tor1-GFP localization. Hygromycin B-treated *s-hhy* mutants showed strong inhibition of growth recovery following hygromycin B treatment relative to their mock-treated counterparts, while *d-hhy* mutants did not. Growth recovery defects of *s-hhy* mutants were confirmed by quantitative growth assays of serially diluted strains on rich media following mock or hygromycin B treatment (Figure 5c). In both liquid and solid media growth assays, *s-hhy* mutant *arf1* exhibited an intermediate growth defect relative to other *s-hhy*'s. To assess whether the growth recovery defect of *s-hhy* mutants is unique to hygromycin B treatment, we also probed their growth recovery following rapamycin treatment (Figure 5d). With the exception of *chc1* and *vps34* which exhibited full inhibition of growth recovery comparable to that observed in the control class C *vps16* mutant, *s-hhy* mutants exhibited a wide range of partial growth recovery defects following rapamycin treatment.

The viability and recovery results implicate compromised cell cycle progression and growth arrest recovery in *s-hhy* mutants, both of which are hallmarks of compromised TORC1 function. *s-hhy* mutants exhibited a wide range of growth recovery defect following rapamycin treatment and uniform severe recovery defect following hygromycin B treatment, suggesting that the observed super-hypersensitivity is unique to hygromycin B.

TORC1 kinase function is compromised in *s-hhy* mutants

Since our results suggest TORC1 signaling defects, we next assessed kinase activity of TORC1 directly and analyzed the phosphorylation of Sch9, a direct substrate of TORC1 (Urban et al. 2007). Sch9 is the yeast equivalent of mTORC1 substrate S6 kinase and a regulator of translational initiation and sphingolipid metabolism in *S. cerevisiae* (Urban et al. 2007; Swinnen et al. 2014). We followed the Sch9 mobility upshift assay approach reported by Urban et al. (2007), where due to the large size of Sch9, partial proteolytic cleavage products of exogenously expressed Sch9-HA were generated by 2-nitro-5-thiocyanobenzoic acid (NTCB), and fragments were assessed for phosphorylation-dependent upshift in SDS-PAGE. Figure 6a shows the migration profile of full length and partial degradation products of Sch9 that retain the HA-tagged c-terminus under various drug treatments for the wild type parental strain. Consistent with previous reports, phosphorylation of both full length and c-terminal partial degradation products of Sch9-HA was severely inhibited in presence of rapamycin. Also consistent with previous reports, Sch9-HA phosphorylation was maximized in the presence of the translation inhibitor cycloheximide (Figure 6A), presumably due to upregulation of TORC1 as free intracellular amino acid levels rise during translation inhibition, as suggested by Urban et al. (2007). As expected, Sch9 phosphorylation was not sensitive to 10µg/ml hygromycin B in wild type, and when wild type cells were treated with hygromycin B and cycloheximide simultaneously, there was no significant difference in Sch9 phosphorylation compared to cycloheximide-only treatment (Figure 6a).

Next, *s-hhy* mutants were assessed similarly in multiple replicates. Different *s-hhy* mutants vary in their growth rates and in protein yields from wild type samples processed in parallel. Therefore, we used the mid-range of partial degradation products highlighted in the box in Figure 6a to compare extent of Sch9 phosphorylation, since they were consistently visible in mutant strains. Low exposure autoradiographs were subjected to densitometry, and percent full phosphorylation was calculated as the top band of the boxed region over the sum of top plus bottom bands. A typical experimental set is presented in Figure 6b and percent full phosphorylation is expressed under each treatment lane. For untreated samples, extent of full phosphorylation from at least two different experimental sets was normalized to that of untreated wild type Sch9 and expressed in Figure 6c. These results show that Sch9 phosphorylation is significantly compromised in *s-hhy* mutants even in absence of hygromycin B treatment. *sac1*, *vps1*, *vps34* and *vps52* showed the largest defect in Sch9 phosphorylation levels at consistently <40% of wild type. As in wild type cells, phosphorylation of Sch9 was lost in *s-hhy* mutants in presence of the TORC1 inhibitor rapamycin and was significantly increased in presence of cycloheximide, indicating that these parameters of TORC1 regulation are not disturbed in *s-hhy* mutants. We were not able to see significant or consistent changes in Sch9 phosphorylation in *s-hhy* mutants upon the same duration and extent of treatment with hygromycin B alone (data not presented), presumably due to their already compromised TORC1 activity shown in Figure 6c. However, when *s-hhy* mutants were treated with hygromycin B while simultaneously stimulating their TORC1 activity with cycloheximide, their Sch9 phosphorylation was further compromised relative to levels seen in cycloheximide treatment alone. The exceptions were *arf1* strain which uniquely showed increased phosphorylation and *drs2* whose Sch9 phosphorylation did not show statistically significant changes. The antagonistic effects of the two translation inhibitors hygromycin B and cycloheximide on almost all *s-hhy* mutants further support a translation independent target for hygromycin B in these mutants.

Thus, Tor1 kinase function, assayed by phosphorylation of its direct substrate, is compromised in *s-hhy* mutants. These results indicate that intact Golgi and late endosome interface is necessary for wild type levels of TORC1 kinase activity. Taken together with defects in Tor1-GFP localization, cell proliferation, and growth recovery in *s-hhy* mutants, our results suggest that efficient Tor1 vacuolar localization is necessary for wild type levels of growth promoting functions of TORC1.

DISCUSSION

We previously reported a genomic screen for hypersensitivity to hygromycin B (*hhy*) mutants and established that *hhy* mutant collection is defective in vacuolar events; the collection is also hypersensitive to rapamycin and caffeine suggestive of compromised TORC1 signaling (Banuelos et al. 2010). Here, we define a super-hypersensitive (*s-HHY*) subgroup of genes that inclusively or exclusively function at the trans-Golgi and late-endosome interface. We also show that Tor1 vacuolar localization, as well as TORC1 growth promoting functions, are compromised and hypersensitive to hygromycin B in *s-hhy* mutants. Together our results establish correlation between compromised Golgi and late endosome interface and super-hypersensitivity to hygromycin B. They also implicate an

intact trans-Golgi and late endosome trafficking interface as a requisite for efficient Tor1 vacuolar localization and TORC1 anabolic functions.

Hygromycin B vulnerability and compromised trans-Golgi and late endosome interface

The *s-HHY* genes are *ARF1*, *CHC1*, *DRS2*, *SAC1*, *VPS1*, *VPS34*, *VPS45*, *VPS52*, and *VPS54*; each has a known role in vesicular trafficking at the trans-Golgi and late endosome interface. Thus, a compromised trans-Golgi and late endosome interface directly correlates with severe hygromycin B hypersensitivity. Undisturbed endosome-independent vacuolar localization of Vam3-RFP in *s-hhy* mutants in presence or absence of hygromycin B indicates that direct Golgi to vacuole trafficking is not compromised nor hypersensitive to the drug. Similarly, undisturbed FM4-64 endocytosis and localization to the vacuole membrane in *hhy* mutants indicates that late endosome to vacuole trafficking is also neither compromised nor hypersensitive to hygromycin in these mutants. Exclusion of all class E and class C *vps* mutants from the uncovered *hhy* collection indicates that compromised multivesicular body formation or vacuolar biogenesis, respectively, are not associated with hypersensitivity to hygromycin B. Lastly, unique hypersensitivity of *hhy* mutants, specifically *s-hhy* mutants, to hygromycin B but not to another translation inhibitor suggests that hygromycin B hypersensitivity is independent of the state of translation machinery; a similar conclusion was reached by Conboy and Cyert (2000) whose mutants were sensitive to hygromycin B but not to additional translation inhibitors. These results, together with the extensive vacuolar morphology defects as well as localization disturbances of Tor1 and the endosomal marker Pep12 in *s-hhy* mutants, support a general trafficking disturbance specifically at Golgi and late endosome interface. Among *s-hhy* mutants, *arf1* is unique in its range of milder or no hygromycin super-hypersensitive phenotypes assayed; this is most likely due to partial functional compensation by Arf2.

What may be the molecular target of hygromycin B in *s-hhy* mutants? While all *s-HHY* gene products function at the trans-Golgi and late endosome interface, they are not part of one molecular complex. Arf1, Chc1 and Vps1 are involved in clathrin coated vesicle formation and budding at trans-Golgi; Vps45 is involved in vesicle docking at late endosome; and Vps52 and Vps54 are two members of the GARP (Golgi Associated Retrograde Protein) complex involved in fusion of late endosome derived retrograde vesicles with Golgi (Bonifacino and Hierro 2011; Conibear and Stevens 2000; Bryant and James 2001). What these gene products have in common is association with membranes during either budding or fusion events at Golgi and late endosome interface. Interestingly, the other two members of GARP complex, Vps51 and Vps53, were not picked up in the screen and did not show *hhy* phenotype upon reexamination (data not presented), suggesting that only GARP complexes missing Vps52 or Vps54 may be compromised sufficiently to be associated with hygromycin B hypersensitivity. The remainder of *s-HHY* gene products function directly in membrane lipid events. Drs2 is the trans-Golgi flippase associated with promoting microdomains and membrane curvature (Graham 2004; Hua et al. 2002.), Sac1p is the phosphoinositide phosphatase involved in generation of PI(3)P, and Vps34p is the kinase that phosphorylates phosphatidylinositol to produce PI(3)P. Interestingly, PI(3)P is the phosphoinositide species enriched in endosomal membranes and implicated in vesicular trafficking (Bhandari et al. 2007; De Camilli et al. 1996); it has also been implicated in

TORC1 regulation (Bridges et al. 2012). Another phosphoinositide, PI(3,5)P₂, has recently been reported to play a role in nutrient sensing by TORC1 (Jin et al. 2014). It is tempting to speculate, therefore, that hygromycin B may be targeting membrane microdomains engaged in fusion and fission events, and that such microdomains may also be important in recruitment and/or trafficking of Tor1. As such, compromised or PI3P-deficient microdomains predicted in *drs2*, *sac1* and *vps34*, or compromised budding/fusion complexes predicted in the remainder of *s-hhy* mutants may be especially vulnerable to hygromycin B. In fact, aminoglycoside drugs have been reported to interfere with coatamer formation and secretion in mammalian cells (Hudson and Draper 1997; Hu et al. 1999). While translation inhibition function of hygromycin B is a cytoplasmic event, the pathway of hygromycin B entry into cells remains unknown. Prior research has suggested that in mammalian cells, aminoglycoside antibiotics are taken up by receptor-mediated endocytosis (Hashino et al. 1995; Hashino et al. 1997), which would not be inconsistent with direct endomembrane interactions at late endosome and trans-Golgi interface. Alternatively, hygromycin B may be indirectly disruptive to a compromised trans-Golgi and late endosome interface.

Late endosome-dependent vacuolar trafficking as a requisite for wild type levels of Tor1 localization and TORC1 function

Our results indicate that late endosome-dependent vacuolar trafficking is compromised in *s-hhy* mutants and necessary for wild type levels of Tor1 vacuolar localization and TORC1 anabolic functions. Since TORC1 functions are compromised in hygromycin B treated *s-hhy* mutants, localization of Tor2, which can functionally substitute for Tor1, is presumably blocked as well. Since our results suggest an intact late-endosome independent trafficking pathway to vacuoles, late-endosome dependent trafficking may be the main pathway utilized by Tor1 and Tor2 for their vacuolar localization. Consistent with that hypothesis, both proteins lack the only two known late-endosome independent and AP-3 dependent trafficking consensus sequences. Alternatively, late-endosome dependent trafficking of other molecules involved in recruitment of Tor1/2 and TORC1 activation may be compromised in *s-hhy* mutants.

s-hhy strains have compromised Tor1-GFP vacuolar localization and Sch9 phosphorylation even in the absence of hygromycin B. This is not surprising considering the direct roles of *s-HHY* gene products in vacuolar trafficking. Furthermore, Vps34p has been shown to directly interact with Tor1p and may be an upstream regulator of TORC1 (Aronova et al. 2007; Jacinto 2008), and physical interactions have been reported between Vps54p and Tor1p (Krogan et al. 2006). Several studies to date have suggested a link between compromised TORC1 function and defects in vacuolar trafficking and function (Zurita-Martinez et al. 2007; Brown et al. 2010; Flinn & Backer 2010). Most recently, Kingsbury et al. (2014) reported that mutations in members of HOPS and CORVET complexes (required for homotypic and heterotypic vacuolar fusion events, endosomal trafficking, and retrograde trafficking from the vacuole) cause defects in TORC1 signaling. While *HHY* gene products and HOPS/CORVET components do not overlap, these results together further implicate intact endomembrane trafficking to the vacuole as a requisite for TORC1 signaling. While mTORC1 is recruited to the lysosomal membrane and activated based on nutrient signaling,

yeast TORC1 localization appears to be constitutively at the vacuole (Betz and Hall 2013; Binda et al 2009). As Tor1 localization remains punctate following treatment in most *s-hhy* mutants, our results support association of Tor1p with the endomembrane at pre-vacuolar compartments, and its subsequent delivery to the vacuole through the late endosome-dependent pathway. This is consistent with a recent report where during Ras-induced senescence, mTOR is recruited to trans-Golgi before transport to a novel lysosomal derived TOR-autophagy spatial coupling compartment (TASCC) (Narita et al. 2011). Interestingly, we don't observe upregulated autophagy in hygromycin B treated *s-hhy* mutants; in fact, they appear to be defective in autophagy under starvation and rapamycin treatment (Ejzykowicz, Ruiz and Gharakhanian, preliminary results). This suggests that vacuolar localization may be required for both anabolic and catabolic functions of TORC1.

Implications

Rapamycin and its analogs (rapalogs) are the main pharmacological agents currently explored to control TORC1 activity as well as mTORC1 hyperactivity in cancer and diabetes (reviewed in Seto 2012; Cornu et al. 2013). However, effective concentrations are highly toxic, and cancer cells rapidly develop resistance to single drug approaches. The search for additional inhibitors of TORC1 is ongoing and the small molecule TORC1 inhibitor CID 3528206 has been reported from a systemic screen (Chen et al. 2012). An interesting implication of our studies is that sub-translation inhibitory levels of hygromycin B can be a potent negative regulator of TORC1. Low doses of hygromycin B in combination with rapamycin or with other drugs that either disturb late endosome-dependent vesicular trafficking or inhibit a particular *s-HHY* gene product may be effective in TORC1 downregulation. We are currently exploring such drug combinations and our preliminary results indicate a synergistic drug combination effect between hygromycin B and rapamycin (Priscilla Bravo and E. Gharakhanian, unpublished results). Controlling TORC1 activity by low-dose drug combinations with hygromycin B offers alternatives for regulating TORC1 in functional studies as well as translational potential in regulating mTORC1 hyperactivity in disease.

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Fig. 1.

hhy mutants exhibit a dichotomy of growth kinetics in presence of hygromycin B. Wild-type and *hhy* strains were grown in presence of 0, 10, 20, and 40 μg/ml of hygromycin B and their OD₅₉₅ were read every 2 hours for 12 hours and then at 24 hours. *hhy* mutants were subsequently divided into two groups: those that did not show growth at any of the tested hygromycin B concentrations (super-*hhy*= *s-hhy*), and those that showed dose-dependent growth (*d-hhy*); they are listed along with their known gene functions in Table 1.

Fig. 2.

hhy mutants do not exhibit parallel increased sensitivities to cycloheximide. Wild-type and *hhy* strains were grown in presence of 0, 0.025, 0.05, and 0.10 μg/ml of cycloheximide and their OD₆₀₀ were read every 2 hours for 24 hours.

Fig. 3.

Hygromycin B treatment alters localization of late endosomal marker Pep12-RFP but not that of the late endosome-independent vacuolar SNARE Vam3-RFP. Localization of Vam3-RFP and Pep12-RFP was assessed in *s-hhy* strains using confocal and nomarski optics microscopy after 4 hours of growth in the presence or absence of 10 μ g/ml hygromycin B. Scale bar = 5 μ m for all panels.

Fig. 4.

Tor1-GFP Localization and Vacuolar Morphology are altered and sensitive to hygromycin B treatment in *s-hhy* mutants. *HHY* genes were individually deleted in a strain in which endogenous *TOR1* was tagged with 3x-GFP (*TOR1-GFP*). Tor1-GFP localization was then assessed by confocal microscopy and nomarski optics after 4 hours of treatment with 10μg/ml hygromycin B. To assess vacuolar morphology and Tor1-GFP vacuolar co-localization, cells were stained with the endocytic vacuolar membrane marker FM4-64. Scale bar = 5μm for all panels. >150 cells were scored for each mutant from multiple trials, and presented in Table 4. a) *s-hhy* strains and b) *d-hhy* strains.

Fig. 5. Hygromycin B treated *s-hhy* strains are viable and defective in recovery from hygromycin B-induced growth arrest a) Viability of *hhy* mutants after 4 hours of treatment with 10µg/ml hygromycin B was assessed microscopically using propidium iodide (PI) staining. Percent viability is the number of live (unstained) cells over total (stained + unstained) cells counted and is shown for treated (black bars) and untreated (white bars) cells. b) Ability of *hhy* strains to recover from hygromycin B induced growth arrest was assessed by determining cell density after 4 hours of treatment or mock-treatment with 10 µg/ml hygromycin B

(white bars) and 24 hours after removal of hygromycin B (black bars). c) *s-hhy* cultures were treated with 10µg/ml hygromycin B for 4 hours, then washed and spotted as 10-fold serial dilutions on YPD plates. d) Ability of *s-hhy* strains to recover from rapamycin-induced growth arrest was assessed by determining cell density immediately and after 4 hours of 200ng/ml rapamycin treatment and also 24 and 48 hours after removal of the drug. Parallel untreated samples are denoted as NT. *vps16* strain served as a control growth recovery defective mutant.

Fig. 6.

Tor1 kinase function is compromised in *s-hhy* mutants. Wild type and *s-hhy* strains expressing a plasmid-based copy of Sch9-HA tag were grown exponentially and treated for 30 min with rapamycin (200ng/ml), 30 min with cycloheximide (25μg/ml), or 4 hours with hygromycin B (10μg/ml) with cycloheximide (25μg/ml) added for the final 30 minutes. Proteins were extracted as described by Urban et al, 2007 and treated or mock-treated with NTCB for cleavage. NTCB Samples were analyzed by SDS-PAGE, and immunoblots were probed with anti-HA antibody. a) Full migration profile of Sch9 in wild type cells under the

various treatments. Left panel contains NTCB mock-treated samples. Right panel contains samples treated with NTCB; the boxed mid-region was consistently visualized in all mutant strains and was used for quantifications. b) The region corresponding to the boxed area in 6A is presented. Extent of phosphorylation was quantified by densitometric analysis of this region using Image J software as described in Materials and Methods. 0= no treatment, R=rapamycin, C=cycloheximide, CH = cycloheximide plus hygromycin B. c) For comparison of phosphorylation among untreated samples, extent of phosphorylation was set to 100% for untreated WT cells and phosphorylation of mutants was expressed relative to WT in parallel experiments. Data include two independent trials per mutant.

TABLE 1

Names and Functions of s-*HHY* and d-*HHY* genes

	Gene	Function
s- <i>HHY</i>	<i>CHC1</i>	clathrin heavy chain, major coat protein of clathrin coated vesicles
	<i>DRS2</i>	trans-Golgi flippase involved in clathrin coated vesicle formation
	<i>SAC1</i>	PI-4-P phosphatase involved in protein trafficking and secretion
	<i>VPS1</i>	dynamain-like GTPase involved in secretory vesicle production
	<i>VPS34</i>	PI-3 kinase; regulates protein sorting and vesicle mediated trafficking
	<i>VPS45</i>	vesicle fusion at Golgi, endosome & vacuole; SNARE complex formation
	<i>VPS52</i>	endosome-late Golgi recycling; GARP complex; Protein sorting to vacuole
	<i>VPS54</i>	endosome-late Golgi recycling; GARP complex; Protein sorting to vacuole
	<i>ARF1</i>	ADP-ribosylation factor; COPI and clathrin coated vesicle formation at Golgi
d- <i>HHY</i>	<i>BUD32</i>	Ser/Thr kinase; tRNA modification; telomere maintenance & transcription
	<i>DHH1</i>	Dead box helicase; mRNA sorting, exporting, decay & translation
	<i>PAF1</i>	Transcription elongation; modifies activity of RNA pol.'s I & II
	<i>TPD3</i>	Reg. subunit of PP2A; required for transcription by RNA pol. III

TABLE 2

Yeast strains used in this study

Strain	Genotype	Reference
BY4742	<i>MATa his3 1 leu2 0 lys2 0 ura2 0</i>	Brachmann et al. 1998
<i>arf1</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 arf1::KAN</i>	Wach et al. 1994
<i>bud32</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 bud32::KAN</i>	Wach et al. 1994
<i>chc1</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 chc1::KAN</i>	Wach et al. 1994
<i>dhh1</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 dhh1::KAN</i>	Wach et al. 1994
<i>drs2</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 drs2::KAN</i>	Wach et al. 1994
<i>paf1</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 paf1::KAN</i>	Wach et al. 1994
<i>sac1</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 sac1::KAN</i>	Wach et al. 1994
<i>tpd3</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 tpd3::KAN</i>	Wach et al. 1994
<i>vps1</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 vps1::KAN</i>	Wach et al. 1994
<i>vps34</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 vps34::KAN</i>	Wach et al. 1994
<i>vps45</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 vps45::KAN</i>	Wach et al. 1994
<i>vps52</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 vps52::KAN</i>	Wach et al. 1994
<i>vps54</i>	<i>MATa his3 1 leu2 0 lys2 0 ura2 0 vps54::KAN</i>	Wach et al. 1994
MP52-2A (TOR1-GFP)	[YL516] <i>MATa; TOR1-D330-3xGFP</i>	Binda et al. 2009
<i>MP52-2A TOR1-GFP+PEP12-RFP</i>		This study
<i>MP52-2A TOR1-GFP+ VAM3-RFP</i>		This study
BY4742 + SCH9-HA		This study
<i>arf1</i> TOR1-GFP	[YL516] <i>MATa; TOR1-D330-3xGFP arf1::KAN</i>	This study
<i>arf1</i> TOR1-GFP+PEP12-RFP		This study
<i>arf1</i> TOR1-GFP+ VAM3-RFP		This study
<i>arf1</i> + SCH9-HA		This study
<i>bud32</i> TOR1-GFP	[YL516] <i>MATa; TOR1-D330-3xGFP bud32::KAN</i>	This study
<i>bud32</i> TOR1-GFP+ PEP12-RFP		This study
<i>bud32</i> TOR1-GFP+VAM3-RFP		This study
<i>bud32</i> + SCH9-HA		This study
<i>chc1</i> TOR1-GFP	[YL516] <i>MATa; TOR1-D330-3xGFP chc1::KAN</i>	This study
<i>chc1</i> TOR1-GFP+ PEP12-RFP		This study
<i>chc1</i> TOR1-GFP+ VAM3-RFP		This study
<i>chc1</i> + SCH9-HA		This study
<i>dhh1</i> TOR1-GFP	[YL516] <i>MATa; TOR1-D330-3xGFP dhh1::KAN</i>	This study
<i>dhh1</i> TOR1-GFP+ PEP12-RFP		This study
<i>dhh1</i> TOR1-GFP+VAM3-RFP		This study
<i>dhh1</i> + SCH9-HA		This study
<i>drs2</i> TOR1-GFP	[YL516] <i>MATa; TOR1-D330-3xGFP drs2::KAN</i>	This study
<i>drs2</i> TOR1-GFP+ PEP12-RFP		This study
<i>drs2</i> TOR1-GFP+VAM3-RFP		This study
<i>drs2</i> + SCH9-HA		This study
<i>paf1</i> TOR1-GFP	[YL516] <i>MATa; TOR1-D330-3xGFP paf1::KAN</i>	This study

Strain	Genotype	Reference
<i>paf1</i>	<i>TOR1-GFP+ PEP12-RFP</i>	This study
<i>paf1</i>	<i>TOR1-GFP+ VAM3-RFP</i>	This study
<i>paf1</i>	+ SCH9-HA	This study
<i>sac1</i>	<i>TOR1-GFP</i> [YL516] <i>MATa</i> ; <i>TOR1-D330-3xGFP sac1::KAN</i>	This study
<i>sac1</i>	<i>TOR1-GFP+ PEP12-RFP</i>	This study
<i>sac1</i>	<i>TOR1-GFP+ VAM3-RFP</i>	This study
<i>sac1</i>	+ SCH9-HA	This study
<i>tpd3</i>	<i>TOR1-GFP</i> [YL516] <i>MATa</i> ; <i>TOR1-D330-3xGFP tpd3::KAN</i>	This study
<i>tpd3</i>	<i>TOR1-GFP+ PEP12-RFP</i>	This study
<i>tpd3</i>	<i>TOR1-GFP+ VAM3-RFP</i>	This study
<i>tpd3</i>	+ SCH9-HA	This study
<i>vps1</i>	<i>TOR1-GFP</i> [YL516] <i>MATa</i> ; <i>TOR1-D330-3xGFP vps1::KAN</i>	This study
<i>vps1</i>	<i>TOR1-GFP+ PEP12-RFP</i>	This study
<i>vps1</i>	<i>TOR1-GFP+ VAM3-RFP</i>	This study
<i>vps1</i>	+ SCH9-HA	This study
<i>vps34</i>	<i>TOR1-GFP</i> [YL516] <i>MATa</i> ; <i>TOR1-D330-3xGFP vps34::KAN</i>	This study
<i>vps34</i>	<i>TOR1-GFP+ PEP12-RFP</i>	This study
<i>vps34</i>	<i>TOR1-GFP+ VAM3-RFP</i>	This study
<i>vps34</i>	+ SCH9-HA	This study
<i>vps45</i>	<i>TOR1-GFP</i> [YL516] <i>MATa</i> ; <i>TOR1-D330-3xGFP vps45::KAN</i>	This study
<i>vps45</i>	<i>TOR1-GFP+ PEP12-RFP</i>	This study
<i>vps45</i>	<i>TOR1-GFP+ VAM3-RFP</i>	This study
<i>vps45</i>	+ SCH9-HA	This study
<i>vps52</i>	<i>TOR1-GFP</i> [YL516] <i>MATa</i> ; <i>TOR1-D330-3xGFP vps52::KAN</i>	This study
<i>vps52</i>	<i>TOR1-GFP+ PEP12-RFP</i>	This study
<i>vps52</i>	<i>TOR1-GFP+ VAM3-RFP</i>	This study
<i>vps52</i>	+ SCH9-HA	This study
<i>vps54</i>	<i>TOR1-GFP</i> [YL516] <i>MATa</i> ; <i>TOR1-D330-3xGFP vps54::KAN</i>	This study
<i>vps54</i>	<i>TOR1-GFP+ PEP12-RFP</i>	This study
<i>vps54</i>	<i>TOR1-GFP+ VAM3-RFP</i>	This study
<i>vps54</i>	+ SCH9-HA	This study

TABLE 3

Primer sequences used in this study

Name		Sequence (5' - 3')
CHC1KO-F	Forward	CCGGATCAGAGTTGAGAGAG
CHC1KO-R	Reverse	TCACCAGCAAGACCTTGATCTGA
CHC1KOCheck-F	Forward	AGGGCTTAAAATGTGTCAGGG
DHH1KO-F	Forward	CCGCATCGCCATTTCGCATAA
DHH1KO-R	Reverse	GCACAGAACTAGAGAAAACGG
DHH1KOCheck-F	Forward	GGGCGATTGTAACATTGGGG
DRS2KO-F	Forward	AACGCCAAATAGAGGTTAGCG
DRS2KO-R	Reverse	ACCGATAACAAAGTGATGGC
DRS2KOCheck-F	Forward	GGGGAATAGTGCAACAAGAAGCC
PAF1KO-F	Forward	ATGACGACTGTAACATTGGA
PAF1KO-R	Reverse	CTTCTACATTGAGGTAATGC
PAF1KOCheck-F	Forward	CACGCAATGAGAACCCTAACC
VPS1KO-F	Forward	AGAGAGGCCTTTTATAGCACC
VPS1KO-R	Reverse	GGATCGTAATGCGAGATAGGA
VPS1KOCheck-F	Forward	CCAGAGGATAGTATGATAGC
VPS16KO-F	Forward	GCAATCGTCTCCATATCAATC
VPS16KO-R	Reverse	CGCACAAACGTAATGGCAGCA
VPS16KOCheck-F	Forward	TCATCGCAGCAGATCGTG
VPS34KO-F	Forward	GCAGATCCGGCATCAAACAC
VPS34KO-R	Reverse	TATGCCACATTAGGGGGAGG
VPS34KOCheck-F	Forward	GTCTGAGCGTTTGCGCTGAG
VPS45KO-F	Forward	GGAGTGAAGAGGTACAGTGA
VPS45KO-R	Reverse	AGAACCTAATTTGCTTGCC
VPS45KOCheck-F	Forward	GGCGTCCGTAACGAGTACTG
VPS52KO-F	Forward	CAAGTGAGAGGAAAGAGGAA
VPS52KO-R	Reverse	GGTAGGTCGTAAACATTTG
VPS52KOCheck-F	Forward	GCTCAAGGGTTGGTAAAGCC
KanMX4-Rev	Reverse	CTGCAGCGAGGAGCCGTAAT

TABLE 4
Vacuolar Morphology and Tor1-GFP Localization in *hhy* mutant strains relative to WT.

		TOR1-GFP Localization Summary Table			
		Untreated		10 µg/ml Hygromycin B	
	Gene	Vacuole Morphology	TOR1-GFP vacuolar Localization	Vacuole Morphology	TOR1-GFP vacuolar Localization
<i>s-hhy</i>	<i>TOR1-GFP</i>	WT	+++	WT	+++
	<i>chc1</i>	Somewhat Fragmented	++	Fragmented	+/-
	<i>drs2</i>	WT-like	++	Fragmented	+
	<i>sac1</i>	WT-like	++	Fragmented	-
	<i>vps1</i>	Large central surrounded by small	++	Large central surrounded by small	-
	<i>vps34</i>	Enlarged	++	Enlarged	-
	<i>vps45</i>	Enlarged	++	Enlarged/fragmented	-
	<i>vps52</i>	Fragmented	+	Fragmented	-
	<i>vps54</i>	Fragmented	++	Fragmented/disordered	-
	<i>arf1</i>	Somewhat Fragmented	++	Somewhat Fragmented	+
<i>d-hhy</i>	<i>bud32</i>	WT-like	++	WT-like	++
	<i>dhh1</i>	WT-like	++	WT-like	++
	<i>pac1</i>	Fragmented	++	Fragmented	+
	<i>tpd3</i>	WT-like	++	WT-like	++