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Genomic analysis of severe hypersensitivity to hygromycin B reveals linkage to vacuolar defects and new vacuolar gene functions in *Saccharomyces cerevisiae*

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Abstract

The vacuole of *Saccharomyces cerevisiae* has been a seminal model for studies of lysosomal trafficking, biogenesis, and function. Several yeast mutants defective in such vacuolar events have been unable to grow at low levels of hygromycin B, an aminoglycoside antibiotic. We hypothesized that such severe hypersensitivity to hygromycin B (*hhy*) is linked to vacuolar defects and performed a genomic screen for the phenotype using a haploid deletion strain library of non-essential genes. Fourteen *HHY* genes were initially identified and were subjected to bioinformatics analyses. The uncovered *hhy* mutants were experimentally characterized with respect to vesicular trafficking, vacuole morphology, and growth under various stress and drug conditions. The combination of bioinformatics analyses and phenotypic characterizations implicate defects in vesicular trafficking, vacuole fusion/fission, or vacuole function in all *hhy* mutants. The collection was enriched for sensitivity to monensin, indicative of vacuolar trafficking defects. Additionally, all *hhy* mutants showed severe sensitivities to rapamycin and caffeine, suggestive of TOR kinase pathway defects. Our experimental results also establish a new role in vacuolar and vesicular functions for two genes: *PAF1*, encoding a RNAP II-associated protein required for expression of cell cycle-regulated genes, and *TPD3*, encoding the regulatory subunit of protein phosphatase 2A. Thus, our results support linkage between severe hypersensitivity to hygromycin B and vacuolar defects.

Keywords

Yeast vacuole; Lysosome; Hygromycin B; Vesicular trafficking

Introduction

The yeast vacuole is a membrane-bounded organelle analogous to the mammalian lysosome. As such, it has multiple functions associated with degradation, receptor down regulation, ion and pH homeostasis, and stress survival (for comprehensive reviews see Jones et al. 1997; Katzmann et al. 2002; Pelham 2002; Bowers and Stevens 2005; Luzio et al. 2007; Mijaljica et al. 2007; Li and Kane 2009). Most recently, vacuoles/lysosomes have been viewed more as

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regulatory organelles responsible for fine tuning of cellular processes and less as simple storage and degradation compartments. Therefore, vacuole trafficking, biogenesis, and function remain intensively explored fields. Like the mammalian lysosome, the yeast vacuole maintains an acidic environment containing vacuolar hydrolases that degrade macromolecules, structural debris, and waste products (Thumm 2000; Martínez-Muñoz and Kane 2008). As with lysosomes, several pathways are responsible for delivery of cargo to the vacuole. The biosynthetic pathway involves sorting of newly synthesized vacuolar proteins away from the secretory pathway in the trans-Golgi network (TGN) and their vesicular delivery to the vacuole; the pathway includes vacuolar delivery both through the late endosome (CPY-pathway) and independent of it (ALP-pathway) (Nothwehr and Stevens 1994; Horazdovsky et al. 1995; Bryant and Stevens 1998; Conibear and Stevens 1998; Bowers and Stevens 2005). The endocytic pathway involves the vesicular delivery of external and cell surface components through the early endosome to late endosome, and through the maturation product of late endosome—the multivesicular body (MVB)—to the vacuole (Katzmann et al. 2002; Pelham 2002; Piper and Katzmann 2007). The autophagic and cytoplasm-to-vacuole targeting (Cvt) pathways involve the transport of cytoplasmic components directly to the vacuole through vesicular delivery. Under starvation conditions, the autophagy pathway is stimulated in order to provide supplementary reserves for the cell; this pathway is also a housekeeping and stress-responsive process and involves the autophagy (ATG) genes (for latest reviews see Shintani and Klionsky 2004; Levin 2005; Huang and Klionsky 2007; Mijaljica et al. 2007; Li and Kane 2009).

Yeast genetics and molecular genetics have been instrumental in deciphering the vesicular trafficking machinery conserved in eukaryotic cells. Genetic screens have resulted in collections of mutants defective in various stages of vesicular trafficking. Over the past two decades, several genetic screens have uncovered mutant collections defective in the CPY-pathway of vacuolar localization including *pep* (Jones 1977), *vps* (Bankaitis et al. 1986; Rothman and Stevens 1986; Bonangelino et al. 2002), *vam* (Wada et al. 1992), and *env* (Takahashi et al. 2008) mutants. Some mutants in these collections have shown sensitivities to low levels of the antibiotic hygromycin B, an aminoglycoside translation inhibitor isolated from *Streptomyces hygroscopicus* (Mcgaha and Champney 2007). Hygromycin B is an atypical aminoglycoside antibiotic in that it has dual inhibitory effects on translation; it interferes with both ribosomal translocation and with aminoacyl-t-RNA recognition (Cabañas et al. 1978). As such, at 200 μg/mL or more, the drug inhibits the growth of most yeast strains. At 50 μg/mL, hygromycin B has been shown to inhibit growth of yeast mutants defective in glycosylation functions (Ali et al. 2004). At hygromycin B concentrations below 50 μg/mL, several vacuolar trafficking and function mutants have shown degrees of growth sensitivities, including *vps1*, *vps11/pep5*, *vps15*, *vps27*, and *vps44/nhx1*, while *vps45* and *vps54/luv1* have shown complete growth inhibition (Conboy and Cyert 2000; Ali et al. 2004; Mukherjee et al. 2006). Finally, *env1* mutants are unable to grow in the presence of 25 μg/mL hygromycin B (Takahashi et al. 2008).

We hypothesized that severe hypersensitivity to hygromycin B may be associated with a specific set of vacuolar defects and may be used to identify new genes involved in vacuolar events. Several comprehensive yeast genomic screens for various drug sensitivities including hygromycin B have been reported in homozygous, heterozygous, or haploid deletion strain collections (Ross-Macdonald et al. 1999; Parsons et al. 2006; Hillenmeyer et al. 2008; for review see Hoon et al. 2008). The screens have not revealed a strong relationship between hygromycin B sensitivity and vacuolar events, yet none of the screens narrowed the scope of the screened phenotype to complete lack of growth. In this study, we performed a genomic screen for growth *h*ypersensitivity to *h*ygromycin B (*hhy* mutants) as scored by complete growth inhibition at 25 μg/mL hygromycin B. Here, we report identification of 14 *hhy* mutants. The corresponding genes include four *VPS* genes—*VPS34*, *VPS45*, *SAC2* (*VPS52)*, and

LUV1 (*VPS54*); five genes with known functions in vesicular trafficking—*DRS2, SBH2, ARF1, CHC1*, and *SAC1*; two genes that have been classified as *VPS* genes in a genomic screen—*DHH1* and *BUD32*; and two genes with published functions in cell cycle-related events—*TPD3* and *PAF1*. The screen also uncovered *hhy1*, a mutant deleted in *YEL059W* which is classified as a dubious hypothetical open reading frame (ORF). A combination of nomarski and fluorescent microscopy, growth sensitivity studies, biochemical assays, and bioinformatics implicate defects in vacuolar function, morphology, and/or trafficking in all the uncovered mutants.

Materials and methods

Media, yeast strains, and antibodies

Yeast cells were grown in yeast extract-peptone-dextrose (YPD) or synthetic complete (SC) medium; YPD or SC media were supplemented with selective components as specified; all yeast and supplemental chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA). The MAT-α haploid *S. cerevisiae* deletion strain library, parental strains *BY4742* (MAT-α, *his3Δl, leu2Δ0, lys2Δ0*, and *ura3Δ0*) and *BY4741* (MAT-a, *his3Δ1*, *leu2Δ0, met15Δ0*, and *ura3Δ0*), and MAT-ahaploids of the 14 hhy strains were gifts fromDr. Greg Payne (UCLA). The collection of 4,828 strains was developed by the *Saccharomyces* Genome Deletion project and contains 80% of the *Saccharomyces cerevisiae* genome; it was generated by PCR-based disruption of all open reading frames by chromosomal integration of a KanMX4 module through homologous recombination (Wach et al. 1994). Mutants were stored in glycerol in fifty-two 96-well plates at −80°C. The mouse monoclonal anti-proCPY antibody was custom ordered from U. Oregon and was initially developed in T. Stevens Laboratory (U.O.). The secondary goat anti-mouse-HPR antibody was purchased from Pierce (Rockford, IL, USA). The parental supersensitive strain used for halo assays was GPY1796 which was generated from GPY404.2 (MAT-a, *ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9*, and *GAL-MEL sst1*::*LYS*).

Yeast genomic screen for hypersensitivity to hygromycin B (hhy)

MAT-α yeast deletion strains in the BY4742 background were transferred from thawed 96-well plates onto YPD and YPD + hygromycin B (25 μg/mL) plates with a pinning tool. Plates were incubated at 30°C for 72 h. hhy phenotype was scored as absence of growth on YPD + hygromycin B plates in comparison to growth on parallel YPD plates. A secondary screen was conducted in order to confirm absence of growth by streaking putative *hhy* strains onto YPD and YPD + hygromycin B (25 μg/mL) plates. Positive results were verified using the corresponding MAT-a knockout strain in the BY4741 background. The deletion of *YEL059W* ORF in *hhy1* strain was confirmed by PCR. YF 5′-TTTAACTATTGGTATATGTGTCCGTGA-3′ binds upstream of the *YEL059W* ORF, YR 5′-ACTTCTAACAAAAGTGACCATGACG-3′ binds within the *YEL059W* ORF, and KR 5′-CTGCAGCGAGGAGCCGTAAT-3′ binds within the kanamycin cassette. As expected, PCR product of primers YF and YR was a 522-bp band from WT samples while no band was observed in *hhy1* samples; PCR product of YF and KR was a 554-bp band in *hhy1* samples, while no band was observed in WT samples (data not presented).

Reintroduction of deleted ORF's into *tpd3*Δ, *paf1*Δ, and *hhy1*Δ

To determine whether the hygromycin B sensitivity of *tpd3*Δ, *paf1*Δ, and *hhy1*Δ resulted from the absence of the deleted ORF, corresponding ORF's were reintroduced by means of CEN and/or 2-μ vectors. YCp-TPD3 (van Zyl et al. 1992) was a gift from Dr. James Broach (Princeton University). pJJ1371 (unpublished) is a uracil selectable 2-μ vector containing *PAF1* and was a gift from Dr. Joan Betz (Regis University). PWO 0577 contains *PRB1* in pRS426 and was a gift from Dr. Dieter Wolf (University of Stuttgart, Germany). pSEY8-PRB1

(Moehle et al. 1986) was a gift from Dr. Scott Emr (Cornell University). PCR-based subcloning was carried out to construct the vectors containing *YELO59W* sequences. The *YELO59W* ORF from BY4742 genomic DNA was amplified using the following primers containing restriction sites on the 5′ ends. F1 (BamHI) 5′-GGGGGGATCCTTGGCGATGAAGCTAATTG-3′; R1 (EcoRI) 5′-GGGGGAATTCTGACCTTTGCCTTGGTCCT-3′; R2 (EcoRI) 5′-GGGGGAATTCGCCAACTCAACACGAAATTC-3′; R3 (KpnI) 5′-GGGGGGTACCTTTAGCTGCAACACTGCATG-3′. pDOG360 was constructed using F1 and R1 which resulted in a 1.2-kb insert containing 661-bp upstream and 273-bp downstream of *YELO59W* and includes *SOM1*. pDOG361 was constructed using F1 and R2 which resulted in a 1.6-kb insert containing 661-bp upstream and 635-bp downstream of *YELO59W* and includes *SOM1*. pDOG362 was constructed using F1 and R3 which resulted in a 3.4-kb insert containing 661-bp upstream and 2.48-kb downstream of *YELO59W* and includes *SOM1* and *PCM1*. PCR reactions were carried out in a final volume of 50 μL containing genomic DNA (100 ng) by using Phusion DNA polymerase (New England Biolabs) according to the manufacturer's instructions. The PCR products were cloned into the uracilselectable CEN vector pRS316 (Sikorski and Hieter 1989) and expanded in Top 10 chemically competent *E. coli* (Invitrogen). Preparation of plasmid DNA was carried out using the Zippy Plasmid Maxiprep and Midiprep Kits (Zymo Research). The yeast deletion strains were transformed using the Frozen EZ Yeast Transformation II kit (Zymo Research). Parent vectors were cloned into parallel cultures to serve as controls. Transformed cells were selected on synthetic minimal media (SM) without uracil. For complementation studies, colonies were streaked on ammonium sulfate free SM-URA + hygromycin B (50 μg/mL) plates.

Invertase secretion assays

Quantitative liquid invertase assays have been described previously (Johnson et al. 1987). The assay was repeated a minimum of four times for each of the strains, and standard deviations were denoted as error bars.

Halo pheromone assays

A single colony of GPY1796 was added to 3 ml of YPD + 0.5% agar; the mixture was poured onto YPD plates and allowed to solidify for 10 min. Single colonies of *hhy* and *wild type* control strains were patched onto the seeded plates with sterile wooden applicators. Plates were incubated at 30°C overnight. Halo assays were repeated six times.

Immunodetection assays

Single colonies of *hhy* and *wild-type* strains were grown and processed for lysis/no lysis and immunodetection with monoclonal anti-pro-CPY antibody as previously described (Takahashi et al. 2008). Immunodetection assays were repeated a minimum of three times for each of the *hhy* strains.

Nomarski and fluorescence microscopy

FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl)pyridium dibromide] fluorescence microscopy and Nomarski microscopy were performed as previously described (Vida and Emr 1995).

Quinacrine [*N*′-(6-chloro-2-methoxy-acridin-9-yl)- *N*,*N*-diethyl-pentane-1,4-diamine] fluorescence microscopy was performed as previously described (Weisman et al. 1987) in YPD plus 50 mM NA_2HPO_4, pH 7.6.

Stained cells were viewed with a Nikon Eclipse E600 fluorescence microscope using corresponding filters. For each strain, prominent phenotypes were determined by scoring 200–300 cells in random fields.

Growth assays in selective media and at high/low temperatures

Selective media were made by addition of NaCl, KCl, $CaCl_2$, $MnCl_2$, $ZnCl_2$, rapamycin, caffeine, sorbitol, or by adjusting pH during preparation of YPD agar plates to achieve final concentrations or pH values specified in figures and tables. Monensin plates were prepared by addition of monensin to synthetic complete media (SC) to a final concentration of 50 μM. Serial tenfold dilutions up to 1:10,000 were made in 96-well microtiter plates starting with OD_{600} = 1.00 of mid-log cells. A pinning tool was used to transfer cells from wells onto selective and replica YPD or SC plates. Plates were incubated at 30°C for 24, 48, or 72 h based on growth rate of strains on YPD or SC plates. For temperature sensitivity studies, two sets of YPD plates were streaked with *hhy* strains and wild type parental BY4742 and were incubated at 15, 30, and 37°C. Growth was observed every 24 h for 4 days for 37°C and for 14 days for 15°C studies. Growth assays were repeated a minimum of three times.

Bioinformatics/statistical significance calculation

To determine significance of Gene Ontology annotation, genes of interest were queried in GO::Term Finder Saccharomyces Genome Database (SGD), which is comprised of object-oriented Perl models (Boyle et al. 2004). The genes were queried under the three different classes available. Default settings for background, feature type, ORF qualifier, annotation methods, annotation source, and evidence codes were used. Statistical significance (*P* value) is calculated using hypergeometric distribution with a cutoff value set at ≤0.01.

Biostatistical analyses

Liquid invertase assay results and microscopic scoring of mutants were subjected to two sample *t* test to assess statistically significant differences from wild type. The statistical tests were conducted using MINITAB software and the alpha *P* value was set at ≤0.05.

Results

In order to test the hypothesis that severe hypersensitivity to hygromycin B is indicative of defects in vacuolar trafficking and/or function, the yeast haploid deletion strain collection of non-essential genes was subjected to a hygromycin B survival screen. The hygromycin B concentration for the screen was established at 25 μg/mL based on growth sensitivity assays within a range of hygromycin B concentrations performed for *env1* mutant, a novel allele of *VPS35* isolated in our laboratory (Takahashi et al. 2008). At this concentration, *env1* consistently showed no growth upon prolonged incubations, while glycosylation defective mutants with growth defects at 50 μg/mL hygromycin B, grew comparable to wild type (data not shown). The primary screen assayed for lack of growth of stamped strains on YPD plates supplemented with 25 μg/mL hygromycin B (Fig. 1a). *H*ygromycin B *h*ypersensitive mutants (*hhy* mutants) were confirmed by a secondary screen of streaked strains on the same selective media (Fig. 1b). Screening of the complete collection resulted in the identification of 14 mutant strains (<0.3% of the total) with complete growth inhibition in the presence of 25 μg/mL hygromycin B. The 14 strains showed no growth under the assay conditions upon prolonged incubations of 1–2 weeks; additionally, they continued to exhibit severe—but not complete—growth sensitivity in hygromycin B concentrations as low as 10 μg/mL (data not shown). Throughout the primary and secondary screens, the putative strains were identified only by their well/plate numbers to minimize any bias for or against specific genes. *HHY* genes were further verified in the MAT-a background. Table 1 lists the *HHY* genes, their biological process, molecular function, and cellular component as gleaned from Saccharomyces Genome Database

(http://www.yeastgenome.org). 24 additional mutant strains (0.5% of the total) showed slowed growth under the screen condition as well as at hygromycin B concentrations up to 50 μg/mL. They are listed at the bottom of Table 1, but were not pursued further since this study was focused on no growth at 25 μg/mL hygromycin B.

Published information on *HHY* gene products allowed classification into one major and one minor category prior to our characterization studies. Eleven genes have known vesicular and/or vacuole trafficking functions and include four original *VPS* genes *VPS34*, *VPS45*, *SAC2* (*VPS52*), and *LUV1* (*VPS54*) (Banta et al. 1988; Robinson et al. 1988; Bowers and Stevens 2005) and three genes that were uncovered in a genomic *vps* screen: *ARF1*, *DHH1*, and *BUD32* (Bonangelino et al. 2002). The remaining genes in this category are *DRS2*, *SBH2*, *CHC1*, and *SAC1* (Sewell and Kahn 1988; Toikkanen et al. 1996; Chen et al. 1999; Hughes et al. 2000; Gruenberg and Stenmark 2004). The second category is comprised of two genes with established roles in cell cycle regulation and progression. Paf1p, a nuclear RNA polymerase II-associated factor, is required for the expression of cell cycle-regulated genes (Shi et al. 1996, 1997; Porter et al. 2002). *TPD3* encodes the regulatory subunit A of protein phosphatase 2A (PP2A); PP2A is required in multiple cell cycle regulation and budding events (Van Zyl et al. 1992; Wang and Burke 1997; Koren et al. 2004). Finally, one previously uncharacterized dubious ORF, *YEL059W*, was uncovered by our screen; the mutant is named *hhy1*.

HHY genes were queried with the use of controlled vocabulary and hierarchical organization provided by Gene Ontology Term Finder from the Saccharomyces Genome Database. The results showed a cluster frequency of five out of 14 genes with GO terms of "post-Golgi vesicle-mediated transport" and "Golgi vesicle transport", with final *P* values of 1.21e–05 and 1.25e–03, respectively. The five genes for both biological processes were *DRS2*, *ARF1*, *VPS45*, *VPS52*, and *VPS54*. The same five genes and *CHC1* were included in a cluster of six out of 14 genes in the GO term "vesicle-mediated transport" with a final P value of 2.35e–03. *VPS45*, *VPS52*, and *VPS54*—three genes shared by all three clusters—are involved in direct "Golgi to vacuole transport" with a final *P* value of 1.21e–03. However, this does not discard the remaining genes that were not in the GO Term Finder results; it suggests that they have not been annotated yet to the particular gene ontology. The total genes in the background distribution were 7,163; the total number of genes annotated within the distribution—directly and indirectly—varied depending on the GO term (24–345 genes). We may say that the relationship of at least six out of 14 genes is statistically significant based on the *P* values; the significant relationship is in vesicular and vacuolar trafficking. The same bioin-formatic analyses were carried out for the combination of 14 *HHY* genes and the 24 genes that showed slow growth under the screen conditions. No additional functional enrichments were uncovered; however, *RVS167*, encoding an actin-associated protein involved in regulation of endocytosis (Lombardi and Riezman 2001), also fell within the "vesicle-mediated transport" GO term.

Thus, based on published literature as well as bioinformatic analyses, genes involved in vacuole function and/or trafficking were overrepresented in *hhy* mutants. We set out to further characterize the *hhy* mutants with respect to vacuole trafficking, morphology, and function, with a focus on genes that have not been linked extensively to vacuolar events in published literature.

Secretion in *hhy* mutants

The state of secretion was assessed in *hhy* mutants, excluding the four *vps* mutants. vps mutants have been well established as lacking secretion defects (Robinson et al. 1988; Bonangelino et al. 2002). A quantitative liquid assay for invertase secretion was performed to assess the vesicular trafficking functions required for secretion (Fig. 2). Statistical analysis of repeated results indicate secretion levels that are significantly lower than wild type in *tpd3*Δ and

*sac1*Δ mutants ($P = 0.008$ and 0.003, respectively). This suggests that vesicular trafficking step (s) including ER to Golgi, intra-Golgi, or Golgi to plasma membrane may be compromised in the two strains but are intact in the remaining *hhy* mutants.

α-factor processing in *hhy* mutants

In order to assess the secretion of active α-factor, pheromone halo assays were conducted on the 14 *hhy* strains. The halo assay measures the responsiveness of lawn cells of the opposite mating type to mating pheromones. Correct anterograde/retrograde trafficking of Kex2p at the late endosome and Golgi interface is essential for processing and activation of α-factor pheromone prior to its secretion. Thus, in the presence of wild type α-factor, lack of halos is indicative of either defects in secretion of the pheromone, or defects in Kex2p trafficking events at the Golgi and late endosome interface. As expected, all four *vps* mutants resulted in minimal or no halos due to established defects at Golgi and late endosome interface (Fig. 3). Also as expected, *sac1*Δ, *tpd3*Δ, and *bud32*Δ strains which showed a range of reduced invertase secretion resulted in minimal or no halos, as did *chc1*Δ and *arf1*Δ strains whose gene products are involved in Golgi and late endosome trafficking events (Sewell and Kahn 1988;Redding et al. 1996). *paf1*Δ, which had not demonstrated any defects in invertase secretion, was completely defective in generating halos. Results of the invertase secretion and halo assays combined are suggestive of defects affecting Golgi and late endosome interface in *paf1*Δ. Finally, *drs2*Δ, *hhy1*Δ, *sbh2*Δ, and *dhh1*Δ resulted in halos equivalent to wild type; these results combined with the intact invertase secretion in the four mutants indicate intact vesicular trafficking at ER to Golgi, intra-Golgi, Golgi to plasma membrane, and Golgi and late endosome interface stages.

Carboxypeptidase Y processing in *hhy* mutants

The vacuolar enzyme carboxypeptidase Y (CPY) transits through the ER, Golgi, and late endosome before arriving at the vacuole. During transit, it is processed from pro-forms (p1CPY and p2CPY glycosylated forms) to the mature CPY through proteolytic cleavage by proteinase A. Vesicular trafficking defects and/or vacuolar function defects lead to persistent forms of pro-CPY. An immunodetection experiment was performed on intact and lysed *hhy* cells using a monoclonal antibody specific for pro-CPY (Fig. 4). Mislocalized and secreted pro-CPY can be detected without lysis (e.g., *vps35*Δ), while internal pro-CPY can only be detected in lysed cells (e.g., *env1*). *vps* mutants were excluded as they have already been established as defective in CPY processing and localization (Robinson et al. 1988;Bonangelino et al. 2002). In repeated experiments, *tpd3*Δ, *chc1*Δ, and *sbh2*Δ showed no significant pro-CPY signal suggesting CPY processing equivalent to wild type; all three showed wild type levels of mature CPY on westerns (data not shown). The results suggest that the three genes are not essential for correct CPY processing. All other *hhy* mutants assayed showed persistent pro-CPY. Three *hhy* mutants showed mislocalization and secretion of pro-CPY. They include deletion strains of *DHH1* and *ARF1* which have been previously classified as vacuolar protein sorting (*VPS*) genes in a genomic screen (Bonangelino et al. 2002) and *hhy1*Δ. The remaining *hhy* strains, *paf1*Δ, *drs2*Δ, *bud32*Δ, and *sac1*Δ, showed internal accumulation of pro-CPY. Thus, 11 of the l4 *HHY* genes affect the CPY pathway to or CPY processing in the vacuole including *PAF1* whose product has not previously been associated with vacuolar events.

Vacuole morphology and endocytosis in *hhy* strains

In order to examine vacuole morphology and integrity of endocytosis pathway in *hhy* strains, Nomarski light microscopy and fluorescent microscopy of cells labeled with FM4-64 were performed (Fig. 5). The lipophylic vital dye FM4-64 is incorporated into the plasma membrane and serves as an endocytic marker that localizes to the vacuole membrane within 60 min in wild type cells at 30°C (Vida and Emr 1995). 200–300 cells from each strain were scored 60

min after staining as summarized in Fig. 5b. In the wild type BY4742 strain, FM4-64 staining and Nomarski optics showed 1–3 prominent vacuoles as the predominant phenotype.

Of the ten *hhy* strains subjected to microscopic studies, six showed vacuole morphology variations from wild type that were statistically significant ($P = 0.0001–0.027$). *sac1Δ*, *arf1Δ*, *luv1Δ*, *hhy1*Δ, and *paf1*Δ strains exhibited a significant increase in fragmented vacuoles compared to wild type. *paf1Δ* and *hhy1*Δ strains had the highest percentage of cells with fragmented vacuoles at 58% and 66%, respectively. Mutants with fragmented vacuoles have been previously categorized as Class B, and those lacking a visible vacuole have been categorized as Class C (Banta et al. 1988; Raymond et al. 1992). Phenotypic scoring did not reveal any Class C *hhy* strains. The mutants were also examined for vacuole acidification using quinacrine staining; *hhy1*Δ was the only mutant with vacuole acidification defect (data summarized in Table 3). The four original *vps* mutants were not included in microscopic studies as they have been thoroughly classified with respect to their vacuole morphologies in previous studies. *vps54* and *vps52* are Class B with fragmented vacuoles; *vps45* and *vps34* are Class D with enlarged vacuoles (Banta et al. 1988; Raymond et al. 1992).

Finally, FM4-64 localization to either prominent or fragmented vacuoles in all examined *hhy* mutants indicates normal endocytic trafficking from plasma membrane, through early and late endosomes, to the vacuole. Thus, none of the l4 *hhy* mutants exhibit defects in bulk endocytosis nor lack of visible vacuoles, while ten of the 14 exhibit vacuole morphology defects including a clustering of eight as Class B mutants with fragmented vacuoles.

Growth of *hhy* mutants under environmental and chemical stress conditions

Growth sensitivities in the presence of certain ions and drugs have been suggestive of defective vacuole function (reviewed in Luzio et al. 2007; Li and Kane 2009). Additionally, several *vps* mutants are sensitive to 37°C, pH extremes, osmotic pressure, and caffeine (Robinson et al. 1988; Conboy and Cyert 2000). *hhy* mutants were assayed for growth under various ionic, pH, hyperosmotic, temperature, and drug conditions associated with vacuolar defects as represented in Fig. 6 and summarized in Tables 2 and 3. All *hhy* mutants exhibited strong growth sensitivities under at least three of the tested stress conditions. Several strains showed increased sensitivities to the divalent ions zinc and/or manganese indicating defects in the vacuolar sequestration of those ions; these included *vps54/luv1Δ*, *vps45Δ*, *vps52/sac2Δ*, *vps34Δ* (zinc only), *chc1Δ*, *bud32Δ*, and *tpd3Δ*. Most showed increased sensitivities to both low and high pH, and cold sensitivity. Most significantly, all *hhy* mutants showed strong sensitivities to rapamycin and caffeine, drugs indicative of a compromised TOR kinase pathway.

In order to further assess vacuole trafficking, we investigated the growth of *hhy* mutants in the presence of monensin, an ionophore that interferes with intracellular trafficking. A recent genomic screen found strong correlation between monensin sensitivity and known genes involved in vacuole trafficking and morphology (Gustavsson et al. 2008). We also found a high correlation between monensin sensitivity and *HHY* genes (Fig. 7). Ten of the 14 *hhy* mutants showed sensitivity to monensin, suggestive of defects in vacuole events. Notably, *hhy1*Δ and *paf1Δ*, both of which had no established connection to vacuolar events prior to this study, were sensitive to monensin.

Phenotypic complementation of *tpd3*Δ, *paf1*Δ, and *hhy1*Δ

TPD3, *PAF1*, and the hypothetical *YEL059W* ORF have not been reported to be involved in vesicular trafficking and/or vacuolar events in previous studies. In order to confirm that the observed vacuolar/vesicular phenotype of the corresponding mutants is due to the deleted ORF, each of the genes were reintroduced into the corresponding mutant (Fig. 8). *PAF1* and *TPD3*

were each able to complement the hhy phenotype of *paf1*Δ and *tpd3*Δ, respectively. *YEL059W* sequences in single copy or multi copy did not complement *hhy1*Δ in neither mating type, despite confirmation of YEL059W deletion and KAN cassette insertion by PCR approaches as detailed in "Materials and methods" (Fig. 8; data not presented). Furthermore, a construct containing *YEL059W* as well as adjacent upstream (*SOM1*) and downstream (*PCM1*) ORF's along with a minimum of 500 bases of flanking sequences also failed to complement *hhy1*Δ (Fig. 8). *PCM1* is an essential gene involved in GlcNAC synthesis, and *SOM1* encodes a mitochondrial inner membrane peptidase essential for respiratory growth. As such, deletion strains of the two genes are not included in the collection. Finally, *PRB1* which encodes the vacuolar protease proteinase B and lies two ORF's upstream of *YEL059W*, also failed to complement *hhy1*Δ; *prb1*Δ of the deletion strain collection did not exhibit *hhy* nor vacuole fragmentation phenotype upon reexamination (data not presented).

Discussion

Several yeast mutants with vacuole trafficking or function defects have recently been shown to exhibit sensitivity to <50 μg/mL hygromycin B (Conboy and Cyert 2000; Ali et al. 2004; Mukherjee et al. 2006; Takahashi et al. 2008). Specifically, *vps54* and *env1* mutants show no growth at such low hygromycin concentrations (Mukherjee et al. 2006; Takahashi et al. 2008). We hypothesized that such severe hypersensitivity to hygromycin B may be indicative of specific vacuole trafficking or function defects. To test this hypothesis and to potentially identify additional genes involved in vacuolar events, a genomic screen of 4,828 yeast haploid deletion strains was conducted for growth sensitivities in 25 μg/mL hygromycin B. 38 mutant strains showed a range of growth sensitivities. Fourteen of the uncovered mutant strains showed no growth upon prolonged incubation times and under a range of *h*ygromycin B concentrations; they were denoted as hypersensitive to hygromycin B (*hhy* mutants) and were pursued in this study (Table 1). Hypersensitivity of these mutants to hygromycin B is not likely to be due to the effect of the drug on protein translation since the mutants did not show increased sensitivity to the translation inhibitor cycloheximide used in our immunodetection screen. Conboy and Cyert (2000) also reached the same conclusion with their mutants which were sensitive to hygromycin B, but did not show additional sensitivity to translation inhibition. Bioinformatics analysis established that the genes showed a statistically significant clustering with GO terms associated with post-Golgi vesicle mediated transport to the vacuole. Microscopic, biochemical, and environmental stress assays established that vacuole morphology, trafficking, and stress survival defects were also highly enriched in the *hhy* collection. Figure 9 summarizes the proposed functions of *HHY* genes in vesicular trafficking and vacuole events based on our results.

Four of the *hhy* mutants were null alleles of the original *vps* collection: *luv1* (*vps54*), *vps45*, *sac2* (*vps52*), and *vps34*. These *vps* mutants have previously been shown to display a defect in protein sorting to the vacuole (Banta et al. 1988; Robinson et al. 1988). Vps52p and Vps54p are members of the Golgi-associated retrograde protein (GARP) and their mutants have class B fragmented vacuoles (Conibear and Stevens 2000). Vps45p is required for Golgi vesicle fusion with the late endosome and its mutant form results in enlarged vacuoles, categorized as class D (Raymond et al. 1992; Bryant and James 2001). Vps34p is a phosphoinositide kinase required for multiple vacuolar trafficking events including vacuole segregation, vacuolar protein localization, multivesicular body formation, and autophagy (Herman and Emr 1990; Schu et al. 1993; Munn and Riezman 1994; Kihara et al. 2001; Wurmser and Emr 2002; Katzmann et al. 2003). Its mutation results in class D enlarged vacuoles (Raymond et al. 1992). Thus, all four uncovered established *VPS* genes affect membrane fission/fusion events at the late endosome and the vacuole.

Three *hhy* mutants have been designated as vacuolar protein sorting mutants through a genomic screen for additional *VPS* genes: *arf1Δ*, *dhh1Δ*, and *bud32Δ* (Bonangelino et al. 2002). Arf1p has been established as a GTPase of the Ras superfamily involved in regulation of coated vesicle formation in vesicular trafficking (Sewell and Kahn 1988). Dhh1p, a dead box protein, regulates Ste12p translation and has a role in recovery from G1/S checkpoint (Bergkessel and Reese 2004; Ka et al. 2008). The *dhh1* mutant displays abnormal cellular morphology and defects in cytokenesis and mitosis, including aberrant "shmoo" formation at mating and abnormal budding morphology (Moriya and Isono 1999). Bud32p is involved in bud site selection and interacts with the KEOPS complex for transcription regulation and telomere maintenance/homeostasis; *BUD32* mutation results in decreased telomere size and defects in cell cycle (Ni and Snyder 2001; Facchin et al. 2007). Our results showing CPY processing and vesicular trafficking defects and temperature sensitivity for the three mutants are consistent with those reported by Bonangelino et al. (2002). Our microscopy results indicate excessive vacuole fragmentation in all three; Bonangelino et al. (2002) also classified *bud32Δ* and *arf1Δ* as having vacuole morphology defects. Additionally, all three mutants showed growth sensitivities to multiple stress conditions tested. Thus, the three mutants show defects in vacuolar trafficking, fusion, and stress survival. Interestingly, *DHH1* and *BUD32* are also involved in cell cycle and cell growth events.

Four of the remaining *hhy* mutants are known to be involved at various stages of vesicular trafficking; those are *drs2Δ*, *chc1Δ*, *sac1Δ*, and *sbh2Δ*. Drs2p is an amino-phospholipid translocase (flippase) that maintains post-Golgi secretory vesicles and contributes to clathrin-coated vesicle formation (Ripmaster et al. 1993; Chen et al. 1999). Deletion mutants of the gene have been reported to accumulate membrane structures analogous to Berkeley bodies and to exhibit a block in endosome-to-vacuole stage of trafficking at non-permissive temperature (Chen et al. 1999; Hua et al. 2002). Interestingly, *drs2* mutant alleles exhibit synthetic lethality with *arf1Δ* (Chen et al. 1999). Chc1p is a clathrin heavy chain coat protein involved in post-Golgi-coated vesicle transport and endocytosis (Gruenberg and Stenmark 2004). Sac1p is a lipid phos-phatase localized to the endoplasmic reticulum and Golgi membranes; it is involved in several vesicular trafficking processes, including cell wall maintenance, Golgi trafficking, and secretion (Hughes et al. 2000; Strahl and Thorner 2007). Additionally, it functions in the control of vacuolar morphology and actin cytoskeleton (Foti et al. 2001). Sbh2p is a component of the Ssh1p-Sss1p-Sbh2p complex involved in protein translocation into the endoplasmic reticulum (Toikkanen et al. 1996). Of the four vesicular trafficking genes, only *SBH2* has an established role limited to the endoplasmic reticulum; consistent with that, its deletion did not result in defects in pro-CPY processing in our studies. It is conceivable that hypersensitivity of *sbh2*Δ to hygromycin B was due to direct effects of the drug on the translation machinery and the post-translational ER translocation of proteins in yeast. However, neither liquid invertase assays nor halo assays revealed compromised secretion—an expected phenotype if the drug was significantly interfering with protein translation/translocation. Instead, *sbh2*Δ cells showed severe growth sensitivities to pH/ionic/osmotic stresses, and multidrug sensitivities suggestive of an essential role in vacuolar function. Taken together, our results suggest a likely role for all four genes in vacuole morphology and/or function; the role of Chc1p and Sbh2p in this process does not appear to affect CPY processing within the limit of our assays.

Two of the uncovered *HHY* genes had no previously established roles in vesicular trafficking —*TPD3* and *PAF1*. Both genes have known rolesincell cycle regulation. Tpd3p is one of two regulatory subunits of the heterotrimeric protein phosphatase 2A (PP2A), a ser/thr phosphatase required for cell morphogenesis, cell cycle progression and cytokinesis, and for transcription by RNA polymerase III (Van Zyl et al. 1992; Wang and Burke 1997). Koren et al. (2004) have suggested that Tpd3p is not essential for the cytokinesis functions of PP2A, but enhances the enzyme activity. We detected multiple defects associated with vesicular trafficking and vacuole

function. *tpd3*Δ mutants were defective in both invertase secretion and halo assays, but not in CPY processing, suggesting a defect at pre-endosomal stages of vesicular trafficking and/or secretion (Fig. 8). However, the mutants exhibited multi drug sensitivities and severe growth defects under various stress conditions suggestive of compromised vacuole function. These results reinforce the notion that Tpd3p regulation of protein phosphatase 2A affects a variety of biological processes in the cell including secretion and stress survival functions.

The second *HHY* gene with no previously established vacuolar function is *PAF1*. Paf1p is a nuclear RNA polymerase II-associated protein that is required for full expression of cell cycle-regulated genes; it acts in the Pkc1p-Slt2p MAP kinase cascade and its human homolog, hPaf1, is associated with tumorigenesis (Shi et al. 1996, 1997; Chang et al. 1999; Porter et al. 2002; Moniaux et al. 2006). Consistent with Shi et al.'s (1996) observations, *paf1Δ* strains in our studies showed larger cell size and severe temperature sensitivity. Additionally, we established severely fragmented vacuoles, CPY processing and halo induction defects, and multi drug sensitivities. Taken together, these results are suggestive of a defect at Golgi to vacuole stage of trafficking affecting vacuole morphology and stress function. Paf1p may regulate the expression of protein(s) affecting vacuolar events including vacuole fusion. We are currently exploring the feasibility of microarray and/or proteomic approaches to investigate global transcriptional and/or translational consequences of *PAF1* and *TPD3* deletion.

Our screen also uncovered one previously uncharacterized dubious ORF, *YEL059W*, whose deletion in *hhy1* mutant was experimentally confirmed. The mutant strain exhibited no defects in secretion or retrograde trafficking at late endosome and Golgi interface based on invertase secretion and halo assays. It exhibited defective CPY processing, extreme sensitivities to pH extremes, divalent cations, and multiple drugs indicative of severely compromised vacuole function. Both mating types of the mutant exhibited enlarged cells and highly fragmented vacuoles in addition to hypersensitivity to hygromycin B. The deleted ORF is located on chromosome V and is not conserved in most yeast or higher eukaryotes. Single copy or multi copy reintroduction of *YEL059W* ORF and its flanking sequences in trans, as well as reintroduction of sequences that include *YEL059W* and its two flanking ORF's, did not rescue *hhy1*. Reintroduction of *PRB1*, an ORF approximately 800 bases upstream of *YEL059W* that encodes vacuolar protease B, also did not rescue the mutant. At this point, we have not identified the locus responsible for the hhy phenotype of *hhy1* mutant. Alternatively, *YEL059W* sequences may have a role in distant cis-acting regulation of other gene(s) involved in vacuolar morphology and function.

FM4-64 localization to vacuoles in *hhy* mutants indicates normal bulk endocytic trafficking. The late endosome to vacuole stage of trafficking is shared between endocytic and biosynthetic CPY pathways, and normal bulk endocytic trafficking to the vacuole would suggest normal late endosome to vacuole trafficking. Therefore, the CPY-processing defects of *drs2*Δ, *sac1*Δ, *bud32*Δ, and *hhy1*, which have no pre-endosomal trafficking defects, are more likely due to defective vacuole function than defective postendosomal vacuolar trafficking.

The combination of bioinformatic and experimental characterizations of *hhy* mutants implicate defects in vacuole trafficking, morphology, and/or stress survival function for all uncovered mutants, including the two mutants deleted in cell cycle regulatory genes *PAF1* and *TPD3* with no previously established connection to vacuolar events. Thus, in agreement with our hypothesis, severe hypersensitivity to hygromycin B was indicative of vacuolar defects and the screen uncovered two genes with no previous known connection to vesicular and/or vacuolar events. The identification of *PAF1* and *TPD3* suggests that there are yet unidentified gene functions in vacuolar events. The fact that all *hhy* mutants were sensitive to a minimum of three stress conditions suggests that hygromycin B hypersensitivity may be due to compromised stress tolerance in these mutants. In one of the most recent large scale genomic

screens for drug sensitivities, multi drug sensitivity to at least 20% of the unique treatments was significantly enriched in deletions of genes associated with vesicular trafficking, and specifically genes associated with vacuole trafficking and function (Hillenmeyer et al. 2008).

Despite >100 genes identified in vacuolar trafficking, function, and morphology, only 14 *hhy* mutants were uncovered. Moreover, the limited number and the functional clustering of mutants that show no growth under low concentrations of hygromycin B versus those that merely show slowed growth suggest that the two phenotypes are associated with functionally distinct classes of mutants. Stress conditions have been documented to result in vacuole fragmentation (Li and Kane 2009; Luzio et al. 2007). Our results support this connection since vacuole fusion/fission defects as well as defects in stress survival are enriched in *hhy* mutants. The only established *vps* mutants uncovered have either excessive fragmentation of vacuoles (Class B) or defects in vacuolar fragmentation (Class D). Among the remaining *hhy* mutants, our microscopic studies confirmed increased vacuole fragmentation in all but *drs2Δ*, *tpd3Δ*, and *chc1Δ*. Other microscopic studies, however, have reported higher percentages of fragmented vacuoles for those three mutants (Chen et al. 1999; Seeley et al. 2002). Significantly, neither Class A *vps* mutants (no vacuole morphology or inheritance defects), nor Class C *vps* mutants (no visible vacuoles) were included in the collection. The lack of Class C mutants is especially interesting since they exhibit the severest pH and osmotic sensitivities within the vps mutant collection (Robinson et al. 1988). Moreover, the Class C genes whose products make up the HOPS complex have been reported to be hypersensitive to gentamicin, another aminoglycoside (Blackburn and Avery 2003; Wagner et al. 2006) and to higher levels of hygromycin B in global multidrug screens (Dudley et al. 2005). Their exclusion from *hhy* collection suggests that our screen was neither simply picking up the most severe *vps* mutants nor those most sensitive to aminoglycoside drugs in general. Thus, while none of the 14 *hhy* mutants exhibit defects in bulk endocytosis or lack of visible vacuoles, ten of the 14 exhibit vacuole morphology defects including a clustering of eight as class B mutants with fragmented vacuoles. Our characterizations suggest that the uncovered mutants share specific defects in vacuole fission/fusion and stress tolerance. All the trafficking pathways to the vacuole involve a final step of membrane fusion at the vacuole; furthermore, the vacuole is a dynamic organelle which undergoes fusion/fission events during the budding stage of the cell cycle and in response to external stress. Thus, the membrane fission/fusion events at the vacuole remain of intense interest (for latest review see Ostrowicz et al. 2008).

As a potential organelle for sequestering hygromycin B from the translation machinery, intact vacuolar function and trafficking may be crucial to cell survival even at low hygromycin B concentrations. However, since only a small fraction of known vacuolar trafficking or function genes were uncovered in our screen, hygromycin B may be directly interfering with a specific step in vacuolar events.Brett et al. (2005) have suggested that pH homeostasis defects may be associated with hygromycin B sensitivity. Our quinacrine staining studies of *hhy* mutants reveal properly acidified vacuoles, except in *hhy1*. Hence, any possible defects in pH regulation in *hhy* mutants may be in other cellular compartments. In mammalian cells, amino-glycoside drugs have been reported to interfere with coatomer formation and secretion (Hu et al. 1999; Hudson and Draper 1997). An attractive model would be that hygromycin B interferes with efficient vacuole fusion/fission events necessary for stress survival and proper cell cycle progression, and that *hhy* mutants are specifically inefficient at membrane fusion/fission. All *hhy* mutants showed severe sensitivities to rapamycin—a phenotype associated with defects in Tor1 kinase pathway which regulates cell growth and vacuolar autophagy under various stress and nutrient conditions (Park et al. 2005; Kuranda et al. 2006; reviewed in Levin 2005; Aronova et al. 2007). Additionally, *hhy* mutants were also hypersensitive to caffeine, 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). Most recently, functional interactions have been reported between TORC1, actin, and vesicular

trafficking (Aronova et al. 2007), and Tor1-GFP fusion proteins were reported to be concentrated near the vacuolar membrane in live yeast cells (Sturgill et al. 2008). Most relevantly, *TPD3* and *TORC1* double mutants show synthetic lethality, and Vps34p has been suggested as an upstream regulator of TORC1 (Aronova et al. 2007; Jacinto 2008); both genes were identified as *HHY* in our screen. The published cell cycle roles of *PAF1*, *TPD3*, *DHH1*, and *BUD32* support a connection between cell cycle regulation and vacuole function and morphology (reviewed in Fagarasanu and Rachubinski 2007). It is conceivable that *HHY* genes may be specifically involved at the interface of TOR kinase pathway and vacuolar events, and hygromycin B may be targeting molecular interactions at that interface.

In this study, a genomic screen for severe hypersensitivity to hygromycin B uncovered 14 *hhy* mutants all of which were implicated in vacuole trafficking, morphology, and/or stress survival defects by a combination of bioinformatic and experimental approaches. The *hhy* collection includes two mutants deleted in cell cycle regulatory genes *PAF1* and *TPD3* with no previously established role in vacuolar events. Additionally, vacuolar fusion/fission defects were enriched in the uncovered collection, and all *hhy* mutants showed hypersensitivity to caffeine and rapamycin, drug sensitivities associated with Tor kinase pathway defects. Additional studies on *hhy* strains relative to Tor kinase pathway and vacuole fusion/fission under stress and during cell cycle progression may shed light on the step affected by hygromycin B.

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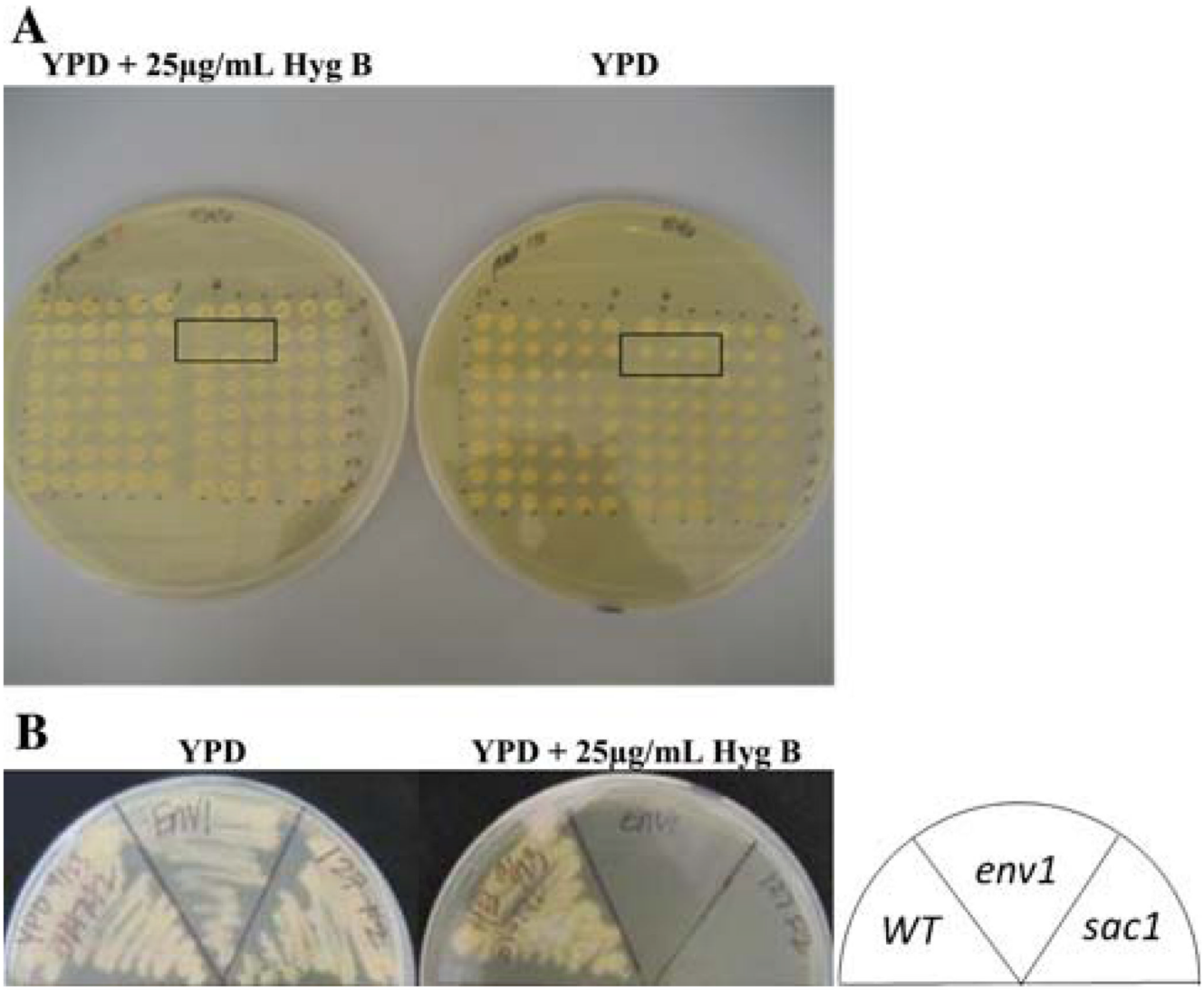


Fig. 1.

Primary and secondary hygromycin B hypersensitivity screens. **a** Strains of haploid Mat-α deletion collection were replica plated from 96-well plates onto YPD and YPD + 25 µg/ml hygromycin B plates, and were incubated for 3 days at 30°C. *Rectangles* depict strains hypersensitive to hygromycin B. **b** Putative *hhy* strains from the primary screen were streaked on YPD and YPD + 25 µg/ml hygromycin B plates, and were incubated for 3 days at 30°C. In this representative plate, *sac1* was confirmed as an *hhy* mutant. *env1* serves as a hypersensitive control

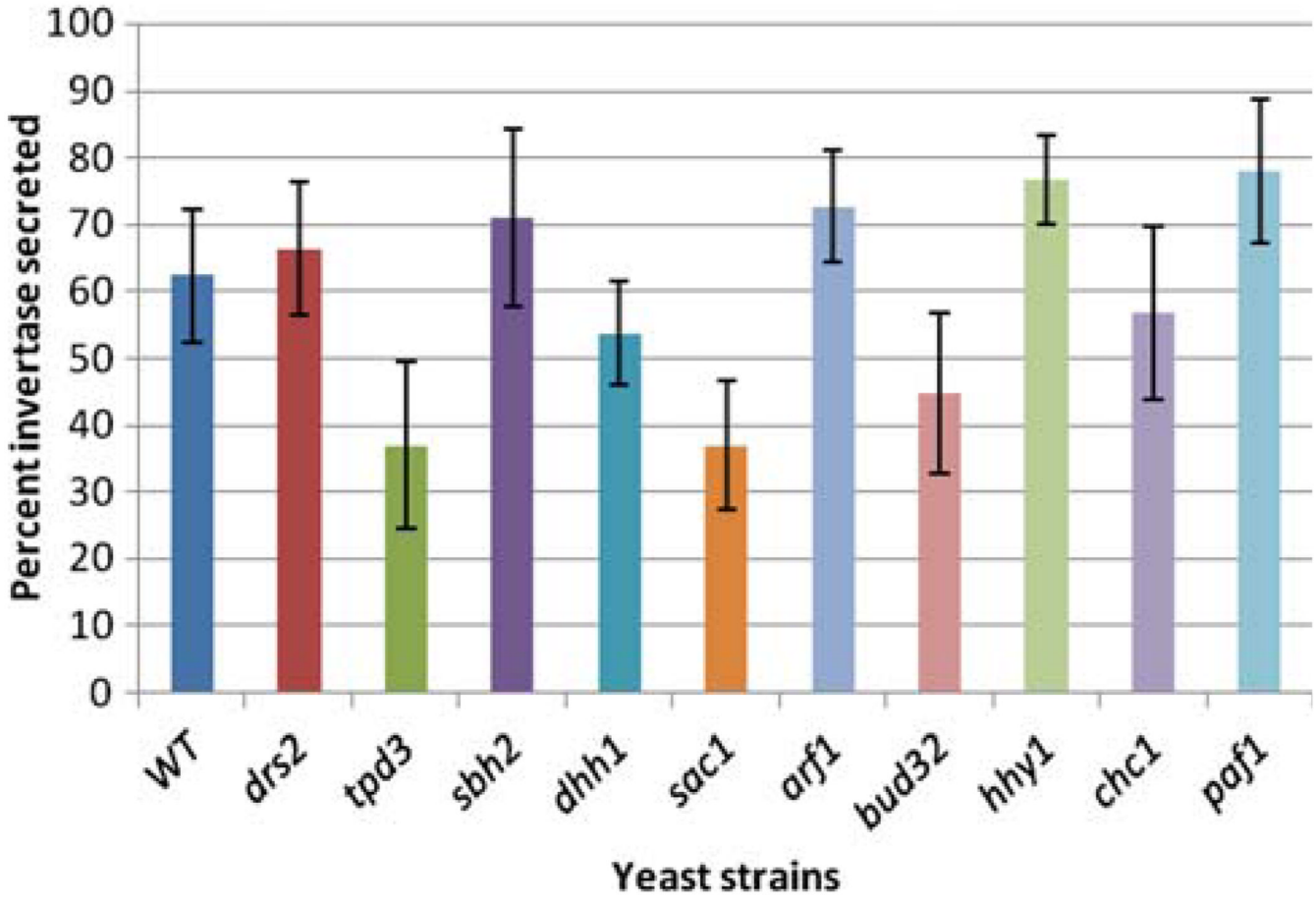


Fig. 2.

State of secretion of invertase in *hhy* mutants. Invertase secretion was assayed in yeast mutants by quantitative liquid invertase assays at 30°C. Strains *tpd3* and *sac1* showed statistically significant defects in secretion relative to *WT* (BY4742). Two-sample *t* test biostatistical analysis was used

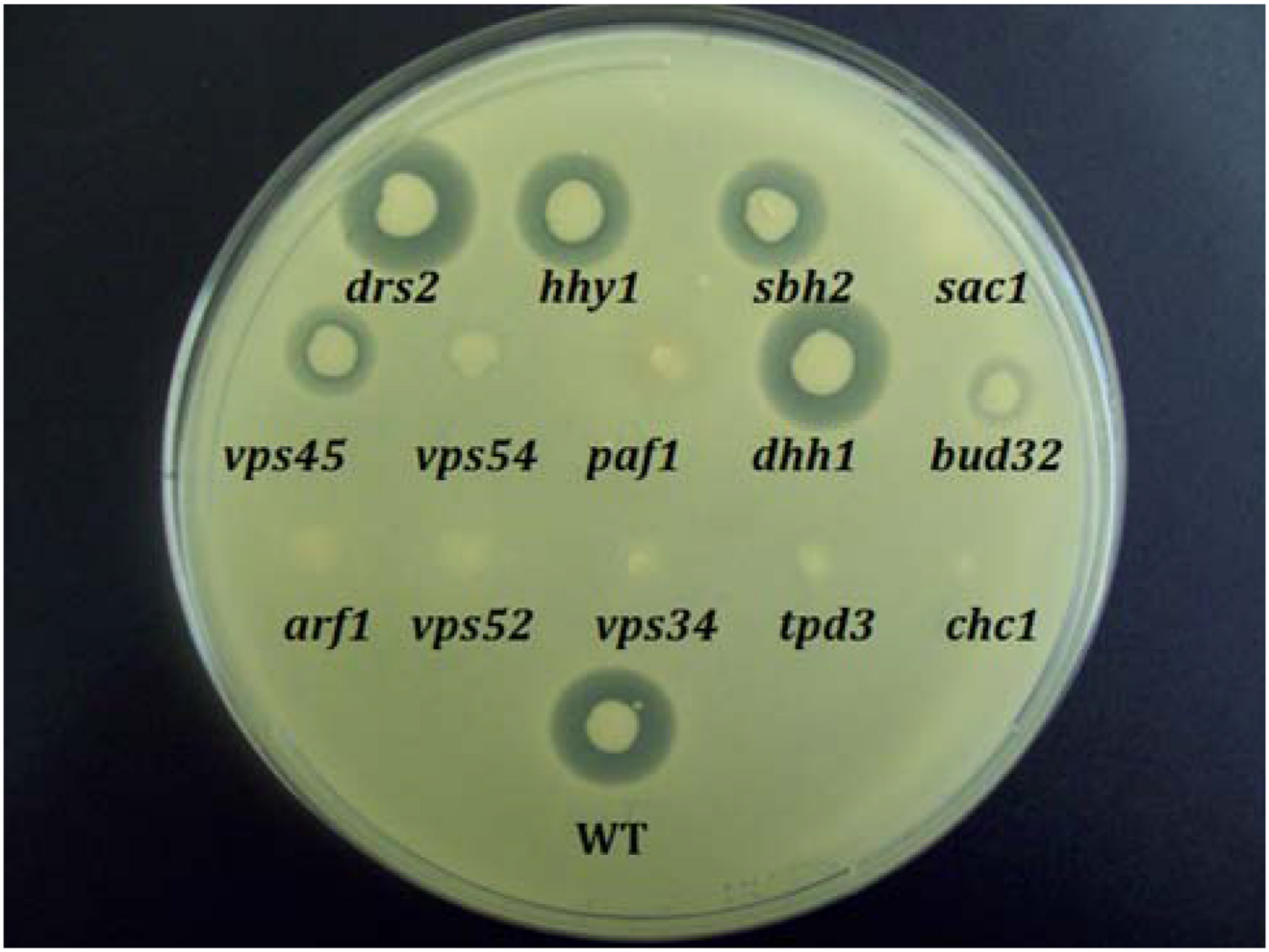


Fig. 3.

Analysis of active α-factor secretion in *hhy* mutants. Active α-factor secretion was assessed with standard halo assays using a lawn of GPY-1796 cells. This experiment was repeated six times with reproducible results

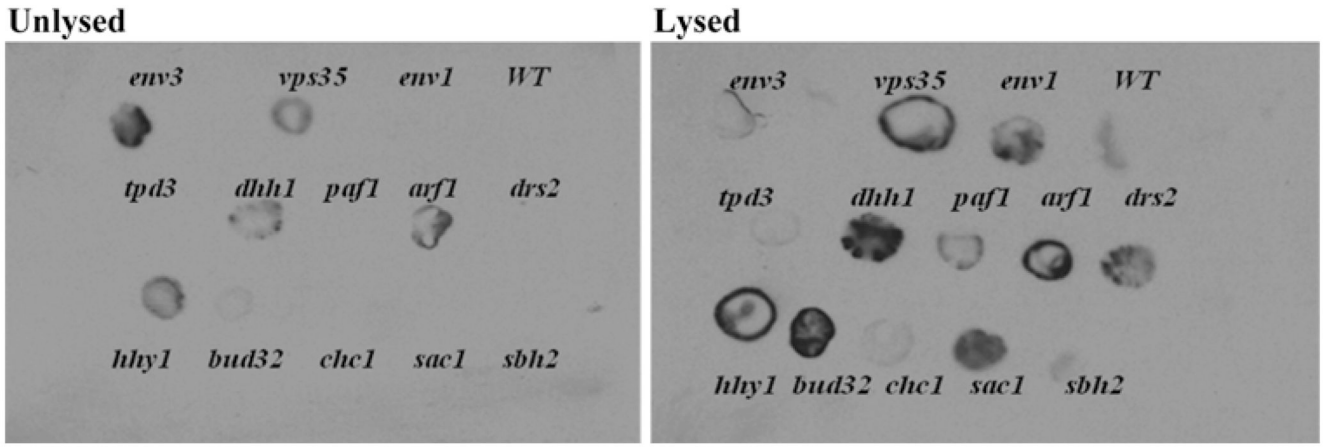
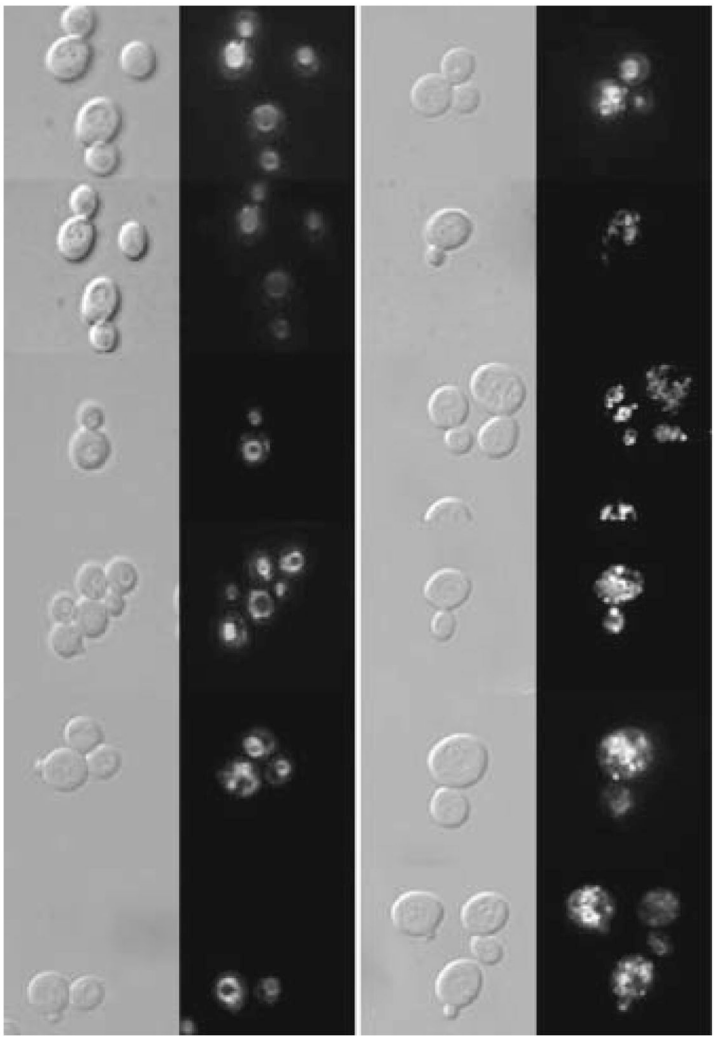


Fig. 4.

State of p2CPY processing in *hhy* mutants. Patched cells were transferred to nitrocellulose membranes for overnight growth, and were probed with anti-proCPY-specific monoclonal antibody either without lysing cells or after lysis. Under the assay conditions, wild type has minimal signal due to efficient processing of proCPY forms into mCPY, while *env1* and *vps35* controls show persistent p2CPY either inside cells or secreted, respectively

A



B

	WT	*sbh2*	*tpd3*	*drs2*	*chc1*	*bud32*	*luv1*	*sac1*	*paf1*	*dhh1*	*hhy1*	*arf1*
Prominent vacuoles	60%	48%	54%	58%	64%	48%	51%	41%	33%	41%	37%	38%
Fragmented vacuoles	34%	41%	36%	33%	32%	42%	49%	51%	66%	49%	58%	54%
No Vacuole	6%	11%	7%	9%	4%	10%	0%	8%	1%	10%	5%	8%

Fig. 5.

Microscopic characterization of *hhy* mutants. **a** Cells were stained with FM4-64 for 60 min and viewed using fluorescence and Nomarksi optics microscopy. The predominant vacuole morphology observed is represented. **b** Percentage of fragmented versus prominent versus no vacuoles in FM4-64 stained cells. 200–300 cells from each strain were scored in random fields. Two-sample *t* test biostatistical analysis was used

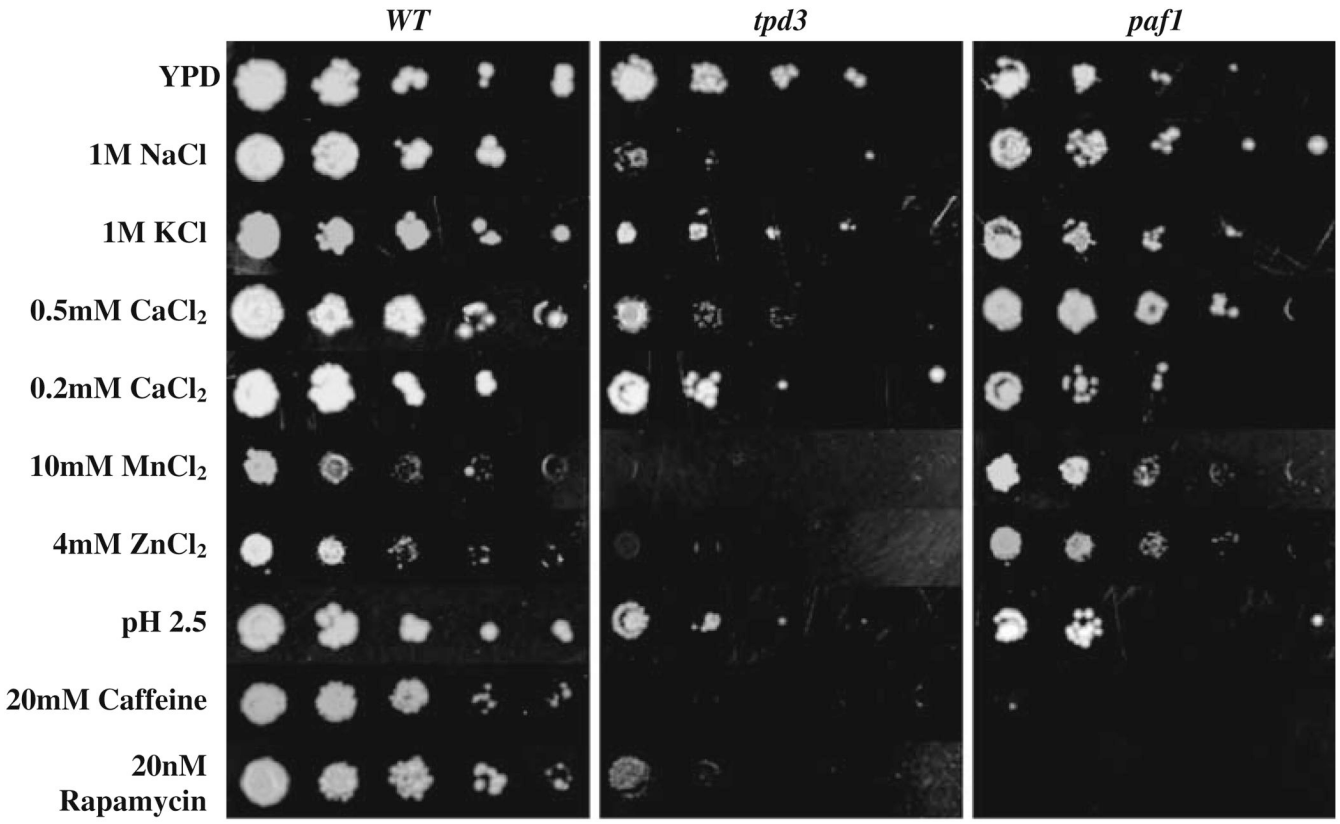


Fig. 6.

Ion and drug sensitivities of *hhy* mutants. Serial tenfold dilutions of log-phase cells were stamped with pinning tool on YPD plates or selective YPD plates with ion or drug concentrations or pH as indicated. Incubations were at 30°C for 48 h for YPD plates and for 72 h for selective YPD plates. *Wild type*, *tpd3*, *paf1* strains are represented in the figure. Data for all *hhy* mutants are presented in Table 2

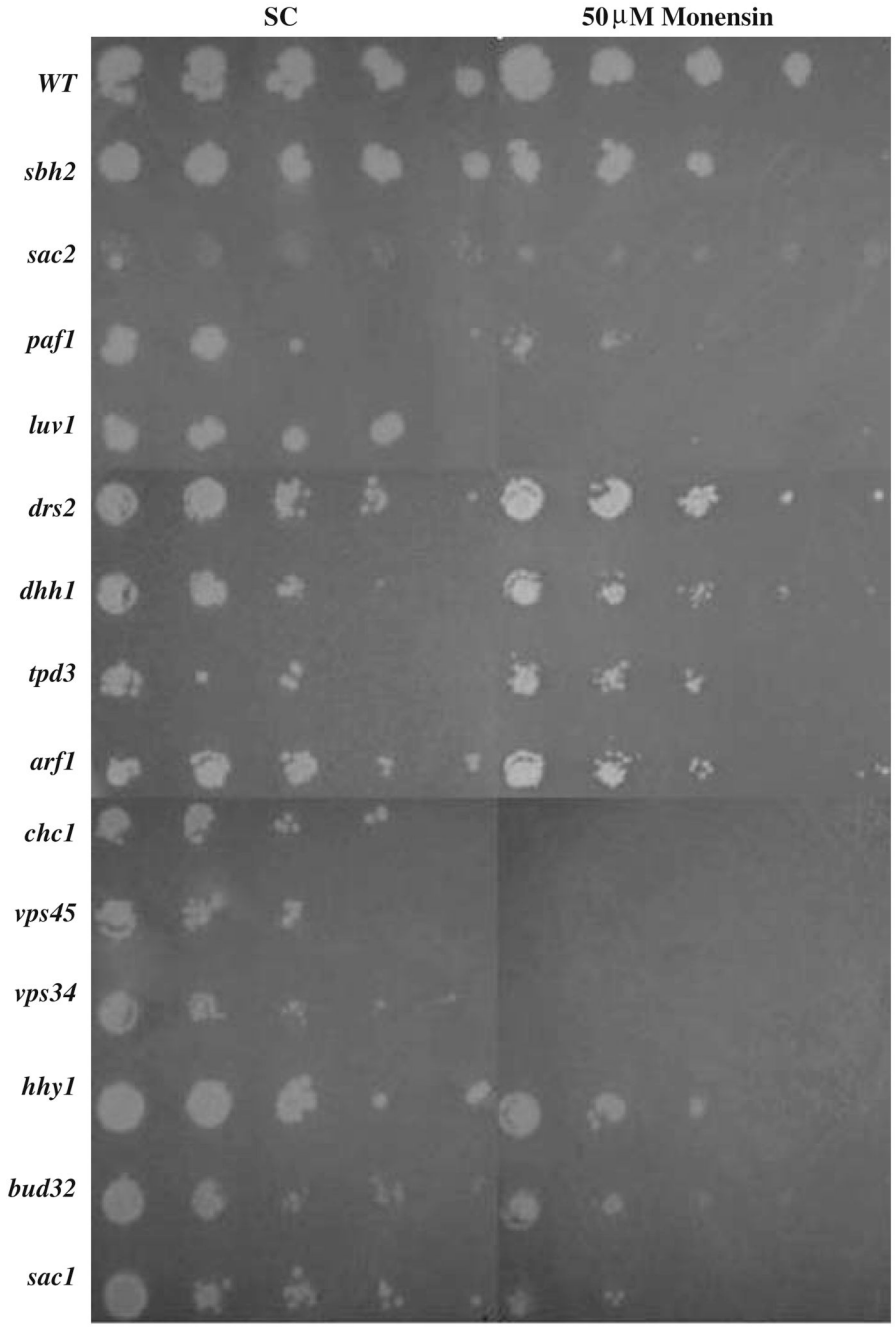


Fig. 7.

Monensin sensitivity assay of *hhy* mutants. Serial tenfold dilutions of log-phase cells were stamped with pinning tool onto synthetic complete (SC) plates in absence or presence of monensin (50 μM). Plates were incubated at 30°C for 48 and 72 h

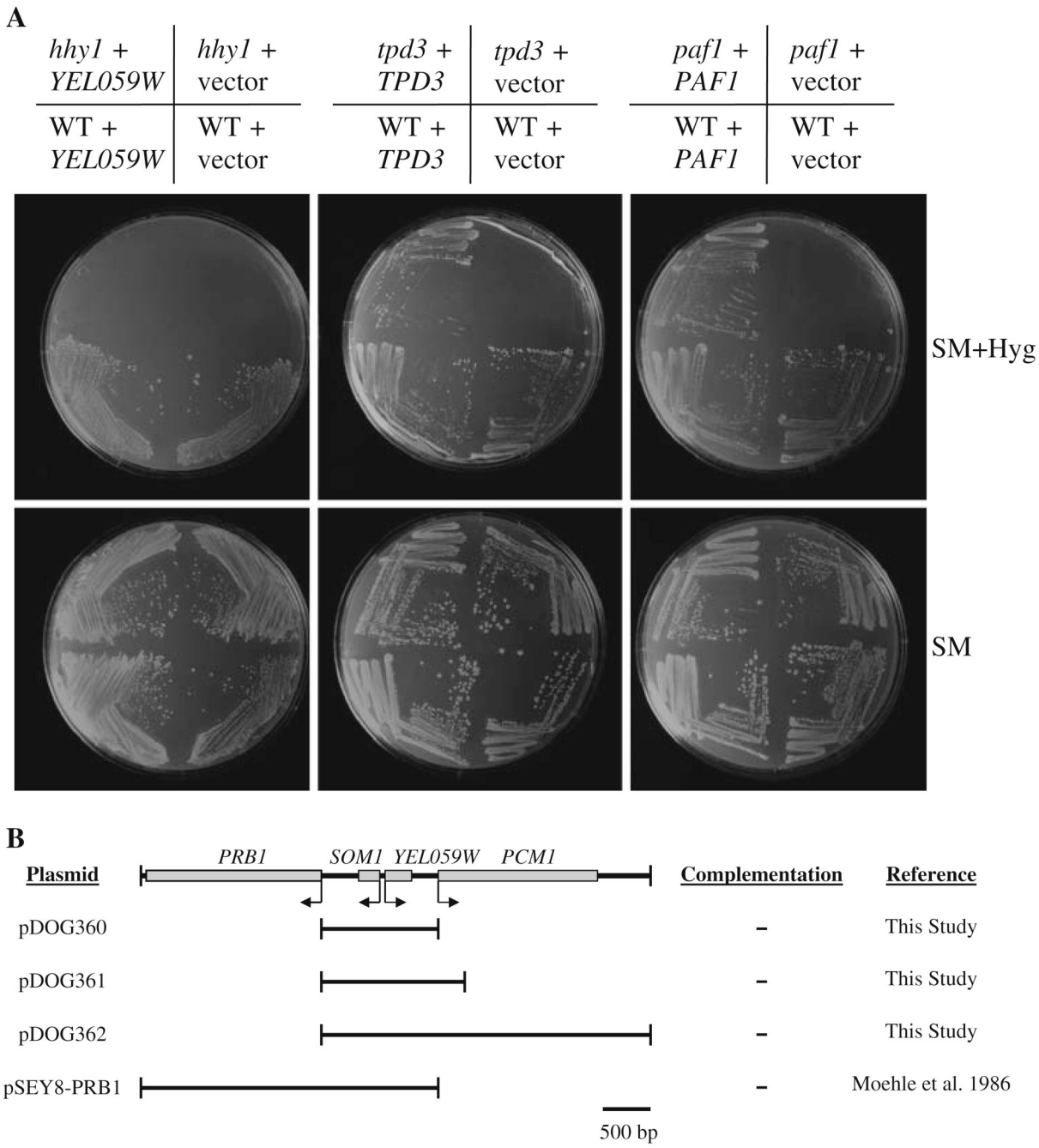


Fig. 8.

Complementation tests for *hhy1*, *tpd3*, and *paf1* mutant strains. **a** Strains were transformed with empty vector or vector containing the corresponding deleted ORF. Transformed cells were streaked onto SM-URA plates with or without 50 μg/ml hygromycin B and grown at 29°C. WT exhibited comparable growth with or without hygromycin B as did *tpd3* and *paf1* strains transformed with their corresponding deleted ORFs. *hhy1* continued to exhibit severe hypersensitivity to hygromycin B when transformed with pDOG362 containing *YEL059W* ORF flanked by its two adjacent ORF's as detailed in b. **b** Diagrammatic representation of constructs used for *hhy1* complementation tests and summary of results

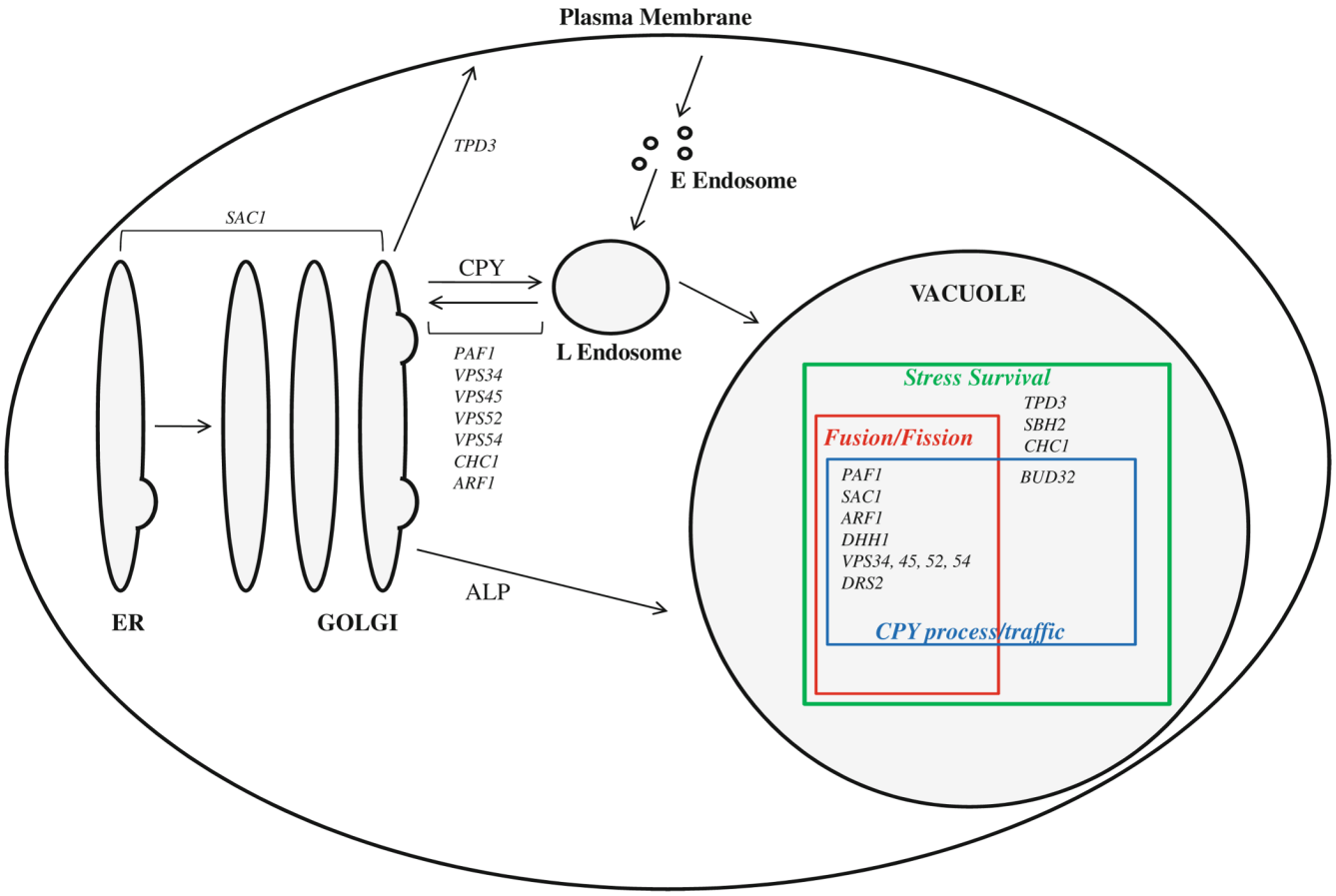


Fig. 9.

Overview of vesicular trafficking in yeast with the proposed site/mode of action of *HHY* genes as suggested by this study. *ER* endoplasmic reticulum; *CPY* carboxypeptidase Y pathways; *ALP* alkaline phosphatase pathway

Table 1

HHY genes and their known biological processes, molecular functions, and cellular components (SGD)

Gene name	Biological process	Molecular function	Cellular component
YEL059W	Unknown	Unknown	Integral to membrane
DRS2	Endocytosis, post-Golgi vesicle- mediated transport, ribosomal small unit assembly	ATPase activity, Phospholipid-translocating ATPase activity	Trans-Golgi network
VPS54/LUV1	Ascospore wall assembly, Golgi to vacuole transport, retrograde transport	Unknown	GARP complex, Golgi apparatus, mitochondrion
SBH2	Cotranslational protein targeting to membrane	ARF guanyl-nucleotide exchange factor activity	Endoplasmic reticulum, translocon complex
SAC1	Phosphoinositide dephosphorylation	Phosphatase activity	Integral to ER membrane and Golgi, mitochondrial outer membrane
ARF1	ER to Golgi and intra-Golgi vesicle-mediated transport, Golgi to plasma membrane transport	GTPase activity	Cytosol, Golgi-associated vesicle
PAF1	DNA recombination, histone methylation, negative regulation of transposition (RNA-mediated)	RNA polymerase II transcription elongation factor activity	Cdc73/Paf1 complex, nucleus, transcription elongation factor complex
DHH1	Cellular morphogenesis (cellular fusion), cytoplasmic mRNA- processing body assembly, deadenylation-dependent deccaping (mRNA), stress granule assembly	Protein binding, RNA helicase activity	Cytoplasm, cytoplasmic mRNA-processing body
VPS45	Golgi to vacuole transport, protein complex assembly, vacuole organization	Unfolded protein binding	Cytosol, extrinsic to membrane, Golgi membrane
VPS52/SAC2	Actin-filament base process, Golgi to vacuole transport, retrograde transport	Protein binding	GARP complex, Golgi apparatus
VPS34	Phosphoinositide phosphorylation, protein amino acid phosphorylation	Protein kinase activity	Endosome, vacuole membrane, phosphatidylinositol 3-kinase complex (I and II), pre-autophagosomal structure
TPD3	Actin filament organization, cell bud growth, mitotic cell cycle spindle assembly checkpoint, translation,	Contributes to serine/threonine phosphatase activity	
CHC1	Endocytosis, vesicle-mediated transport	Structure molecule activity	Clathrin vesicle coat
BUD32	Positive regulation of transcription from RNA polymerase II promoter, telomere maintenance, cellular bud site selection	Serine/threonine kinase activity	EKC/KEOPS protein complex, cytoplasm, nucleus

Strains deleted in the following genes exhibited slow growth under the screen conditions: *ERG6, YM1010W-A, CPA1, CIN5, MRP51, MSY1, TAF14, PET100, YDR065W, RVS167, RMD7, GLN3, BUR2, YGR043C, YOR200W, MCT1, MRPL15, ISM1, DBP7, YDL129W, NAM2, TUP1, VAN1*, and *YPR022C*

Table 2

Drug and ion sensitivities of uncovered *hhy* mutants

Strain	YPD	1M NaCl	1M KCl	0.5 M KCl	0.5 mM $CaCl_2$	0.2 mM $CaCl_2$	10 mM $MnCl_2$	4 mM $ZnCl_2$	pH 2.5	pH 8.0	20 nM rapamycin	20 mM caffeine
WT	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
hhy1	+++	+++	+++	+++	∓	∓	∓	∓	∓	∓	∓	∓
drs2	+++	+++	+++	+++	++	++	++	+++	+++	++	∓	∓
luv1	+++	+++	+++	+++	+++	+++	∓	∓	∓	∓	−	−
sbh2	++	++	++	++	++	++	++	++	++	∓	∓	∓
sac1	+++	++	+++	+++	+++	+++	+++	++	++	∓	∓	−
arf1	++	++	++	++	++	++	∓	++	++	++	++	∓
paf1	+++	+++	+++	+++	+++	+++	++	+++	+	++	−	−
dhh1	+++	++	+++	+++	+++	+++	++	+++	+++	++	∓	−
vps45	+++	++	+++	+++	+++	+++	∓	∓	++	∓	∓	−
sac2	+++	++	+++	+++	+++	+++	∓	∓	−	∓	∓	−
vps34	++	∓	++	++	++	++	++	−	∓	−	−	−
tpd3	++	∓	+	+	+	++	−	−	+	∓	−	−
chc1	+++	+++	+++	+++	+++	+++	−	∓	∓	−	−	−
bud32	+++	+++	+++	+++	+++	+++	∓	∓	∓	+++	∓	−

BY4742 (WT) and 14 mutants were grown on YPD and selective media with the indicated concentrations. Following 48–96-h growth, colony number and size on plates were scored as follows: +++ strong growth; ++ moderate growth; ∓ weak growth; − no growth

Table 3

Summary of various characterizations of *hhy* mutants (this study)

Gene name	Secretion	Halo assays	CPY processing	Ion/pH sensitivity	Caff./Rap. sensitivity	Vacuole morphology	Acidification	Temperature sensitivity at 37°C	Temperature sensitivity at 15°C
tpd3	Defective	Defective	WT	Yes	Yes	WT	WT	Yes	Yes
drs2	WT	WT	Defective	Slight	Yes	WT	WT	No	Yes
sbh2	WT	WT	WT	Yes	Yes	WT	WT	No	Yes
sac1	Defective	Defective	Defective	Yes	Yes	Fragmented	WT	No	Yes
arf1	WT	Defective	Defective	Yes	Yes	Fragmented	WT	No	Yes
chc1	WT	Defective	WT	Yes	Yes	WT	WT	Slight	Yes
paf1	WT	Defective	Defective	Yes	Yes	Fragmented	WT	Yes	Yes
dhh1	WT	WT	Defective	Yes	Yes	Fragmented	WT	No	Yes
bud32	WT	Defective	Defective	Yes	Yes	WT	WT	Yes	Yes
hhy1	WT	WT	Defective	Yes	Yes	Fragmented	Defective	Yes	No

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