A Genome-Wide Immunodetection Screen in *S. cerevisiae* Uncovers Novel Genes Involved in Lysosomal Vacuole Function and Morphology

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**Abstract**

Vacuoles of yeast *Saccharomyces cerevisiae* are functionally analogous to mammalian lysosomes. Both are cellular organelles responsible for macromolecular degradation, ion/pH homeostasis, and stress survival. We hypothesized that undefined gene functions remain at post-endosomal stage of vacuolar events and performed a genome-wide screen directed at such functions at the late endosome and vacuole interface – *ENV* genes. The immunodetection screen was designed to identify mutants that internally accumulate precursor form of the vacuolar hydrolase carboxypeptidase Y (CPY). Here, we report the uncovering and initial characterizations of twelve *ENV* genes. The small size of the collection and the lack of genes previously identified with vacuolar events are suggestive of the intended exclusive functional interface of the screen. Most notably, the collection includes four novel genes *ENV7, ENV9, ENV10*, and *ENV11*, and three genes previously linked to mitochondrial processes – *MAM3, PCP1, PPE1*. In all *env* mutants, vesicular trafficking stages were undisturbed in live cells as assessed by invertase and active α-factor secretion, as well as by localization of the endocytic fluorescent marker FM4-64 to the vacuole. Several mutants exhibit defects in stress survival functions associated with vacuoles. Confocal fluorescence microscopy revealed the collection to be significantly enriched in vacuolar morphologies suggestive of fusion and fission defects. These include the unique phenotype of luminal vesicles within vacuoles in the novel *env9* mutant and severely fragmented vacuoles upon deletion of *GET4*, a gene recently implicated in tail anchored membrane protein insertion. Thus, our results establish new gene functions in vacuolar function and morphology, and suggest a link between vacuolar and mitochondrial events.

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**Introduction**

A hallmark of eukaryotic cells is their ability to meticulously organize and compartmentalize tasks within membrane bounded organelles. An example of such a specialized organelle is the dynamic lysosome. The lysosome is involved in vital cellular functions including macromolecular degradation, receptor down regulation, pH/ion homeostasis, and stress survival [1–4]. The yeast vacuole is functionally analogous to the mammalian lysosome and serves as an elegant genetic model for studying lysosomal function, biogenesis, and trafficking. Yeast genes involved in these processes often have conserved orthologues in higher eukaryotes [5,6].

Three conserved 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 trans Golgi and their vesicular delivery to the vacuole [7–11]. 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 [1,2,12]. The conserved macroautophagic and the yeast-specific cytoplasm-to-vacuole targeting (Cvt) pathways involve the transport of cytoplasmic components directly to the vacuole through vesicular delivery [4,13–16]. All three trafficking pathways involve a final step of heterotypic membrane fusion at the vacuole. Furthermore, the vacuole itself is a dynamic organelle which undergoes homotypic fusion/fission events during budding and in response to environmental stress and nutrient levels. Due to limited available mutants, heterotypic fusion remains less understood than homotypic fusion. Nonetheless, all membrane fusion/fission events at the vacuole remain of intense interest [17,18]. Several models have been presented to elucidate late endosome to lysosome trafficking, including endosomal maturation, direct fusion of endosome to lysosome, and carrier transport [19,20]. Two models that support direct fusion include the “kiss and run” model where fusion and fission of endosomes and lysosomes occur to exchange cargo, and the “hybrid” model where a hybrid organelle is formed by heterotypic fusion of late endosome and lysosome [21–24]. The continued persistence of multiple models is
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largely due to the limited gene functions identified at the final stages of lysosomal delivery and processing. We are interested in exploring such gene functions in yeast.

As in mammalian cells, the biosynthetic pathway delivers the bulk of the resident hydrolytic enzymes of the vacuole including the well characterized carboxypeptidase Y (CPY). CPY is synthesized as an inactive zymogen and undergoes core glycosylation in the rough endoplasmic reticulum to form p2CPY precursor. It is then transported through Golgi compartments where it is further glycosylated into p2CPY form. In trans Golgi, p2CPY is sorted from the secretory pathway by its receptor, Vps10p, and is delivered to the late endosome [11,25]. At the late endosome, p2CPY is uncoupled from its receptor and is delivered to the vacuole where it is processed into mature CPY in a Proteinase A (PrA) dependent manner. Vps10p receptor is recycled back to trans Golgi via the retromer complex and is engaged in repeated cycles of p2CPY sorting and delivery at the trans Golgi and late endosome interface. Several laboratories have reported genetic screens for yeast mutants defective in the CPY pathway including pef1, isp1, and ecm27 mutants [26–30]. These mutant collections generally secrete p2CPY. We hypothesized that in mutants exclusively defective at post-endosomal stage of vacuole delivery/function, Vps10p would retain its ability to cycle back to trans Golgi and direct p2CPY to late endosomes. Based on this hypothesis, we developed a novel immunodetection screen for mutants that internally accumulate p2CPY at the late endosome and vacuole interface – env mutants [31]. In the current study, we modified and applied the approach on a genome-wide scale using the MAT-a haploid deletion strain library, which uncovered 12 ENV genes. This study ascribes new vacuolar processing and morphology functions to several formerly characterized genes and uncovers four novel ENV genes, ENV7, ENV9, ENV10, and ENV11.

Results

Genome-wide immunodetection screen

Previously, we reported a novel colony Immunodetection genetic screen for internal accumulation of precursor CPY [31]. Here, we modified and applied the screen at a genome-wide level to the MAT-a haploid deletion strain library in two stages (Figure 1). In the primary immunodetection screen, we uncovered 188 mutants that indicated any proCPY presence above wild type levels in lysed patched samples. Uncovered strains were further pursued in a secondary screen of lysed and unlysed patched strains to confirm persistent proCPY and identify mutants that specifically accumulate proCPY internally. The secondary screen was repeated at least four times for each of the 188 mutants and only the 166 mutants which gave persistent and strong proCPY signal in repeated experiments were pursued beyond the secondary screen. During primary and secondary screens, the strains were identified only by grid/plate number to minimize bias. The mutants were matched to systematic/gene names at the completion of the two stage screen. 151 of the 166 strains secreted proCPY and 150 of those were, in fact, previously identified isp mutants in the original mutant screens [27,32] and the genome-wide screen of deletion mutants [30]. Additionally, one previously unreported isp mutant, ecm27A, was uncovered. Fifteen putative env mutants were identified at the end of the secondary screen stage. Since errors and extraneous mutations have been reported with the haploid deletion strain collection, MATa counterparts of the putative mutants were also assessed for env phenotype via patch immunoblots. Aside from the deletion mutant for proteinase A (PrA), the vacuolar hydrolase responsible for CPY maturation [33], thirteen env mutants were confirmed in their MAT-a counterparts, and their deleted genes were confirmed by PCR. One of the fourteen mutants, hhy1/yel059w, was also uncovered by us in a genome-wide screen for hygromycin B hypersensitivity. The ORF is designated as a “dubious ORF, unlikely to encode a protein” in the Saccharomyces Genome Database (SGD), and the reintroduction of the ORF does not result in either phenotype ([34], unpublished results). Thus, the responsible locus for the phenotype remains unmapped. Table 1 lists the 12 uncovered ENV genes and their annotated biological process, molecular function, and cellular component as gleaned from SGD (http://www.yeastgenome.org).

The collection does not include any genes previously identified with vacuolar events. DCR2 overlaps 34 bases of the FPS38 C-terminus coding sequences. As such, it may signify a weak FPS38 allele. A previous genome-wide study aimed at identifying endosomal transport factors conducted quantitative immunoblot assays to assess secretion of CPY in the deletion strain library [35]. Of the 12 ENV genes, DCR2 was the only one consistently scored in the top 200 hits. However, since DCR2 encodes a protein involved in ER unfolded protein response, it was pursued as part of the ENV collection. The uncovered twelve genes are not allicic to the loci implicated in env1-env3 allelic mutants, and complementation assays in diploids of conditional env4 and env5 haploids and the non-isogenic deletion env mutants have been inconclusive ([31], unpublished results). GO TermFinder analysis displayed no significant enrichments. However, based on published information on ENV gene products, we note a few key groupings. Three ENV genes function in mitochondrial morphology and/or organization. MAM3 was originally identified to be involved in mitochondrial morphology in a systematic study [36]. More recent studies reveal that it is an integral vacuolar membrane protein whose degrees of expression mark the degree of manganese toxicity [37]. PCP1 encodes a rhomboid-like mitochondrial intramembrane serine protease that is essential for normal mitochondrial morphology and DNA maintenance [38,39]. At least two functions have been noted for PPE1. Its gene product has been previously identified as a mitochondrial ribosomal protein [40] and also as a carboxyl methylesterase that inactivates the catalytic subunit of phosphoprotein phosphatase 2A (PP2A) [41].

Three ENV gene functions are associated with the endomembrane system. SBE22 encodes a Golgi protein that functions in transporting components necessary for cell wall formation from the Golgi to the cell surface and aids in cell wall integrity [42]. DCR2 encodes a phosphoesterase that acts as both a dosage-dependent cell cycle regulator [43] and a down regulator in the ER unfolded protein response [44]. During our studies, we failed to characterize ENV gene, ENV8, was identified by others as a member of the GET complex genes involved in tail-anchored protein insertion and named GET4 [45,46].

Two ENV genes are cytoplasmic residents. RPS27B encodes the protein component of the small (40s) ribosomal subunit and may be involved in the structural dynamics of the ribosome [47,48]. HOG1 has been extensively studied as the central node in the HOG signaling pathway involved in osmotic stress response. Hog1p is a mitogen-activated protein (MAP) kinase; under hyperosmotic stress it localizes to the nucleus and mediates upregulation of genes by phosphorylating transcription factors [49,50]. Its mammalian homolog, MAP kinase p38, also functions in hyperosmotic and stress responses [51,52].

Lastly, four ENV genes are novel and have no previously established functions – ENV7, ENV9, ENV10, and ENV11. To confirm that the env phenotype in the four mutants is a result of the deleted genes, we constructed recombinant CEN vectors encoding
each of the novel ORF’s, introduced each into the corresponding deletion mutant, and assayed for phenotypic complementation by means of colony immunodetection (Figure 2). In each case, the mutant phenotype is rescued with the reintroduction of the corresponding ORF and its endogenous upstream/downstream regulatory sequences. Thus, \textit{ENV7}, \textit{ENV9}, \textit{ENV10}, and \textit{ENV11} are four novel genes whose individual deletion leads to internal accumulation of precursor CPY.

Secretion and \(\alpha\)-factor processing in \textit{env} mutants

Internal accumulation of p2CPY in putative \textit{env} mutants could be conceivably due to blocks in the pre-endosomal stages of trafficking including secretion. To assess early trafficking events as well as trans Golgi to plasma membrane trafficking, we conducted quantitative invertase secretion and qualitative active pheromone secretion assays (Figure 3, A and B). When yeast is grown on sucrose as the sole carbon source, secreted invertase catalyzes the disaccharide to glucose and fructose for uptake through their corresponding permeases and can be quantitatively measured via a colorimetric assay for glucose. Such quantitative assays showed no statistically significant secretion defect in \textit{env} mutants relative to the isogenic wild type (Figure 3A). Thus, secretion and all vesicular trafficking events shared between the vacuolar biosynthetic and secretion pathways remain intact in \textit{env} mutants. As such, \textit{ENV}
Table 1. Uncovered genes and their known biological processes, molecular functions, and cellular components (Saccharomyces Genome Database).

| ENV Genes | Biological Process | Molecular Function | Cellular Localization |
|-----------|--------------------|--------------------|-----------------------|
| MAM3 (YOL060C) | Required for normal mitochondrial organization and cell Mn²⁺ homeostasis | Unknown | Vacuole membrane |
| PCP1 (YGR110W) | Required for normal mitochondrial organization, protein import into mitochondrion IM space, signal peptide process | Peptidase activity | mitochondrion |
| PPE1 (YHR075C) | Protein modification process; small subunit mitochondrial ribosome protein | Protein with carboxyl methylesterase activity | Soluble fraction |
| DCR2 (YLR361C) | ER unfolded protein response, protein aa dephosphorylation activity, pt of start for mitotic cycle | Hydrolase, phosphoesterase involved in down regulation of the unfolded protein response | Unknown |
| SBE22 (YHR103W) | Involved in the transport of Cell wall components (bud growth), functionally redundant with sbe2p | Unknown | Cytoplasm |
| HOG1 (YLR113W) | Hypersomatic response, osmosensory signaling pathway regulation of RNA pol II transcription | MAP kinase activity | Cytoplasm, nucleus |
| RP527B (YHR021C) | Cleavage of tricistronic rRNA transcript and maturation, translation, small ribosomal subunit assembly | Structural constituent of ribosome | Cytosolic small ribosomal subunit |
| ENV7 (YPL236C) | Unknown | Protein serine/threonine kinase activity | Fungal-type vacuole membrane |
| GET4/ENV8 (YOR164C) | Posttranslational protein targeting to membrane; protein insertion into ER membrane | Unknown | Cytoplasm |
| ENV9 (YOR246C) | Unknown | Oxidoreductase activity | Lipid particle |
| ENV10 (YLR065C) | Unknown | Unknown | unknown |
| ENV11 (YGR071C) | Unknown | Unknown | Nucleus |
| VPS Gene | Biological Process | Molecular Function | Cellular Localization |
|------------|------------------|--------------------|-----------------------|
| ECM27 | Cell wall organization | Unknown | Unknown |

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gene deletions do not result in defects at ER/Golgi, intraGolgi, or Golgi/plasma membrane interfaces. The pheromone halo assay was used next to evaluate protein trafficking at the late endosome and trans Golgi interface in the env mutants as well as the new vps mutant, env27A by assessing protease dependent activation of α-factor in trans Golgi. Mature α-factor is a small peptide pheromone that arrests the cell cycle and cell growth of MAT-a yeast cells in preparation for mating [53]. It transits the early stages of vesicular trafficking as a pro-pheromone and is processed in trans Golgi in a protease dependent manner to its mature, active form prior to secretion. Several proteases that recycle between trans Golgi and late endosome have been implicated in pro-α-factor maturation and activation in trans Golgi including Kex1p, Kex2p, and DPAP-A [54–57]. Active pheromone is capable of arresting the cell cycle of a lawn of supersensitive MAT-a cells in halo assays. As intact secretion was confirmed by invertase assays, any defect in halo formation would indicate defects at trans Golgi and late endosome interface. This assay has been used repeatedly in our laboratory to assess both major and minor defects in α-factor secretion [34,58]. From equivalent inocula, equivalent zones of growth inhibition relative to patch size were seen in env mutants, ccm27A, and the isogenic wild type in repeated experiments (Figure 3B). As expected, a severely compromised halo is seen in vps35A, which lacks the Vps35p component of the retromer complex involved in retrograde trafficking from late endosome to trans Golgi. Due to their slower growth, fob1Δ and vps27Δ exhibited both smaller patches and halos relative to wild type. Thus, defects were not noted in vesicular trafficking stages leading to secretion nor in anterograde/retrograde trafficking at trans Golgi and late endosome interface in env mutants.

Bulk endocytosis and vacuolar morphology in the uncovered mutants

To assess the integrity of bulk endocytosis and vacuolar morphology, uncovered strains were stained with FM4-64 and

Figure 2. ENV7, ENV9, ENV10, ENV11 ORF’s complement env phenotype of their corresponding deletion mutants. The four strains deleted in each of the orphan genes were transformed with parent CEN vector or CEN vector containing the corresponding deleted ORF and its flanking 300–500 upstream and downstream sequences. Transformed cells were patched onto SM-URA plates for selection and were subjected to colony immunodetection with anti-proCPY specific mAb. WT(BY4742) transformed with parent or recombinant vectors containing each of the four ORF’s were grown and processed in parallel as controls. doi:10.1371/journal.pone.0023696.g002
viewed with DIC and confocal microscopy (Figure 4A); 300–400 cells were scored per strain for statistical analysis (Figure 4B). FM4-64 is a vital fluorescent styryl dye that embeds itself into the plasma membrane and eventually localizes to the vacuole membrane through the endocytic pathway. As such, it serves as both an endocytic marker and a vacuole membrane marker in live cells. In wild type cells, the vital dye localizes to the vacuole within 60 minutes of incubation at 30°C [59]. Since FM4-64 traverses the cell along the endocytic pathway, localization of the dye to the vacuole membrane requires intact vesicular trafficking at the interfaces of plasma membrane, early endosome, late endosome, and vacuole. We observed that FM4-64 localizes to vacuole membranes in all uncovered mutants, indicating intact bulk endocytosis. Vacuolar localization of FM4-64 in mutants with drastic vacuolar morphology defects was confirmed by costaining with the vacuolar dye Cell Tracker Blue (Figure 4C).

In the isogenic wild type BY4742 strain, the predominant vacuole morphology is 1–4 prominent vacuoles seen in 64% of cells, while 30% have fragmented vacuoles (Figure 4, A and B). The new vps mutant, *ecm27Δ*, displays a wild type vacuole morphology pattern and can be classified as Class A (Figure 4, A and B). Six of the 12 env deletion strains have statistically significant vacuolar morphology differences from wild type (Figure 4, A and B). *pcp1Δ* and get4/env8Δ strains exhibit predominantly fragmented vacuoles at 63.5% and 50.5%, respectively; *env11Δ* exhibits a
significant increase in cells with fragmented vacuoles at 41.4%. Additionally, both *pcp1*Δ and *get4/env8*Δ cell sizes are significantly larger than that of wild type in the MAT-a background. The average size of the wild type cells observed is approximately 4 μm in diameter, whereas *pcp1*Δ and *get4/env8*Δ strains are approximately 8–10 μm in diameter. However, the larger cell sizes were not observed in the MAT-a counterparts (data not shown) and may be specific to MAT-a mating type.

*ppc1*Δ and *dcr2*Δ exhibit statistically significant reduction in number of cells with fragmented vacuoles at 17%, and 20.2%, respectively. Lastly, the prominent vacuole morphology in the novel *env9*Δ mutant is one or more lumenal vesicles within the vacuole in 47% of scored cells in both mating types (Figure 4, A–C). The vacuolar morphology phenotype was complemented upon reintroduction of *ENV9* gene (data not presented). To our knowledge, lumenal vesicles have not been previously reported in vacuolar morphology studies. Thus, six of the twelve env mutants have vacuolar morphologies suggestive of vacuole fusion and fission defects.

**Growth of env mutants under various stress conditions**

(env mutants were assayed for pleiotropic growth defects under several drug, pH/ion and temperature conditions (Figure 5; Table 2). Growth characterizations were formulated based on previous studies of conditions that affect mutants defective in vacuolar trafficking and/or function. Since wild type growth was retarded under several tested conditions, mutant growth was scored relative to the isogenic wild type for each specific condition. Three strains show growth sensitivities to high salt suggestive of defects in osmoregulation, a vacuolar function. *pcp1*Δ and *rps27b*Δ are sensitive at 1.5 M NaCl, and *hog1*Δ is unable to grow at both 1.0 M and 1.5 M NaCl consistent with the established role of its MAP kinase product in osmoregulation [50]. *hog1*Δ is also sensitive to potassium and low pH. *pcp1*Δ, *sbe22*Δ, and *rps27b*Δ exhibit severe sensitivities to manganese, while *mam3*Δ grows more robustly in presence of manganese relative to wild type. *mam3*Δ phenotype is consistent with previous studies that attribute increased manganese tolerance.
to MAM3 deletion [37], mam3Δ, pep1Δ, and rps27bΔ also exhibit growth defects in presence of both rapamycin and caffeine. Growth sensitivities to both drugs are hallmarks of defects in Tor kinase complex 1 (TORC1) mediated signal transduction, and Tor1p kinase localizes to vacuolar membranes in living yeast cells [60].

env9Δ and sbe22Δ show growth sensitivities to caffeine but not rapamycin, suggestive of defects in cell wall integrity pathway. sbe22Δ has been previously identified as playing a significant role in cell wall formation [42]. Growth at 37°C and 15°C were also assayed. env mutants exhibit no growth sensitivities at 37°C (data not presented). Cold sensitivity is suggestive of compromised dynamics in large, multi-subunit complexes that render them “frozen” at low temperatures. The novel mutants env10Δ and env11Δ, and the only two mutants with predominantly fragmented vacuoles, pep1Δ and get4/env8Δ, are cold sensitive (Table 2).

Lastly, monensin sensitivity has been linked to acidification defects in Golgi and post-Golgi compartments [61]. Recently, a genomic screen for monensin sensitivity (mon mutants) was heavily enriched for post-Golgi trafficking, which also included a subset of genes involved in vacuole biogenesis [61]. None of the env mutants were included in the mon collection, and our assays for monensin sensitivity did not uncover any growth defects (Table 2).

Thus, several members of env collection exhibit defects in vacuolar related stress survival that includes survival under rapamycin, caffeine, manganese, and osmotic stress.

### Steady state accumulation of p2CPY in env mutants

We performed western blot analysis of cell lysates to confirm the internal accumulation of p2CPY in env mutants using the pro-specific monoclonal antibody (Figure 6A). pep4Δ mutants are completely defective in CPY processing and were used as a positive control for p2CPY accumulation. Steady state persistence of p2CPY could be confirmed in nine out of twelve env mutants in repeated experiments. No proCPY species could be detected in rps27bΔ, pep1Δ, and hog1Δ lysates under the initial growth and western blot analysis conditions used. The three mutants are unique in the collection for exhibiting growth sensitivities to high salt conditions (Table 2). Hence, the three strains were subjected to western analysis subsequent to logarithmic growth under 0.75 M NaCl, which was the highest NaCl concentration that supported WT and mutant growths in liquid YPD cultures. Following high salt treatments, persistent p2CPY could be reproducibly seen in pep1Δ, but not in rps27bΔ or hog1Δ strains. The two strains were also analyzed for p2CPY accumulation in westerns following treatments to which each had shown growth sensitivities in our studies (Table 2), including potassium, manganese, and caffeine, but no persistent p2CPY was detectible (data not presented). Thus, within the resolution of our western analyses, steady state intracellular accumulation of the 69 kDa p2CPY could be confirmed for mam3Δ, pep1Δ, pep1Δ, dcr2Δ, sbe22Δ, env7Δ, get4/env8Δ, env9Δ, env10Δ, and env11Δ.

### Discussion

Here, we have described a yeast genome-wide screen designed to uncover mutants with exclusive defects at the late endosome and vacuole interface – env mutants. We report a collection of twelve ENV genes whose deletion results in defects in vacuolar function and morphology. A combination of quantitative invertase
and qualitative active α-factor secretion assays reveal no secretion or vesicular trafficking defects prior to the late endosome. The bulk endocytic pathway is also intact in all env mutants as assessed microscopically. As the biosynthetic CPY-pathway and the endocytic pathway converge at the late endosome, intact endocytosis also implicates normal trafficking from late endosome

Table 2. Growth characterizations of uncovered mutants under selective conditions.

| BY4742     | YPD | 1.0 M Na+ | 1.5 M Na+ | 1.0 M K+ | 10 mM Mn2+ | 4 mM Zn2+ | 0.5 mM Ca2+ | pH 8.0 | pH 2.5 | 20 nm rapamycin | 20 mM caffeine | SM+ 0.5% ethanol | SM+ 50 μM monensin | YPD 15°C |
|------------|-----|-----------|-----------|---------|-----------|-----------|-----------|-------|-------|----------------|----------------|-----------------|-------------------|---------|
| WT         | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| env7A      | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| get4/env8A | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| env9A      | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| env10A     | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| env11A     | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| mam3A      | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| pcp1A      | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| ppe1A      | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| da2A       | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| sbe22A     | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |
| hog1A      | ++  | +         | +         | +       | +         | +         | +         | +     | –/+   | –/+            | –/+            | –/+             | –/+               | –/+    |
| rps27bA    | ++  | +         | +         | +       | +         | +         | +         | +     | –/+   | –/+            | –/+            | –/+             | –/+               | –/+    |
| ecm27A     | ++  | ++        | ++        | ++      | ++        | ++        | ++        | ++    | ++    | ++             | ++             | ++              | ++                | ++      |

+++ stronger growth than WT
++ WT growth
+ weaker growth than WT
–/+ significantly weaker growth than WT
– no growth

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Figure 6. Steady-state accumulation of p2CPY in env mutants. Strains were grown in YPD overnight and were then diluted for further growth to logarithmic phase in YPD (A), or YPD+0.75 M NaCl (B). Cell extracts were normalized for protein content and subjected to western analysis. pep4A mutants are completely defective in CPY processing and a 1:5 dilution of cell lysate was used as control for more equivalent visualization of signal. Membranes were probed with anti-proCPY mAb; hexokinase I was used as loading control and detected with pAb’s. Two separate gels in A are marked by a vertical bar.
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to vacuole. Together, these data spatially map the defect observed in *env* mutants to the vacuole. Such defects would be expected to include homotypic and/or heterotypic fusion/fission defects at the vacuole, or vacuolar function defects such as macromolecular processing or stress survival. Indeed, *env* mutants exhibit such defects. First, based on the screen itself, *env* mutants show CPY processing defects. Second, half the collection exhibits vacuolar morphologies suggestive of fusion/fission defects including a novel morphology of vesiculated vacuoles in *env*Δ – a novel mutant itself. Third, a partially overlapping group of five mutants exhibit severe defects in various stress survivals associated with vacuolar function. Thus, pleiotropic phenotypes associated with defects at the vacuole are the common thread in this mutant collection. The fact that none of the *ENV* genes overlap with vacuolar protein sorting (*VPS*), vacuolar morphology (*VAM*), or monensin sensitivity (*MON*) genes suggests that they represent previously unappated and distinct vacuolar related gene functions. Accordingly, one third of the uncovered *ENV* genes are previously uncharacterized genes – *ENV7*, *ENV9*, *ENV10*, and *ENV11*. Genome-wide localization studies of GFP-tagged strains have reported Env7p at the vacuole membrane, Env9p in lipid particles, and Env11p in the nucleus [62]. To date, our preliminary microscopic and biochemical studies confirm localization of HA- and GFP-tagged Env7p to the vacuole membrane and Env11p to the nucleus (Manandhar, Oliveira, Ricarte, and Gharakhanian, unpublished results). Our bioinformatics analyses suggest that Env7p is a putative kinase with 29% identity with the human serine/threonine kinase STK16 [63–65], and Env9p is a putative oxidoreductase with 40% identity with the human retinol dehydrogenase RDH12, a gene associated with retinal degeneration [66]. The cellular functions of both human proteins remain unknown. Consistent with its nuclear localization, Env11p includes a putative zinc-finger DNA binding domain. These findings support a combination of enzymatic roles in vacuolar events by *ENV7* and *ENV9* gene products, as well as an indirect role through transcriptional regulation of other gene products by *ENV11*. Localization studies as well as functional and phylogenetic characterizations of novel *ENV* genes are in progress.

A persistent steady-state internal pool of p2CPY species could not be confirmed for rps27bΔ and hog1Δ in western analyses under normal or high salt suspended growth conditions. Since the same monoclonal antibody recognizes a pro-fragment containing species in patch immunoblots, the processing defect in rps27bΔ and hog1Δ may be in degradation of the ~9 kDa pro-fragment following its cleavage from p2CPY. In repeated experiments, we did not detect such a species in 8–12% polyacrylamide gels as assessed by appropriate molecular weight markers (data not presented). Alternatively, the *env* phenotype in rps27bΔ and hog1Δ may be minimized in liquid cultures due to differences in vacuole dynamics, osmotic support or transcription/translation rates between liquid and semi-solid growth conditions. As the vacuole structurally and functionally responds to the environment, such phenotypic plasticity would not be inconsistent with the osmotic stress sensing role of Hog1p and the general translation role of the ribosomal subunit Rps27bp. Alternatively, their mutant strains may represent false positives of the screen at 2 out of total of 166 (1.2%) that were denoted as *env* or *vps* at the end of the secondary screen.

Additionally, we identified *ECM27* as a new *VPS* gene and established its deletion mutant as Class A with respect to vacuole morphology. *ECM27* was first uncovered in a screen for calcufluor hypersensitivity [67]. More recently, it was included in the top 20 genes whose deletion leads to CPY secretion in a genome-wide study [35]. Unlike most *vps* mutants, *ecm27Δ* does not exhibit a defect in retrograde trafficking as assessed in our halo assays. This difference may be why the gene was not picked up in *vps* screens and suggests that the involvement of Ecm27p in vesicular trafficking of CPY may be distinct from known Vps proteins. Bioinformatic analyses predict a signal peptide, multiple transmembrane domains, and homologies to sodium/calcium exchangers. The cellular function and localization of Ecm27p remain unknown.

**ENV genes and vacuole morphology**

Microscopic studies of the *env* mutants reveal previously unreported vacuolar morphology functions for *PCP1, GET4, ENV11*, *PP1, DCR2*, and *ENV11*. The null mutants of the genes have statistically significant increases or decreases in fragmented vacuoles relative to the isogenic wild type strain suggestive of in vivo vacuole fusion/fission defects. The products of *PCP1* and *PP1* genes are involved in mitochondrial events, and *ppl1Δ* mutants have been reported to exhibit fragmented mitochondria [39,40]. Like the vacuole, mitochondria are dynamic organelles that engage in fusion and fission events. Furthermore, the product of *MAM3*, the third uncovered *ENV* gene to have a previously reported mitochondrial function, has been reported to be localized to the vacuole membrane [37]. Recently, phospholipid mediated signaling between mitochondria and vacuoles has been proposed [68], and mitochondrial contribution has been suggested in biogenesis of the autophagosome membrane in macroautophagy [69]. Together, these and our findings suggest that mitochondrial and vacuolar events may be deeply interconnected. Assessment of mitochondrial morphology in *env* mutants, particularly mutants exhibiting abnormal vacuolar morphology, may shed light on more global organelar fusion/fission gene functions.

Most interestingly, the novel mutant *env*Δ exhibits lumenal vesicles within vacuoles – a previously unreported phenotype in vacuolar morphology studies. The in vivo lumenal vesicles are reminiscent of luminal vesicles reported following in vitro homotypic fusion of isolated vacuoles [70]. Staining of the luminal vesicle membranes with the endocytic and vacuole membrane marker FM4-64 suggests that their origin may be an endocytic compartment. Defects in breakdown of internalized vacuolar membranes following microautophagy (invagination of vacuole membrane), heterotypic fusion of late endosomal multivesicular bodies (MVBs) with vacuoles, or homotypic vacuole fusion would be consistent with the observed phenotype. Interestingly, high throughput genetic interaction studies report negative interactions between *ENV9* and *ENV10* as well as between *ENV9* and *GET4*/*ENV8* [71]. Assessment of CPY processing and vacuolar morphology in these double mutants may be especially instructive.

**ENV genes and stress survival**

Several *env* mutants are sensitive to environmental stress conditions suggestive of defects in vacuolar stress survival. *hog1Δ* exhibits severe growth sensitivity to sodium consistent with the established role of its MAP kinase product in osmoregulation [50]. The mutant also exhibits growth sensitivities to potassium and low pH suggestive of HOG pathway mediated response under a range of stress conditions. A subset of *env* mutants exhibit tolerance or hypersensitivity to manganese. *mam3Δ* shows increased tolerance to manganese consistent with previous reports [37], while *she22Δ* and *rps27bΔ* exhibit no growth in its presence. The response to manganese does not apply generally to metals since no tolerance/hypersensitivity was observed with zinc. The two yeast manganese transporters, Smf1p and Smf2p, are ubiquitinated and directed to the vacuole for degradation through the MVB pathway at both physiological and toxic levels [72–74]. As such, tolerance or
lysosomal gene functions in health and disease. Yeast will likely contribute to further understanding of conserved unknown cellular function. As such, their functional elucidation in ENV7 organization and function. Several degradation as well as possible connections to mitochondrial events including fusion/fission dynamics and macromolecular gene collection is poised to offer new insights into vacuolar contribution of this study is identification of four novel genes stress survival defects at the vacuole. The most significant enriched for heterotypic and/or homotypic fusion/fission and indirectly at the vacuole. The uncovered genes affect vacuolar in stress survival functions orchestrated at the vacuole. Thus, the env collection is enriched for defects in stress survival functions orchestrated at the vacuole.

Our results suggest that ENV genes affect events directly or indirectly at the vacuole. The uncovered genes affect vacuolar CPY processing at a post-endosomal stage, and the collection is enriched for heterotypic and/or homotypic fusion/fission and stress survival defects at the vacuole. The most significant contribution of this study is identification of four novel genes involved in vacuolar events– ENV7, ENV9, ENV10, ENV11. The ENV gene collection is poised to offer new insights into vacuolar events including fusion/fission dynamics and macromolecular degradation as well as possible connections to mitochondrial organization and function. Several ENV genes, including the novel ENV7 and ENV9, appear to be orthologues of human genes with unknown cellular function. As such, their functional elucidation in yeast will likely contribute to further understanding of conserved lysosomal gene functions in health and disease.

Materials and Methods

Yeast media and strains

Yeast strains were grown on yeast extract-peptone-dextrose (YPD) or synthetic minimal (SM) media from Difco Chemicals (St. Louis, MO) and were prepared as described [83]. YPD or SM media were supplemented with selective components as specified. The MAT-a haploid S. cerevisiae deletion strain library, MAT-a haploids of the env and ips strains, and parental strains BY4742 (MAT-a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) and BY4741 (MAT-a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) were gifts from Dr. Greg Payne (UCLA). The collection of 4828 strains was developed by the Saccharomyces Genome Deletion project and contains 80% of the Saccharomyces cerevisiae genome, which was generated by PCR-based disruption of all open reading frames by chromosomal integration of a KanMX4 module through homologous recombination [84]. Mutants were stored in 15% glycerol in fifty-two 96-well plates at −80°C.

Plasmids

PCR-based cloning was carried out to insert DNA sequences of previously uncharacterized ENV genes into the uracil-selectable CEN vector pRS316 [85]. Each ORF was amplified from BY4742 genomic DNA using custom-made forward (F1) and reverse (R1) primers (Operon; Huntsville, AL) that introduced restriction sites specified in parenthesis as follows:

pMSG370 was constructed using F1 (EagI) 5’-GGGGGCTCAGTGGGAGAGAAGTTATCTATTTG-3’; R1 (SacI) 5’-GGGGGCTCAGTGGGAGAGAAGTTATCTATTTG-3’, which resulted in a 2.1-kb insert containing 791-bp upstream and 298-bp downstream of YOR246CENV7.

pDOG3100 was constructed using F1 (EcoRI) 5’-GGGGGCTCAGTGGGAGAGAAGTTATCTATTTG-3’; R1 (SacI) 5’-GGGGGCTCAGTGGGAGAGAAGTTATCTATTTG-3’, which resulted in a 1.6-kb insert containing 588-bp upstream and 583-bp downstream of YLR656CENV10.

pLOG3110 was constructed using F1 (KpnI) 5’-GGGGGCTCAGTGGGAGAGAAGTTATCTATTTG-3’; R1 (SacI) 5’-GGGGGCTCAGTGGGAGAGAAGTTATCTATTTG-3’, which resulted in a 3.8-kb insert containing 626-bp upstream and 628-bp downstream of YGR071CENV11.

PCR reactions were carried out with Phusion DNA polymerase according to the manufacturer’s instructions (New England Biolabs; Ipswich, MA). Recombinant plasmids were amplified in Top10 competent E. coli (Invitrogen; Carlsbad, CA). Preparation of plasmid DNA was carried out using Zymy Plasmid Maxiprep and Midiprep Kits (Zymo Research; Orange, CA).

Genomic screen for internal accumulation of p2CPY

The genomic screen is summarized in Figure 1A and consisted of sequential primary and secondary immunoblot assays that were modifications of an approach first described in Takahashi et al. [31]. In the primary screen, yeast strains were transferred from 96-well plates onto YPD plates with a pinning tool and incubated for 48 hours at 30°C. Cells were replica-plated onto nitrocellulose membranes and incubated at 30°C overnight. Following overnight incubation, membranes were washed with double-distilled water (ddH2O) and subjected to a 40 minute chase with 20 mg/ml cycloheximide (Sigma-Aldrich; St. Louis, MO) at 30°C. This translation inhibition step minimizes p1CPY and p2CPY species that are normal intermediates in the emergence of mature CPY in wild type cells. Membranes were washed three times for five minutes with ddH2O and were exposed to lysis buffer (0.1% SDS, 0.2 M NaOH, and 0.5% β-mercaptoethanol) for 40 minutes at room temperature. Membranes were then washed with ddH2O and blocked in a solution of tris buffered saline (50 mM Tris–HCl, pH 8.0, 150 mM NaCl) and 0.05% (v/v) Tween-20 in 5% (w/v) non-fat dry milk for 1 hour. Following blocking, membranes were probed with anti-proCPY specific mAb (Mab-4-H10-E6, U. Oregon) and were subjected to chemiluminescent autoradiography (Thermo Scientific; Rockford, IL).

In the secondary immunoblot assay, positive strains from the primary screen were replica plated onto two YPD plates and then transferred to two nitrocellulose membranes. Both membranes were incubated with 20 mg/ml cycloheximide for 40 minutes. One membrane was treated with lysis buffer for 40 minutes (lysed immunoblots) and the other was treated with ddH2O (non-lysed immunoblots) for 40 minutes. Membranes were then probed and developed as described. Following the secondary screen, deleted ORF’s of uncovered env strains were confirmed by PCR using GoTaq (Promega; Madison, WI) and ORF-specific and KAN-cassette specific primers corresponding to those used for original library confirmations by the Saccharomyces Genome Deletion Project consortium (Operon; Huntsville, AL).

Following confirmation of deleted ORF’s, env phenotype of each mutant was assessed in its MAT-a counterpart by immunodetection as described above. Only mutants whose env phenotype was confirmed in MAT-a were pursued further.

Functional complementation of uncharacterized env mutants

To confirm linkage of the env phenotype to the deleted ORF’s in the novel mutants env7A, env9A, env10A, and env11A, correspond-
ing ORF’s were reintroduced by means of transformation with recombinant CEN vectors pMSG370, pTHG390, pDOG3100, and pLOG3110, respectively, using Frozen EZ Yeast Transformation II kit (Zymo Research; Orange, CA). WT (BY4742) with parent and recombinant vectors containing each ORF were used as controls. Transformed cells were selected on SM media without uracil. Transformed cells were subjected to colony immunodetection as described in the primary screen, with slight modifications. Cells were patched onto SM-URA plates and incubated at 30°C for 72 hours. Following incubation, cells were replica-plated onto nitrocellulose membranes and incubated on YPD plates at 30°C overnight. Subsequent steps were as described for primary screen.

Liquid invertase secretion assay

Quantitative liquid invertase assays were performed as described [86]. Secreted invertase is expressed as the percentage of extracellular invertase over total intracellular and extracellular invertase. Each strain was assayed a minimum of 3 times and statistical analysis was conducted using Minitab15 to determine if secretion was statistically different from wild type (Two-Sample t-Test, N = 3, confidence interval 95%, p<0.05).

Halo pheromone assay

A single colony of GPY-1796 was resuspended in 1 mL YPD and added to 4 mL of 0.5% agar (Sigma; St. Louis, MO). Resuspended cells in YPD + agar solution were poured evenly onto a YPD plate and allowed to solidify for 10 minutes. Single colonies of env and wild type control strains were diluted to OD<sub>600</sub> = 0.26 and patched onto seeded plates using a sterile pinning tool. Plates were incubated at 30°C for 3 days. Halo assays were repeated a minimum of three times.

Confocal microscopy

FM4-64 [N-(3-triethylammoniumpropyl)-4-p-diethylo- minophenylhexatrienyl]pyridium dibromide] staining was performed as described [59]. Prior to staining, overnight cultures were diluted in YPD and harvested during mid-logarithmic phase (OD<sub>600</sub> = 0.4–0.6). For containing experiments, vital dye Cell Tracker Blue CMAC was added in addition to prescribed content and subjected to western analysis [87]. Membranes were blocked in a solution of tris buffered saline (50 mM Tris–HCl, pH 8.0, 150 mM NaCl) and 0.05% (w/v) Tween-20 in 5% (w/v) non-fat dry milk. Following blocking, membranes were probed with anti-proCPY specific mAb (Mab-4-H10-E6, U. Oregon) and loading control anti-hexokinase I specific pAB (Abcam; Cambridge, MA). Membranes were then subjected to chemiluminescent autoradiography (Thermo Scientific; Rockford, IL). Yeast strains exposed to high salt conditions were grown overnight at 30°C in YPD, washed once in YPD+0.75 M NaCl, and grown to logarithmic phase at 30°C in YPD+0.75 M NaCl. All subsequent steps were performed as described above.

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Author Contributions

Conceived and designed the experiments: EG. Performed the experiments: FR RM SC TS MS TH LO EG. Analyzed the data: EG FR RM SC TS MS TH LO EG. Wrote the paper: FR EG.

References

1. Katsumata DJ, Odorizzi G, Emr SD (2002) Receptor downregulation and multivesicular-body sorting. Nat Rev Mol Cell Biol 3: 893–905.
2. Pelham HR (2002) Insights from yeast endosomes. Curr Opin Cell Biol 14: 454–462.
3. Luzio JP, Bright NA, Pryor PR (2007) The role of calcium and other ions in sorting and delivery in the late endocytic pathway. Biochem Soc Trans 35: 1081–1091.
4. Li SC, Kane PM (2009) The yeast lysosome-like vacuole: endpoint and crossroads. Biochim Biophys Acta 1793: 650–663.
5. Mullins C, Bonafacino JS (2001) The molecular machinery for lysosome biogenesis. Bioessays 23: 333–343.
6. Luzio JP, Poupon V, Lindsay MR, Mullock BM, Piper RC, et al. (2005) Membrane dynamics and the biogenesis of lysosomes. Mol Membr Biol 22: 141–154.

Growth assays in selective media and at high/low temperatures

YPD was supplemented with ions or drugs by adding NaCl, KCl, CaCl<sub>2</sub>, MnCl<sub>2</sub>, ZnCl<sub>2</sub>, rapamycin or caffeine to achieve concentrations specified in Figure 6 and Table 2. To prepare plates with pH of 2.5 or 8.0, HCl or NaOH was added to YPD, respectively. Monensin was resuspended in ethanol; SM Monensin plates and SM control plates containing ethanol were prepared as described [61]. Yeast colonies grown on YPD were suspended in sterile water to a concentration of OD<sub>600</sub> = 0.26 (approximately 3.0 x 10<sup>5</sup> cells/ml). Undiluted cells and serial 10-fold dilutions of up to 1:1,000 were transferred using a pinning tool onto selective media. Plates were incubated at 30°C, and assessed for growth after 2 to 9 days. For temperature sensitivity assays, env and wild type strains were streaked onto pre-equilibrated YPD plates and incubated at 15°C for 7 days or 37°C for 3 days.

Western blotting

Yeast cultures were grown at 30°C to logarithmic phase in YPD and whole cell lysates were prepared using Y-PER Yeast Protein Extraction Reagent, as directed by the manufacturer (Pierce; Rockford, IL). Protease inhibitor cocktail specific to yeast was added as directed by the manufacturer (Sigma; St. Louis, MO). Protein content was measured using BCA Protein Assay Kit (Bio-Rad; Hercules, CA). Lysates were normalized for protein content and subjected to western analysis [87]. Membranes were blocked in a solution of tris buffered saline (50 mM Tris–HCl, pH 8.0, 150 mM NaCl) and 0.05% (w/v) Tween-20 in 5% (w/v) non-fat dry milk. Following blocking, membranes were probed with anti-pro-CPY specific mAb (Mah-4-H10-E6, U. Oregon) and loading control anti-hexokinase I specific pAB (Abcam; Cambridge, MA). Membranes were then subjected to chemiluminescent autoradiography (Thermo Scientific; Rockford, IL). Yeast strains exposed to high salt conditions were grown overnight at 30°C in YPD, washed once in YPD+0.75 M NaCl, and grown to logarithmic phase at 30°C in YPD+0.75 M NaCl. All subsequent steps were performed as described above.

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7. Nothwehr SF, Stevens TH (1994) Sorting of membrane proteins in the yeast secretory pathway. J Biol Chem 269: 10183–10188.
8. Horazdovsky BF, DeWald DB, Emr SD (1995) Protein transport to the yeast vacuole. Curr Opin Cell Biol 7: 544–551.
9. Bryant NJ, Piper RC, Weiman LJ, Stevens TH (1998) Retrograde traffic out of the yeast vacuole to the TGN occurs via the prevacuolar/endoosomal compartment. J Cell Biol 142: 651–663.
10. Conibear E, Stevens TH (1998) Multiple sorting pathways between the late Golgi and the vacuole in yeast. Biochim Biophys Acta 1404: 211–230.
11. Bowers K, Stevens TH (2005) Protein transport from the late Golgi to the vacuole in the yeast Saccharomyces cerevisiae. Biochim Biophys Acta 1744: 430–454.
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12. Piper RC, Katzmann DJ (2007) Biogenesis and function of multivesicular bodies. Annu Rev Cell Dev Biol 23: 319–347.
13. Levine B (2005) Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. Cell 120: 159–162.
14. Huang J, Klionsky DJ (2007) Autophagy and human disease. Cell Cycle 6: 1837–1849.
15. Mijaljica D, Prescott M, Klionsky DJ, Devenish RJ (2007) Autophagy and vacuole homeostasis: a case for self-degradation? Autophagy 3: 417–421.
16. Simonsen A, Tooze SA (2009) Coordination of membrane events during autophagy by multiple class II PI3-kinase complexes. J Cell Biol 186: 773–782.
17. Ostromow CW, Meiringer CT, Ungermann C (2008) Yeast vacuole fusion: a model system for eukaryotic endomembrane dynamics. Autophagy 4: 3–19.
18. Wickner W (2010) Membrane fusion: five lipids, four SNAREs, two cholesterol, two tetraspans, and a Rab, all dancing in a ring on yeast vacuoles. Annu Rev Cell Dev Biol 26: 115–136.
19. Mellman I (1996) Endocytosis and molecular sorting. Annu Rev Cell Dev Biol 12: 573–625.
20. Futter CE, Pearse A, Hewlett LJ, Hopkins CR (1996) Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. J Cell Biol 132: 1011–1023.
21. Bright NA, Reaves BJ, Mullock BM, Luzio JP (1997) Dene core lysosomes can fuse with late endosomes and are re-formed from the resultant hybrid organelles. J Cell Sci 110 (Pt 17): 2027–2040.
22. Wickner W (2002) Yeast vacuoles and membrane fusion pathways. Embio J 21: 1241–1247.
23. Luzio JP, Pryor PR, Gray SR, Gratian MJ, Piper RC, et al. (2005) Mammalian traffic to and from lysosomes. Biochim Soc Symp. pp 77–86.
24. Prati RA, Wickner W (2007) Distinct targeting and fusion functions of the PX and SNARE domains of yeast vacuolar Vam7p. J Biol Chem 282: 1133–1138.
25. Maleutterna EG, Horvathova BF, Ereghin NL, Galahazhann K, Emr SD (1998) The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the VPS10 gene. Cell 77: 579–586.
26. Jones EW (1977) Proteinase mutants of Saccharomyces cerevisiae. Genetics 85: 32–33.
27. Bankaitis VA, Johnson LM, Emr SD (1986) Isolation of yeast mutants defective in membrane protein sorting genes in Saccharomyces cerevisiae. Mol Biol Cell 13: 4286–2501.
28. Takahashi MK, Frost C, Oyadomari K, Pinho M, Sao D, et al. (2008) A novel immunodetection screen for vacuolar defects identifies a unique allele of VPS35 protein kinase. J Biol Chem 283: 13211–13221.
29. Vida TA, Emr SD (1995) A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J Cell Biol 129: 779–792.
30. Steurgill TW, Cohen A, Dieringer M, Martin DD, et al. (2008) TOR1 and TOR2 have distinct locations in live cells. Eukaryot Cell 7: 1819–1830.
31. Gustavsson M, Barmark G, Larsson J, Maren E, Ronne H (2008) Functional genomics of mossomin sensitivity in yeast: implications for post-Golgi traffic and vacuolar H+-ATPase function. Mol Gen Genomics 279: 233–248.
32. Ha HK, Falvo JV, Gerke LC, Carroll AS, Howson RW, et al. (2003) Global analysis of protein localization in budding yeast. Nature 425: 696–701.
33. Snaider DB, Peryy Gardner M, Copeland NG, Gilbern DJ, et al. (1998) Cloning and characterization of Krt, a member of a novel subfamily of serine/threonine kinases. Hum Mol Genet 7: 2157–2166.
34. Lajoie JM, Gerwin N, Fernandez P, Gutierrez-Ramos JC, Berna A (1998) Cloning, expression analysis, and functional characterization of Pkl12, a member of a new subfamily of ser/thr kinases. Biochem Biophys Res Commun 249: 380–384.
35. Berson AE, Young C, Morrison SL, Fujii GH, Sherg J, et al. (1999) Identification and characterization of a myristoylated and palmitoylated serine/threonine protein kinase. Biochem Biophys Res Commun 259: 533–538.
36. Selska OV, Korkina OV, Stenmanov AV, Kim T, Nelson PS, et al. (2005) Biochemical properties of purified human retinol dehydrogenase 12 (RHD12); catalytic efficiency toward retinoids and C9 aldehydes and effects of cellular retinol-binding protein type I (CRBP1) and cellular retinalexhde-binding protein (CRALBP) on the oxidation and reduction of retinoids. Biochemistry 44: 7057–7064.
37. Lusier M, White AM, Sheraton J, de Paola T, Treadwell J, et al. (1997) Large scale identification of genes involved in cell surface biosynthesis and architecture in Saccharomyces cerevisiae. Genetics 147: 435–450.
38. Chen S, Tarsio M, Kane PM, Greenberg ML (2006) Cardiolipin mediates cross-talk between mitochondria and the vacuole. Mol Biol Cell 17: 5047–5057.
39. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, et al. (2010) Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell 141: 656–667.
40. Wang L, Seeley ES, Wickner W, Merz JR (2002) Vacuole fusion at a ring of vertex docking sites leaves membrane fragments within the organelle. Cell 108: 357–369.
41. Costanzo M, Baryshnikova A, Bellay J, Kim T, Spear ED, et al. (2010) The yeast protein Atlas: a integrated encyclopedia of the proteome. Cell 141: 228–239.
73. Sullivan JA, Lewis MJ, Nikko E, Pelham HR (2007) Multiple interactions drive adaptor-mediated recruitment of the ubiquitin ligase Rsp5 to membrane proteins in vivo and in vitro. Mol Biol Cell 18: 2429–2440.

74. Jensen LT, Carroll MC, Hall MD, Harvey CJ, Beese SE, et al. (2009) Down-regulation of a manganese transporter in the face of metal toxicity. Mol Biol Cell 20: 2810–2819.

75. Soulard A, Cohen A, Hall MN (2009) TOR signaling in invertebrates. Curr Opin Cell Biol 21: 825–836.

76. Stanfel MN, Shamich LS, Kaeberlein M, Kennedy BK (2009) The TOR pathway comes of age. Biochim Biophys Acta 1790: 1067–1074.

77. Kapahi P, Chen D, Rogers AN, Katewa SD, Li PW, et al. (2010) With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell Metab 11: 453–465.

78. Neufeld TP, Arsham AM (2010) tRNA trafficking along the TOR pathway. Cell Cycle 9: 3146–3147.

79. Hall MN, Tamanoi F (2010) The Enzymes: Structure, Function and Regulation of TOR Complexes from Yeasts to Mammals. Maryland Heights, MO: Academic Press.

80. Sancak Y, Peterson TR, Shaull YD, Lindquist RA, Thoreren CC, et al. (2008) The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320: 1496–1501.

81. Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, et al. (2010) Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141: 290–303.

82. Flinn RJ, Backer JM (2010) mTORC1 signals from late endosomes: taking a TOR of the endocytic system. Cell Cycle 9: 1869–1870.

83. Sherman F (2002) Getting started with yeast. Methods Enzymol 350: 3–41.

84. Wach A, Brachat A, Pohlmann R, Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast 10: 1793–1808.

85. Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122: 19–27.

86. Johnson LM, Bankaitis VA, Emr SD (1987) Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. Cell 48: 875–885.

87. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350–4354.