Ypt4 and lvs1 regulate vacuolar size and function in Schizosaccharomyces pombe

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ABSTRACT

The yeast vacuole plays key roles in cellular stress responses. Here, we show that deletion of lvs1, the fission yeast homolog of the Chediak-Higashi Syndrome CHS1/LYST gene, increases vacuolar size, similar to deletion of the Rab4 homolog ypt4. Overexpression of lvs1-YFP rescued vacuolar size in ypt4Δ cells, but ypt4-YFP did not rescue lvs1Δ, suggesting that lvs1 may act downstream of ypt4. Vacuoles were capable of hypotonic shock-induced fusion and recovery in both ypt4Δ and lvs1Δ cells, although recovery may be slightly delayed in ypt4Δ. Endocytic and secretory trafficking were not affected, but ypt4Δ and lvs1Δ strains were sensitive to neutral pH and CaCl₂, consistent with vacuolar dysfunction. In addition to changes in vacuolar size, deletion of ypt4 also dramatically increased cell size, similar to tor1 mutants. These results implicate ypt4 and lvs1 in maintenance of vacuolar size and suggest that ypt4 may link vacuolar homeostasis to cell cycle progression.

Introduction

Vacuoles are important players in numerous cellular processes in yeast. They regulate storage of water, ions, and nutrients; recycle organelles and macromolecules in response to damage or nutrient deprivation; and play a key role in the osmotic shock and other stress responses through regulated fusion and fission events.1,2 Despite these common functions, the fission yeast vacuole differs substantially in morphology compared with the budding yeast vacuole. Schizosaccharomyces pombe cells are characterized by numerous small vacuoles, much like human lysosomes, rather than one or few large, central vacuoles observed in Saccharomyces cerevisiae.3 Although the molecular machinery that drives vacuolar-protein sorting is largely conserved between the two types of yeast,4 the identities of the molecular players that mediate differences in vacuolar structure between the two organisms are currently unknown. Elucidation of these poorly explored mechanisms in fission yeast could shed new light on evolutionarily conserved lysosomal biogenesis pathways in mammalian systems.

A large body of evidence has identified a set of conserved genes that regulate the biogenesis of lysosomes and lysosome-related organelles (LROs) in mammalian systems.4 Many of these genes have been shown to be associated with clinical disorders characterized by hypopigmentation and platelet storage pool deficiency in mice and humans,5 although the precise cellular functions of their protein products are unclear in some cases. One of the least characterized of these pigment dilution genes is CHS1/LYST, the causative gene in Chediak-Higashi Syndrome (CHS) type 1.6 CHS patients are characterized by hypopigmentation, immune and coagulation defects, and neurologic issues at the phenotypic level and enlarged, lysosomes and lysosome-related organelles at the cellular level.6,7 The cellular function of Lyst is unclear; Lyst and its homologs, all of which are characterized by the presence of a conserved BEACH domain,8 have been proposed to serve in multiple functions related to lysosomal biogenesis and homeostasis. They have been suggested to act as positive regulators of lysosomal fission,9 negative regulators of lysosomal fusion,10 as well as positive regulators of post-lysosome and LRO biogenesis and fusion with the cell surface.11-13 Importantly, the end result in each of these scenarios is the hallmark enlargement of lysosomes, post-lysosomes, and LROs observed in CHS. In contrast to LYST homologs in other organisms, deletion of the budding yeast LYST homolog, BPH1, does not result in enlarged vacuoles and only causes a mild defect in sorting of vacuolar proteins, suggesting that the BEACH domain may serve a different function in budding yeast.14 The role of the fission yeast homolog of LYST, lvs1, in control of vacuolar size and function has not previously been characterized. However, lvs1p is 22.16% identical to human LYST, while Bph1p is 14.86% identical to human LYST and 29.46% identical to lvs1p, suggesting that fission yeast lvs1p could share more conservation of function with the human protein.15

In fission yeast, antagonistic control of vacuolar size by the sty1 mitogen-activated protein kinase (MAPK) pathway and protein phosphatase 2C plays an important role in the response to environmental stresses, such as osmotic shock or starvation.16,17 The sty1 homolog Hog1p has also been implicated in the osmotic response in budding yeast.18 Similarly, homologs of Rab7 (ypt7/ypt71/YPT7) have been implicated in regulation...
of vacuole and vacuole-endosome fusion in both types of yeast, suggesting some level of mechanistic conservation between the two species. However, considering the differences in vacuolar morphology between fission and budding yeast, species-specific mechanisms that contribute to vacuolar homeostasis must also exist. One potential player in a fission yeast-specific mechanism that regulates vacuolar size is the Rab ypt4p. Deletion of ypt4 has been shown to result in increased vacuolar size, but ypt4 has no clear ortholog in S. cerevisiae according to Pombase (www.pombase.org).

The goal of this exploratory study was to characterize the unique mechanisms that contribute to vacuolar homeostasis in fission yeast. We compared the phenotypes of cells lacking ypt4 and lvs1p, the fission yeast homolog of LYST. By assessing cell and vacuolar morphology, membrane trafficking, and vacuolar function in these cells, we show that lvs1p likely acts downstream of ypt4p in regulation of vacuolar size and also link ypt4p to control of cell size. Our results establish fission yeast as a novel model for CHS. Fission yeast lacking lvs1 has enlarged vacuoles, consistent with the enlarged lysosomes and lysosomal organelles that are the hallmark of this disorder.

**Results**

To characterize the molecular pathways that control vacuolar homeostasis in fission yeast, we explored the phenotypes of strains with deletion of lvs1, the fission yeast homolog of human LYST, or ypt4, a gene previously shown to be associated with changes in vacuole size in fission yeast. To assess vacuolar morphology, wildtype (WT), lvs1Δ, and ypt4Δ cells were stained with the yeast vacuole membrane stain MDY-64. Both ypt4Δ and lvs1Δ cells had larger vacuoles than WT cells, with ypt4Δ cells appearing to have the largest vacuoles of the 3 strains (Fig. 1A). To quantify these results, we measured the pixel area of the largest vacuole in each cell for all 3 strains and calculated the distribution of cells with vacuoles falling within one of four size-based bins. Quantification confirmed that the ypt4Δ cells had the largest vacuoles, although both lvs1Δ and ypt4Δ cells had vacuoles that were significantly larger than that of the WT cells (Fig. 1B).

In addition to enlarged vacuoles, initial morphological observations of ypt4Δ cells suggested that they were larger than WT or lvs1Δ cells. A similar phenotype was observed in cells lacking TORC2 activity, suggesting the potential for a link between ypt4 and control of cell division. To quantitatively assess the phenotype, we measured the lengths and widths of WT, ypt4Δ, and lvs1Δ cells captured using a differential interference contrast (DIC) filter. Results from this analysis revealed that ypt4Δ cells (Fig. 2A, C), but not lvs1Δ cells (Fig. 2B, D) exhibited a significantly greater length, width, and length/width ratio than WT cells.

To better understand the relationship between ypt4p and lvs1p, we next assessed vacuolar size in WT, ypt4Δ, and lvs1Δ cells overexpressing ypt4-YFP or lvs1-YFP. To visualize the vacuoles, cells were stained with the lipophilic dye FM4-64, which transits the endocytic pathway to reach the vacuole. Quantification of vacuolar size using Image J revealed that, similar to results with MDY-64 (Fig. 1), both ypt4Δ and lvs1Δ cells had significantly larger vacuoles than the WT strain, with ypt4Δ vacuoles being the largest (Fig. 3A). Initial rescue experiments using ypt4p tagged at the N-terminus (YFP-ypt4p) generated a non-functional protein that did not rescue the ypt4Δ deletion mutant. In contrast, overexpression of C-terminally-tagged ypt4-YFP rescued the vacuolar phenotype in ypt4Δ cells. However, overexpression of ypt4-YFP did not reduce vacuolar size in WT or lvs1Δ cells, actually resulting in a slight enlargement in vacuolar size. Overexpression of lvs1-YFP rescued vacuolar size to WT levels in ypt4Δ cells and reduced vacuole size to smaller than WT in both WT and lvs1Δ strains. Because overexpression of lvs1p rescued loss of ypt4, but the converse was not true, these data suggest that lvs1p may act downstream of ypt4p to drive vacuolar fission (Fig. 3A). However, it is important to note that the C-terminally tagged ypt4-YFP did not properly localize to membranes, instead localizing throughout the cytoplasm, consistent with inhibition of geranylgeranylation on the 2 adjacent C-terminal cysteines of ypt4, complicating the interpretation of these results.

To further explore the functional roles of ypt4 and lvs1, we characterized the response of ypt4Δ and lvs1Δ cells to hypertonic shock by exposing FM4-64-stained cells to dH2O for 90 minutes (Fig. 3B). Consistent with previous studies, vacuoles in ypt4Δ cells underwent fusion to form larger vacuoles (compare Fig. 3A, C). Similarly, enlarged vacuoles in the lvs1Δ strain were also capable of fusing to create larger vacuoles (compare Fig. 3A, C). However, although WT and lvs1Δ cells subjected to 90 minutes of recovery in normal media returned to normal vacuole size, ypt4Δ cells still possessed enlarged vacuoles after 90 min, suggesting that passive fission may be partially impaired in these cells (compare Fig. 3A, C; 17).

Ypt4p shares 47% identity with human Rab4, which has previously been implicated in endosomal trafficking. Furthermore, membrane trafficking pathways have been implicated in control of vacuolar size in yeast. Therefore, we next

![Figure 1](Image) Deletion of ypt4 or lvs1 results in an increase in vacuole size. (A) Wildtype (WT), ypt4Δ, and lvs1Δ cells were stained with MDY-64, and vacuoles were visualized by fluorescence microscopy. (B) Quantification of A. The pixel area of the largest vacuole in each cell was measured using ImageJ software. The percentage of cells containing vacuoles of the indicated pixel areas were calculated for each strain. Data are presented as the mean ± SEM. *p < 0.001 vs. WT by Student's t-test.
analyzed general endocytic and secretory trafficking pathways in cells lacking \textit{ypt4} or \textit{lvs1}. Endocytosis was quantitatively analyzed by monitoring FM4–64 uptake using flow cytomtery. Inhibition of endocytosis using NaN3 and NaF has previously been shown to inhibit FM4–64 uptake.\textsuperscript{23} Over 30 minutes, we observed no differences in FM4–64 uptake between WT, \textit{ypt4Δ}, and \textit{lvs1Δ} cells (Fig. 4A). Secretion of the enzyme cargo acid phosphatase was also similar between the three strains (Fig. 4B). Brefeldin A (BFA) is a fungal metabolite that has been shown to inhibit protein secretion in many organisms, including fission yeast.\textsuperscript{29} Consistent with the absence of defects in secretion of acid phosphatase, neither \textit{ypt4Δ} or \textit{lvs1Δ} cells exhibited sensitivity to BFA (Fig. 4C). These results suggest that changes in vacuolar size in \textit{ypt4Δ} and \textit{lvs1Δ} cells are not due to general alterations in membrane trafficking pathways.

In CHS, defective biogenesis of lysosomes and lysosome-related organelles has been shown to result in impaired function,\textsuperscript{2} and fission yeast strains with impaired vacuolar biogenesis or acidification have been shown to be sensitive to CaCl\textsubscript{2} (\textit{vps33Δ}, \textit{vma1Δ}, \textit{vma3Δ}) and/or neutral pH (\textit{vma1Δ}, \textit{vma3Δ}).\textsuperscript{30,31} Therefore, to test whether vacuolar function was impaired in the \textit{ypt4Δ} and \textit{lvs1Δ} strains, we assessed growth on neutral (pH 7) media and media containing 50 mM CaCl\textsubscript{2}. Both the \textit{ypt4Δ} and \textit{lvs1Δ} strains were sensitive to neutral pH and CaCl\textsubscript{2} (Fig. 4D). These results suggest that vacuolar function is impaired by loss of \textit{ypt4} or \textit{lvs1}.

**Discussion**

Our results demonstrate that both \textit{ypt4} and \textit{lvs1} play key roles in maintaining vacuolar function and homeostasis in fission yeast. These findings also establish fission yeast as a model for Chediak-Higashi Syndrome by showing that fission yeast \textit{lvs1} functions similarly to the mammalian \textit{LYST},\textsuperscript{32} with \textit{lvs1} deletion resulting in enlarged vacuoles. In contrast to these observations in fission yeast, deletion of the budding yeast \textit{LYST} homolog \textit{bph1} did not affect vacuolar morphology, although vacuolar protein sorting was mildly impaired.\textsuperscript{14} These data establish key differences between budding yeast and fission yeast with respect to vacuolar biogenesis pathways. These distinct pathways may contribute to the differences in vacuole morphology between the two organisms.

Several mechanisms have been suggested to contribute to the development of enlarged lysosomes and LROs in CHS. \textit{LYST} and its homologs in other organisms have been proposed as a potential pathway.\textsuperscript{1} It will be interesting to explore a potential role in post-vacuolar sorting or fusion of these organelles (and other LROs) with the cell surface.\textsuperscript{11-13} Although our results do not differentiate between these possibilities, we do show that overexpression of \textit{lvs1}–YFP resulted in the presence of smaller vacuoles, suggesting that a role in vacuolar fusion is likely. Based on observations in higher eukaryotes mentioned in the third mechanism above,\textsuperscript{11-13} it will be interesting to explore a potential role in post-vacuolar sorting in fission yeast in future studies. To our knowledge, no evidence of post-vacuolar traffic has been shown in yeast, although it has been proposed as a potential pathway.\textsuperscript{1}

Although our results do not define the downstream mechanisms regulated by \textit{lvs1}, they do suggest that \textit{ypt4} may act upstream of \textit{lvs1} in control of lysosomal homeostasis. In mammalian cells, Rab4 isoforms have primarily been implicated in endosomal traffic.\textsuperscript{33,34} However, a recent report shows that inhibition of endosomal sorting by expression of a dominant-negative Rab4 allele affects lysosomal biogenesis in \textit{Drosophila}.\textsuperscript{35} Furthermore, proper endosomal biogenesis has been implicated in development of secretory lysosomes in CHS.\textsuperscript{13} Thus, the role of \textit{ypt4} in maintenance of vacuolar homeostasis...
in fission yeast may be conserved in higher eukaryotes. However, the interpretation of these results is complicated by the lack of membrane targeting of overexpressed ypt4-YFP, potentially implicating ypt4-YFP in a signaling role that contributes to vacuolar homeostasis, rather than a trafficking role.

The precise mechanistic connection between ypt4p and lvs1p remains unclear. A previous study has shown that activation of the sty1 MAPK pathway can drive vacuolar fission in ypt4Δ cells. This raises the question of whether lvs1p may be a direct or indirect target of the sty1 MAPK pathway. Bioinformatic
analysis suggests that lvs1p possesses three potential sty1 phosphorylation sites (S147, S1157, and T1210). Furthermore, in Arabidopsis, Lyst-interacting protein 5 (Lip5) is phosphorylated by the MAP kinases MPK3/6 to regulate multivesicular body biogenesis in the plant's defense against pathogens. Thus, lvs1p and/or its regulators may be downstream target(s) of the stress-responsive sty1 MAPK pathway. Interestingly, in mammalian cells, the p38 pathway (analogous to sty1) is also activated in response to stress and has been implicated in regulation of lysosomal biogenesis, as well as some types of autophagy. These observations suggest the potential for a conserved eukaryotic pathway that regulates lysosomal/vacuolar homeostasis in response to environmental stress and suggest a new avenue of exploration to better understand the regulation and function of mammalian LYST proteins.

Another interesting phenotype noted in this report is the increase in length and width of ypt4Δ cells. This fairly uncommon phenotype has also been observed in the tor1-L2045D mutant in response to low glucose, and in yeast overproducing the ppe1p or ppa2p phosphatases. These results suggest that ypt4p may play a role in a signaling pathway that coordinates cell growth and cell division in response to nutrient availability. Consistent with this idea, the highly enlarged vacuoles observed in ypt4Δ cells are reminiscent of those observed in fission yeast cells subjected to glucose deprivation, and glucose deprivation also regulates TORC2 activity. Furthermore, TOR1 has been shown to regulate a vacuole inheritance cell cycle checkpoint in S. cerevisiae, and the vacuole has been suggested to scale with cell size in budding yeast. These observations suggest that ypt4p may connect a vacuole-dependent, nutrient sensing and scaling mechanism to control of the cell cycle. Thus, the increased size of ypt4Δ mutant cells may result from uncoupling of cell division from control of cell size and nutrient availability.

This potential role for ypt4 and its homologs may be conserved in higher eukaryotes. The human Rab4 protein has been shown to undergo reversible phosphorylation at a consensus site for Cdk1/p34cdc2 kinase to regulate its membrane association during entry into mitosis. Importantly, Cdk1/p34cdc2 has also been shown to phosphorylate Raptor, a key regulator of mTOR activity, suggesting that Rab4 and mTOR may be coordinately regulated at the G2/M transition in mammalian cells. Thus, Rab4 may also play an important role in mTOR-dependent cell cycle transitions in mammalian cells, although this hypothesis requires further investigation.

Together, these observations suggest interesting new directions to explore in both yeast and mammalian systems. Are LYST/lvs1 MAPK substrates that are activated in response to cell stress? Are ypt4p/Rab4A/Rab4B master regulators that integrate lysosomal/vacuolar function and nutrient sensing with cell cycle progression? These are interesting questions that could lead to novel directions to better understand Chediak-Higashi syndrome.

Figure 4. Deletion of ypt4 or lvs1 affects vacuolar function. (A) WT, lvs1Δ, and ypt4Δ cells were incubated with 32 μM FM4-64 for the indicated times at 30 °C, followed by extensive washing. FM4-64 uptake was measured by flow cytometry. Mean fluorescence was normalized to the 0 min timepoint for each strain. (B) Equal numbers of WT, lvs1Δ, and ypt4Δ cells were used to inoculate YES media. At the indicated times, an aliquot of the media was subjected to spectrophotometric determination of acid phosphatase activity by incubation with 2 mM p-nitrophenyl phosphate for 5 min, followed by measurement of the optical density of the sample at 405 nm. OD405 values were normalized to the 0 h timepoint for each strain. Results are reported as mean ± SEM for (A) and (B). C, D. Cells were subjected to a 10-fold serial dilution and were spotted on YES or YES + 10 μg/mL BFA (C) or YES + 10 mM Tris-HCl, pH 7; or YES + 50 mM CaCl2 (D). Plates were incubated for 3–4 d at 30 °C.
Syndrome, as well as the general role of the lysosome and vacuole as a stress-responsive organelle in eukaryotes.

Materials and methods

Strains, yeast culture, and DNA manipulations

Strains used in this study are listed in Table 1. All strains were purchased from Bioneer (Alameda, CA). Cells were cultured at 30°C in Yeast Extract plus Supplements (YES; Sunrise Science Products; San Diego, CA) or Edinburgh Minimal Media (EMM; Sunrise Science Products) containing appropriate nutritional supplements. Deletion mutants were selected by growth on YES media containing 200 μg/mL G418.

pDUAL-YFH1c vectors carrying ypt4-YFP (SPAC1B3.11c) and lvs1-YFP (SPBC28E12.06c) under control of the full-strength mnt1+ promoter were purchased from the Riken Biosource Center DNA Bank (Ibaraki, Japan, deposited by M. Yoshida49-51). Plasmids were transformed into the WT, ypt4Δ, and lvs1Δ strains by the lithium acetate method, as described previously.52 Transformants were selected on EMM containing appropriate nutrients and G418.

MDY-64 staining and fluorescence microscopy

Wildtype (WT), lvs1Δ, and ypt4Δ cultures were resuspended in HEPES/glucose buffer (10 mM HEPES, pH 7.4; 5% glucose) containing 10 μM MDY-64 (ThermoFisher Scientific; Wal-tham, MA). After incubating 3 minutes at room temperature, the cells were washed in HEPES glucose buffer. Vacuoles were imaged using a Zeiss Axioskop 2 fluorescence microscope (Zeiss; Oberkochen, Germany). Experiments were repeated a minimum of 3 times without independent cultures, and 10–15 images were captured per sample. Image J was used to quantify vacuole size as described previously.27 Results are reported as the mean ± standard error of the mean (SEM).

Quantification of cell size

To quantify cell size, images of the indicated cells were captured on the Zeiss Axioskop 2 described above using a differential interference contrast (DIC) filter. Cell length and width were measured in pixels using Image J. Results are reported as the mean ± standard deviation (SD) of 3 independent experiments.

FM4-64 staining, vacuolar fusion, and endocytosis assays

FM4-64 staining was performed as described previously.17,27 Briefly, cultures (WT, ypt4Δ, lvs1Δ, and ypt4-YFP and lvs1-YFP overexpression, as indicated) were resuspended in fresh YES media containing 32 μM FM4-64 (ThermoFisher Scientific) and incubated at 30°C for 20 minutes. After washing, the cells were resuspended in fresh YES media and incubated at 30°C for 30 min to facilitate delivery of FM4-64 to the vacuole. After washing, cells were imaged as described above.

For vacuolar fusion and recovery assays, WT, ypt4Δ, and lvs1Δ cells were stained with FM4-64, as described above. To induce fusion, instead of resuspending cells in fresh YES media, cells were resuspended in dH2O and incubated for 90 min,27 followed by washing. Cells that underwent recovery were incubated for an additional 90 min at 30°C before imaging and vacuolar quantification as described above.

For quantification of FM4-64 endocytosis, WT, ypt4Δ, and lvs1Δ cultures were resuspended in fresh YES media containing 32 μM FM4-64 (ThermoFisher Scientific; Wal-tham, MA) and incubated at 30°C for 0, 15, or 30 minutes. Cells were then washed 3 times with ice-cold YES media. FM4-64 fluorescence per cell was measured using a BD Accuri C6 Flow Cytometer (BD Biosciences; San Jose, CA). Mean fluorescence intensities were calculated for samples of at least 10,000 cells. Results represent the mean ± SEM from 3 independent experiments.

Acid phosphatase secretion

Secretion of acid phosphatase activity was assayed as described previously.27,28 Equal numbers of WT, ypt4Δ, and lvs1Δ cells were resuspended in fresh YES media and incubated at 30°C for the indicated times. At each timepoint, a media sample was collected for acid phosphatase secretion analysis and immediately subjected to centrifugation at 25000 × g for 1 min. Supernatants were stored at 4°C until all samples were collected. Acid phosphatase activity was assayed by incubating each sample with an equal volume of substrate solution (2 mM p-nitrophenyl phosphate, 0.1 M sodium acetate, pH 4.0) for 5 min at 30°C. The reaction was stopped by the addition of 0.33 M NaOH. Phosphatase activity was quantified by measuring the absorbance of each reaction at 405 nm (OD405). Results represent the mean ± SEM from 3 independent experiments.

Spot assays

Equal numbers of WT, lvs1Δ, and ypt4Δ cells were subjected to a 10-fold serial dilution, and 5 μL of the resulting dilutions were spotted on the following plates: YES; YES + 10 μg/mL Brefeldin A (BFA); YES + 10 mM Tris-HCl, pH 7; and YES + 50 mM CaCl2. The plates were incubated at 30°C for 3–4 d before imaging.

Statistical analyses

Statistical analyses were performed on a minimum of 3 independent experiments, comprising a minimum of 60 cells per strain. Significant differences between paired samples were analyzed using Student’s t-test, and p-values less than 0.05 were deemed statistically significant. For multiple comparisons, or one-way analysis of variance (ANOVA) was used, followed by post-hoc Student’s t-test using Bonferroni’s correction.

Table 1. *S. pombe* strains used in this study.

| Strain    | Genotype                          | Source          |
|-----------|-----------------------------------|-----------------|
| Wildtype  | ade6-M210 ura4-D18 leu1–32 h+     | Bioneer         |
| ypt4Δ     | ypt4Δ::kanMX4 ade6-M210 ura4-D18 leu1–32 h+ | Bioneer         |
| lvs1Δ     | lvs1Δ::kanMX4 ade6-M210 ura4-D18 leu1–32 h+ | Bioneer         |
Bioinformatic analyses

Identification of putative sty1p phosphorylation sites in Yvs1p was performed using NetPhosYeast 1.036 and NetPhos 3.1.37 Putative sty1p phosphorylation sites were defined as residues predicted to be phosphorylated in yeast using NetPhosYeast and predicted to be p38 consensus sites using NetPhos 3.1.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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