

Membrane contact sites regulate vacuolar fission via sphingolipid metabolism

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Abstract

Membrane contact sites (MCSs) are junctures that perform important roles including coordinating lipid metabolism. Previous studies have indicated that vacuolar fission/fusion processes are coupled with modifications in the membrane lipid composition. However, it has been still unclear whether MCS-mediated lipid metabolism controls the vacuolar morphology. Here we report that deletion of tricalbins (Tcb1, Tcb2, Tcb3), tethering proteins at endoplasmic reticulum (ER)-plasma membrane (PM) and ER-Golgi contact sites, alters fusion/fission dynamics and causes vacuolar fragmentation in the yeast *Saccharomyces cerevisiae*. In addition, we show that the sphingolipid precursor phytosphingosine accumulates in tricalbin-deleted cells, triggering the vacuolar division. Detachment of the nucleus vacuole junction (NVJ), an important contact site between the vacuole and the perinuclear ER, restored vacuolar morphology in both cells subjected to high exogenous phytosphingosine and Tcb3-deleted cells, supporting that phytosphingosine transport across the NVJ induces vacuole division. Thus, our results suggest that vacuolar morphology is maintained by MCSs through the metabolism of sphingolipids.

eLife assessment

This manuscript presents **valuable** findings about how sphingolipids and membrane contact sites are involved in promoting vacuole fission in *S. cerevisiae*. While a connection between the levels of sphingolipids and vacuole homeostasis is interesting, the data pertaining to this issue are **incomplete** and do not fully support the claim for causality between altered lipid composition and organelle dynamics. The work will be of interest to cell biologists working on organelle biogenesis and lipid metabolism.

Cellular organelles are formed by membranes with unique lipid compositions, morphology, and functions. The vacuole of budding yeast is an organelle that shares many similarities with mammalian lysosomes and plant vacuoles. Vacuoles possess degradative and storage capacities, and are essential for maintaining cellular homeostasis, including maintaining pH or ion homeostasis, cellular detoxification, and responses to osmotic shock and nutrient environments. Vacuoles are liable to change their morphology (size, volume, and number) through cycles of fusion and fission, to adapt to the intracellular and extracellular environments or upon vacuole inheritance to daughter cells. The steady-state morphology of the vacuole is maintained by a balance of constitutive vacuolar fusion and fission processes [1] [2].

The homotypic vacuolar membrane fusion process occurs in four stages: priming, tethering, docking and fusion [3] [4] [5] [6]. Each stage is defined as follows. “Priming” In the priming reaction, inactive fusion factors that are still assembled from previous fusion events are recycled. The yeast N-ethylmaleimide-sensitive factor (NSF), Sec18p, is activated by ATP and releases the NSF attachment protein (SNAP), Sec17p, from the protease complex, thereby activating the SNAP receptor (SNARE) molecule [3] [4] [7]. “Tethering” The HOPS/Class C Vps complex and the Rab- GTPase Ypt7p mediate the reversible contact of opposing vacuoles [4] [8]. “Docking” The coupling of three Q-SNAREs (Vam3p, Vam7p, and Vti1p) with an opposing R-SNARE (Nyv1p) to form a trans-SNARE complex between the two corresponding vacuoles. “Fusion” The final stage in which the lipid bilayer fuses and the contents of the lumen are mixed [5] [7]. For stable vacuolar fusion, several factors are required such as vacuolar acidification by V-ATPase proton pump activity [1] [9] [10] [11], ions [12] and ion transporters [13].

Furthermore, vacuolar fission is required for proper vacuolar inheritance during mitosis and acute response to osmotic shock in yeast, however the molecular mechanism of fission is not fully understood, and limited knowledge is available. Vacuolar fission is performed in two steps that require proteins and lipids [14]. The first step is the contraction and invagination of the vacuolar membrane through the involvement of Vps1p, a dynamin-like GTPase [15] and V-ATPase, which drives the proton gradient [1]. Next, vacuolar fission is promoted by Vac14p, Vac7p, and Fab1p [16], which are required for the generation of PtdIns[3,5]P₂, and Atg18p, an effector of Fab1p and a sensor of PtdIns[3,5]P₂ levels [17] [18]. Recently, it has also been shown that vacuolar fission is associated with nutrient status and responds to ER stress via TORC1 targets [19] [20]. Moreover, it has been proposed that vacuolar fusion and fission dynamics are regulated by the Yck3p-Env7p kinase cascade, which maintains an equilibrium between fusion and fission activities [21].

Organelles construct a dynamic network by associating with each other through membrane-contact sites (MCSs), which play an important role in lipid transport and metabolism [22] [23]. Lipids (phosphatidic acid (PA), diacylglycerol, ergosterol, phosphatidylinositol (PtdIns) phosphate (PtdInsP)) and lipid raft domains have been shown to be important as fusion and fission effectors and discussed in a variety of recent reviews [2] [17] [24] [25] [26] [27]. However, it remains unclear if MCS-mediated lipid metabolism controls the morphology of the vacuole. Vacuoles have contact sites with other organelles. The MCS where the nuclear ER meets the vacuole is called the nucleus-vacuole junction (NVJ), and the ER-localized Nvj1p and vacuole-membrane-localized Vac8p act as tethers to form the NVJ [28]. Vacuoles also form MCS with mitochondria called vacuole and mitochondria patches (vCLAMP). vCLAMP is mediated by the soluble (cytosol- localized) HOPS complex subunit Vps39p, the vacuole-localized small Rab-GTPase Ypt7p, and by an unknown mitochondrial factor [29] [30]. The translocase of the mitochondrial outer membrane (TOM) subunit Tom40p has been identified as a direct binding partner of Vps39p, suggesting a mechanism for the formation of vCLAMP by the Ypt7p-Vps39p-Tom40p tether [31]. Although it has been suggested that MCSs with vacuoles are involved in the

transport of lipids such as sterols and precursors of sphingolipids that are involved in vacuolar morphology [32] [33] [34] [35], the roles of those MCSs-mediated lipid metabolisms in the regulation of vacuole morphology are not understood.

In this study, we found that tether proteins of MCS formation are involved in the regulation of vacuolar morphology. We show that the deletion of tricalbins, tethers of the MCS between the ER and the plasma membrane (PM) [36] and between the ER and the Golgi apparatus [37], affects vacuolar division. Phytosphingosine, a precursor of ceramide, accumulated in the tricalbin-deleted strain and was revealed as the underlying cause that triggers vacuolar fission. Moreover, deletion of key tethering proteins at the NVJ recovered vacuolar morphology of cells subjected to high exogenous phytosphingosine and tricalbin-deleted cells, indicating that the NVJ is required for phytosphingosine-induced vacuolar fission. In summary, we propose that vacuolar morphology is regulated by MCSs through regulation of sphingolipid metabolic pathways.

Results

Deletion of tricalbins causes vacuole fragmentation

Tricalbins (Tcb1p, Tcb2p, and Tcb3p) are ER membrane tethering proteins that connect the cortical ER (cER) with the PM [38], and contribute to PI4P turnover [36], sterol flux and transport [39] and maintain other lipid homeostasis [40]. It is also suggested that tricalbins create ER membrane curvature to maintain PM integrity [41] [42]. Tricalbins also localize to the ER-Golgi contacts and are responsible for the non-vesicular transport of ceramide from ER to Golgi apparatus [37]. However, their role in other organelle morphology and function remains unknown. Interestingly, the *NVJ1* gene, which encodes a major component of the NVJ, showed a negative synthetic interaction with the absence of all three tricalbins (*tcb1Δ2Δ3Δ*) [41]. Moreover, HOPS subunit Vam6p and Rab-GTPase Ypt7p, which are both involved in vacuolar fusion, also showed negative synthetic interaction with *tcb1Δ2Δ3Δ* [41]. Based on the findings, we assumed that tricalbins may be involved in the regulation of vacuolar morphology or function linked with the NVJ. To investigate the role of tricalbins in vacuole morphology, we analyzed the number of vacuoles per cell by a fluorescent probe FM4-64 that selectively stains yeast vacuolar membranes. We observed that compared to wild type cells, the *tcb1Δ2Δ3Δ* strain showed a phenotype characterized by a decreased percentage of cells with one vacuole and an increased percentage of cells with two or more vacuoles (**Fig 1A**). We refer to this phenotype as vacuolar fragmentation. In addition, analysis with single- and double deletion strains revealed that single deletion of *TCB1* or *TCB3* already exhibited strong vacuole fragmentation (**Fig 1B**). These results indicate that tricalbins are important to maintain vacuole morphology.

We next examined whether deletion of tricalbins affects vacuolar acidification. As homotypic vacuole-vacuole membrane fusion requires the vacuolar H⁺-ATPase (V-ATPase) [11] [43] [44], tricalbins could be indirectly involved in maintaining vacuole morphology through V-ATPase. Therefore, we addressed whether tricalbin mutant strains could grow in alkaline media, because V-ATPase-deficient mutants can grow in acidic media but fail to grow in alkaline media [43]. As a negative control, we used *vma3Δ* mutant cells in which one of the V0 subunits of V-ATPase had been deleted. The *vma3Δ* mutant cells grew in acidic medium (pH 5.0), but failed to grow in alkaline medium (pH 7.5) (**Fig S1A**). In contrast, all tricalbin mutant strains grew like wild type in both conditions, suggesting that the V-ATPase in these mutants remains functionally normal. Thus, these results suggest that vacuole fragmentation caused by tricalbin deletion is not due to a loss of function of V-ATPase.

Furthermore, we investigated the effects of tricalbin deletion on delivery of a vacuolar protease, carboxypeptidase S (Cps1p) that is sorted into the vacuole lumen upon endosome–vacuole fusion. Deletion of HOPS complex components that mediate endosome–vacuole fusion results in vacuole

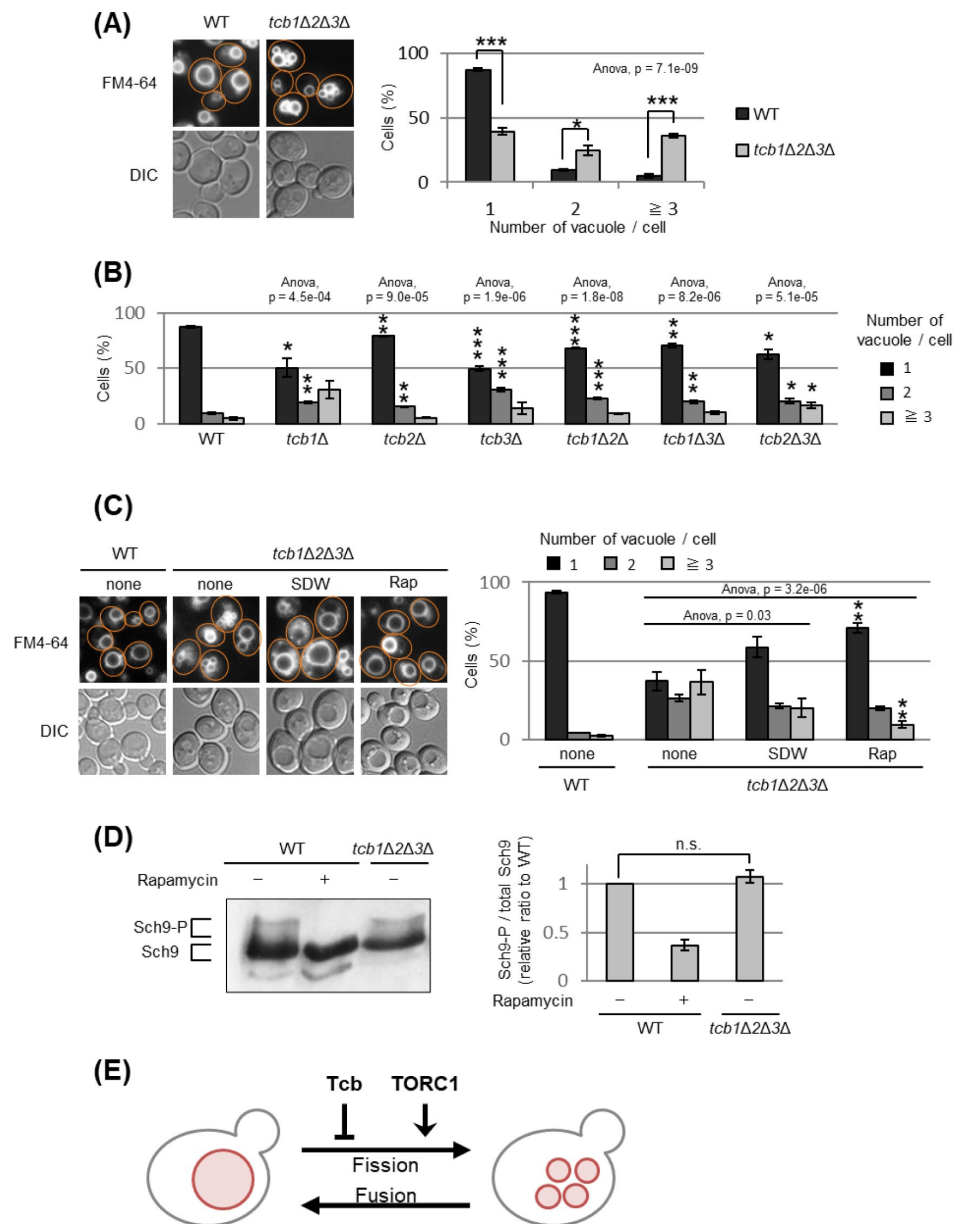


Figure 1.

Deletion of tricalbin proteins causes vacuole fragmentation

(A, B) Cells (FKY2577 and FKY2927 in A; FKY2577, FKY2909, FKY3819, FKY2924, FKY3023, FKY3820 and FKY3008 in B) were grown overnight at 25 °C in YPD. Then vacuoles were stained with FM4-64 and imaged by fluorescence microscopy. The number of vacuoles per cell was counted and categorized into one of three groups. The data represent mean $\pm$ SE of three independent experiments, each based on more than 100 cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test compared with WT.

(C) Cells (FKY2577 and FKY2927) were grown overnight at 25 °C in YPD. Cells were then incubated in sterile distilled water (SDW) for more than 45 minutes or YPD with 200 nM of rapamycin for 2 hours. Vacuoles were stained with FM4-64 and imaged by fluorescence microscopy. The number of vacuoles per cell was counted and categorized into one of three groups. The data represent mean $\pm$ SE of seven (SDW) or three (Rap) independent experiments, each based on more than 100 cells. ** $p < 0.01$ by Student's t-test compared with none treated *tcb1Δ2Δ3Δ* cells.

(A-C) Significant differences analysis between the pairwise combination of groups was performed using two-way ANOVA.

(D) Cells (FKY2577 and FKY2927) transformed with pRS416-SCH9-5HA were cultured in YPD, treated with 200 nM rapamycin (control) or untreated. The extracts from cells expressing Sch9- 5HA were reacted with 2-nitro-5-thiocyanobenzoic acid and analyzed by immunoblotting using anti- HA. Phosphorylated Sch9 relative to the total Sch9 was calculated and shown in comparison to untreated WT cells. The data represent mean $\pm$ SE of three independent experiments. * $p < 0.05$ by Student's t-test.

(E) Illustration shows that tricalbin proteins negatively regulate the vacuole fission in a TORC1- independent manner.

fragmentation [45]. To test the possibility that vacuole fragmentation in tricalbin mutant cells is caused by a defect of endosome–vacuole fusion, we analyzed the localization of GFP-Cps1p in tricalbin mutant cells. In both of WT and *tcb3Δ* cells, GFP-Cps1p was observed in the vacuole lumen (Fig S1B), suggesting that the tricalbin mutant exhibits a normal delivery of vacuolar proteins via endosomes to the vacuole. In addition, the fact that tricalbin deletion does not affect the maturation of CPY, a vacuolar hydrolase carboxypeptidase Y [37], indicates that the vacuolar degradation ability as well as protein delivery to the vacuole is normal. Taken together, tricalbins are required for maintaining vacuole morphology, but not due to an indirect action through vacuolar acidification or endosome-vacuole fusion.

Tricalbins negatively regulate vacuole fission in a parallel pathway with TORC1

The number and size of vacuoles within a cell are regulated by coordinated cycles of vacuolar fission and fusion. To address whether vacuole fragmentation in the tricalbin mutant is caused by facilitation of vacuole fission or an impaired homotypic vacuole fusion, we examined the effect of hypotonic stress on vacuole fragmentation in *tcb1Δ2Δ3Δ* mutant cells, because low- osmotic stimuli such as water promote homotypic vacuole fusion to maintain cytoplasmic osmolarity [46]. We observed that addition of water to *tcb1Δ2Δ3Δ* cells partially restored vacuole fragmentation, suggesting that vacuolar fusion machinery is functional in *tcb1Δ2Δ3Δ* cells (Fig 1C). This result suggests that loss of tricalbins alters fusion-fission dynamics by primarily affecting the fission machinery rather than fusion.

As the target of rapamycin complex 1 (TORC1) is a positive regulator of vacuole fission in response to hyperosmotic shock and ER stress [19] [20], we next examined if rapamycin, which is an inhibitor of TORC1, affects the vacuole fragmentation in *tcb1Δ2Δ3Δ* cells. As shown in Figure 1C, we observed that the vacuole fragmentation in *tcb1Δ2Δ3Δ* cells was suppressed by rapamycin, suggesting that TORC1 may be required for tricalbin deletion-induced vacuolar fragmentation. We attempted to characterize further the relationship between tricalbin and TORC1, and showed that *tcb1Δ2Δ3Δ* cells had no significant effect on phosphorylation levels of Sch9p, a major downstream effector of TORC1 (Fig 1D). These results suggest that deletion of tricalbins does not activate TORC1. Therefore, we conclude that tricalbins and TORC1 act in parallel and opposite ways to regulate vacuole fission (Fig 1E).

The TM domain of Tcb3 contributes to mediating protein interactions between the tricalbin family to maintain vacuolar morphology

Tcb3p possesses an N-terminal TM domain, a central SMP domain, and multiple C-terminal C2 domains (Fig 2A). To identify which domain of Tcb3p is essential for regulating vacuole fission, we replaced the C-terminal cytoplasmatic sequence of Tcb3p with a GFP binding protein (GBP). This way, we created three strains expressing either full-length Tcb3-GBP; Tcb3(Full)-GBP, Tcb3-GBP lacking C2 domains; Tcb3(TM-SMP)-GBP or Tcb3-GBP lacking both SMP and C2 domains; Tcb3(TM)-GBP. Vacuole morphology in cells expressing Tcb3(Full)-GBP (Fig 2B (iii)) was almost similar to that in WT cells (Fig 2B (i)), indicating that the addition of GBP has no effect on vacuole morphology. Remarkably, vacuoles remained non-fragmented even in cells expressing Tcb3(TM)- GBP, which lacked most of the C-terminal region (Fig 2B (v)), as well as Tcb3(TM-SMP)-GBP (Fig 2B (iv)). In an attempt to address the question of why the TM domain of Tcb3p is sufficient to suppress vacuolar division, we found that cells expressing Tcb3(TM)-GBP and lacking Tcb1p and Tcb2p (Fig 2B (vi)) showed vacuolar fragmentation to the same extent as *tcb3Δ* mutant cells (Fig 2B (ii)). These results suggest that the TM domain of Tcb3p requires Tcb1p and Tcb2p to suppress vacuole fission. According to the structural simulation by AlphaFold2, each TM domain of Tcb1p, Tcb2p, and Tcb3p formed alpha-helical structure, which is known to be typical for membrane proteins (Fig 2C). It is likely that Tcb1p and Tcb2p directly interact with Tcb3p by

their TM domains. Interestingly, the complex of these three TM domains was constructed with Tcb3p between Tcb1p and Tcb2p in most cases (**Fig 2D**). Together these data suggest that the TM domain of Tcb3p contributes sufficiently to the maintenance of vacuolar morphology by mediating the tricalbin complex formation (**Fig 2E**).

Accumulated phytosphingosine in tricalbin-deleted cells causes vacuole fragmentation

To understand how the deletion of tricalbins leads to vacuole fragmentation, we examined the involvement of lipids. Previous reports showed that levels of ceramides and acylceramides increased in *tcb1Δ2Δ3Δ* cells [37], and levels of long-chain bases (LCBs) increased in Δ tether cells lacking six ER-PM tethering proteins [47]. Here we measured lipids in *tcb1Δ2Δ3Δ* cells by in vivo labeling with [³H] dihydrosphingosine (DHS), which is a precursor of phytosphingosine (PHS), and observed significant increases in ceramide, phosphatidylethanolamine (PE), PHS, phosphatidylinositol (PI), complex sphingolipids such as inositolphosphorylceramide (IPC) and mannosyl-inositolphosphorylceramide (MIPC) and LCB-1P (DHS-1P/PHS-1P) levels (**Fig 3A**).

We first tested whether increased levels of PHS cause vacuolar fragmentation. Loss of ceramide synthases could cause an increase in PHS levels. Our analysis showed that vacuoles are fragmented in *lag1Δlac1Δ* cells, which lack both enzymes for LCBs (DHS and PHS) conversion into ceramides (**Fig 3B**). This suggests that ceramide precursors, LCBs or LCB-1P, can induce vacuolar fragmentation. Therefore, we tested if exogenously added PHS induces vacuolar fragmentation in WT cells. As shown in **Fig 3C**, exogenous addition of PHS induced vacuolar fragmentation. PHS are converted into ceramide by the ceramide synthase Lag1p, which is the main enzyme synthesizing phytoceramide [48]. Thus, we next examined whether PHS-induced vacuolar fragmentation occurs in *lag1Δ* cells. Our analysis showed that vacuolar fragmentation in *lag1Δ* cells treated with PHS was comparable to that for WT cells (**Fig 3C**). These results confirm that the increases in ceramide and subsequent product IPC/MIPC are not the cause of vacuolar fragmentation, but rather its precursors induce vacuolar fragmentation.

Because our lipid analysis showed a strong increase in LCB-1P in *tcb1Δ2Δ3Δ* cells, phosphorylation of PHS may be involved in PHS-induced vacuolar fragmentation. Although exogenously added LCBs move slowly from the PM to the ER if they are not phosphorylated, efficient utilization of exogenously added LCBs in sphingolipid synthesis requires a series of phosphorylation and dephosphorylation steps for LCBs [49]. The major yeast LCB phosphate phosphatase Lcb3p dephosphorylates LCB-1P (DHS-1P/PHS-1P) to yield DHS/PHS [50], while Lcb4p and Lcb5p catalyze the reverse reaction producing DHS-1P/PHS-1P [51]. Lcb3p, Lcb4p, and Lcb5p are localized to the ER, PM or Golgi [49] [52] [53] [54]. We examined the ability of exogenous PHS to fragment vacuoles in cells lacking these processing factors. Our results showed that PHS-induced vacuolar fragmentation was completely blocked in the *lcb3Δ* cells in which PHS-1P is not dephosphorylated (**Fig 3D**), suggesting that PHS-induced vacuolar fragmentation requires the reaction of dephosphorylation of PHS-1P by Lcb3p. On the other hand, we observed that vacuoles were still fragmented in *lcb4Δ lcb5Δ* and *lcb3Δ lcb4Δ lcb5Δ* cells (**Fig 3D**). These results suggest that elevated levels of non-phosphorylated PHS induces vacuolar fragmentation (**Fig 3E**). Additionally, we tested whether overexpression of Rsb1p, which has been reported as a translocase that exports LCBs from the inner to the outer leaflet of the PM [55], rescues vacuole fragmentation in *tcb1Δ2Δ3Δ* cells. As shown in **Fig 3F**, we observed that Rsb1p overexpression results in decreased vacuole fragmentation. Collectively, these results support the model in which vacuole fragmentation in tricalbin-deleted cells is caused by increased levels of PHS.

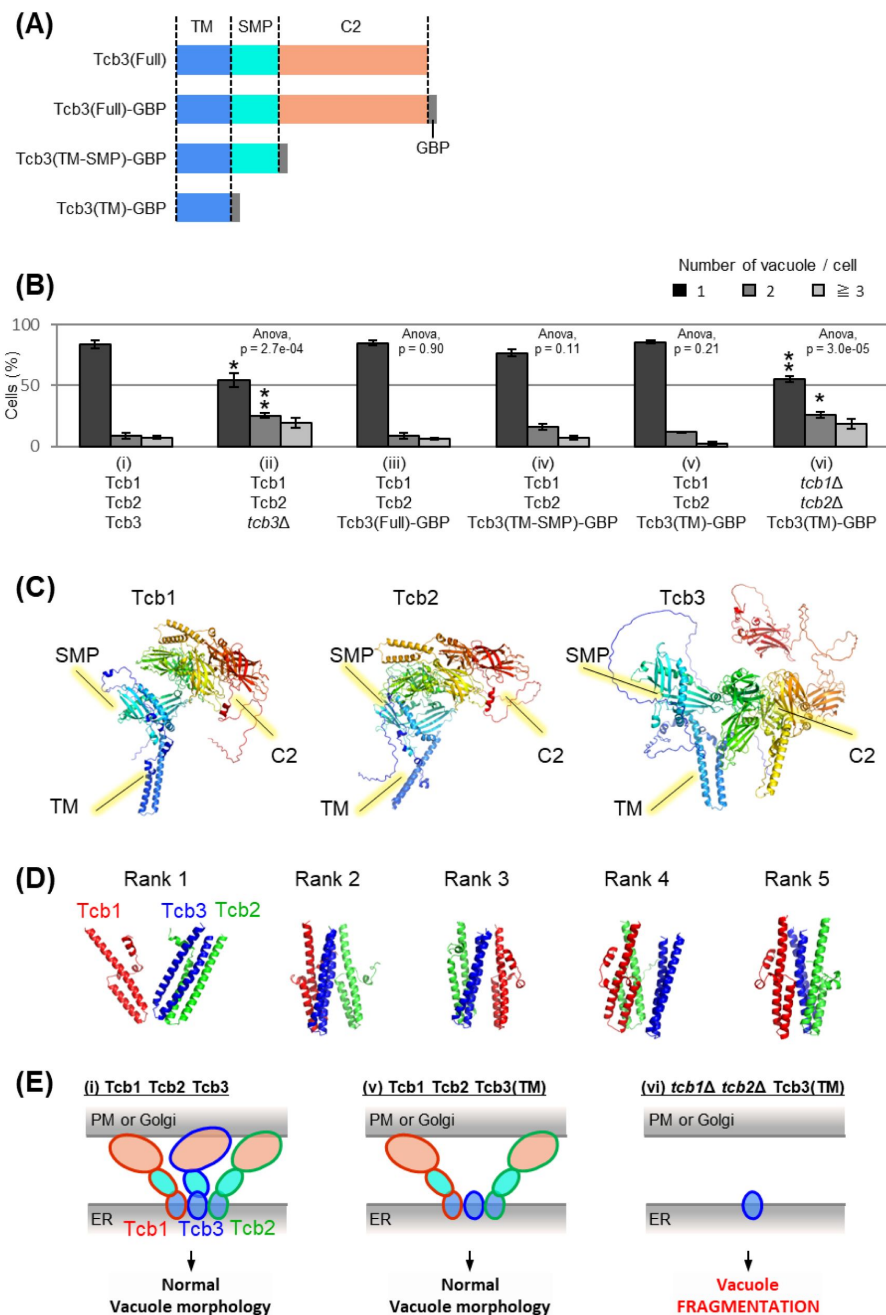


Figure 2.

Effects of domain deletion and artificial tether on vacuole morphology

(A) Diagram of domain organization of Tcb3 protein. TM, transmembrane domain; SMP, synaptotagmin-like mitochondrial lipid-binding protein; C2, calcium-dependent lipid-binding domain; GBP, GFP-binding protein.

(B) Cells (FKY2577 (i), FKY2924 (ii), FKY3903 (iii), FKY3904 (iv), FKY3905 (v) and FKY4754 (vi)) were grown overnight at 25 °C in YPD. Then vacuoles were stained with FM4-64 and imaged by fluorescence microscopy. The number of vacuoles per cell was counted and categorized into one of three groups. The data represent mean $\pm$ SE of three independent experiments, each based on more than 100 cells. * $p < 0.05$ and ** $p < 0.01$ by Student's t-test compared with Tcb1 Tcb2 Tcb3

(i). Significant differences analysis between the pairwise combination of groups was performed using two-way ANOVA.

(C) The modeled structures of the Tcb1p, Tcb2p and Tcb3p proteins. The ribbons and arrows indicate alpha-helices and beta-sheets, respectively.

(D) TM domain complex in Tcb1p (red), Tcb2p (green), and Tcb3p (blue). The rank "X" indicates the order in which the complexes are most likely to form.

(E) Illustrations show that TM domain of Tcb3 contributes to mediating protein interactions between the tricalbin family to maintain vacuolar morphology.

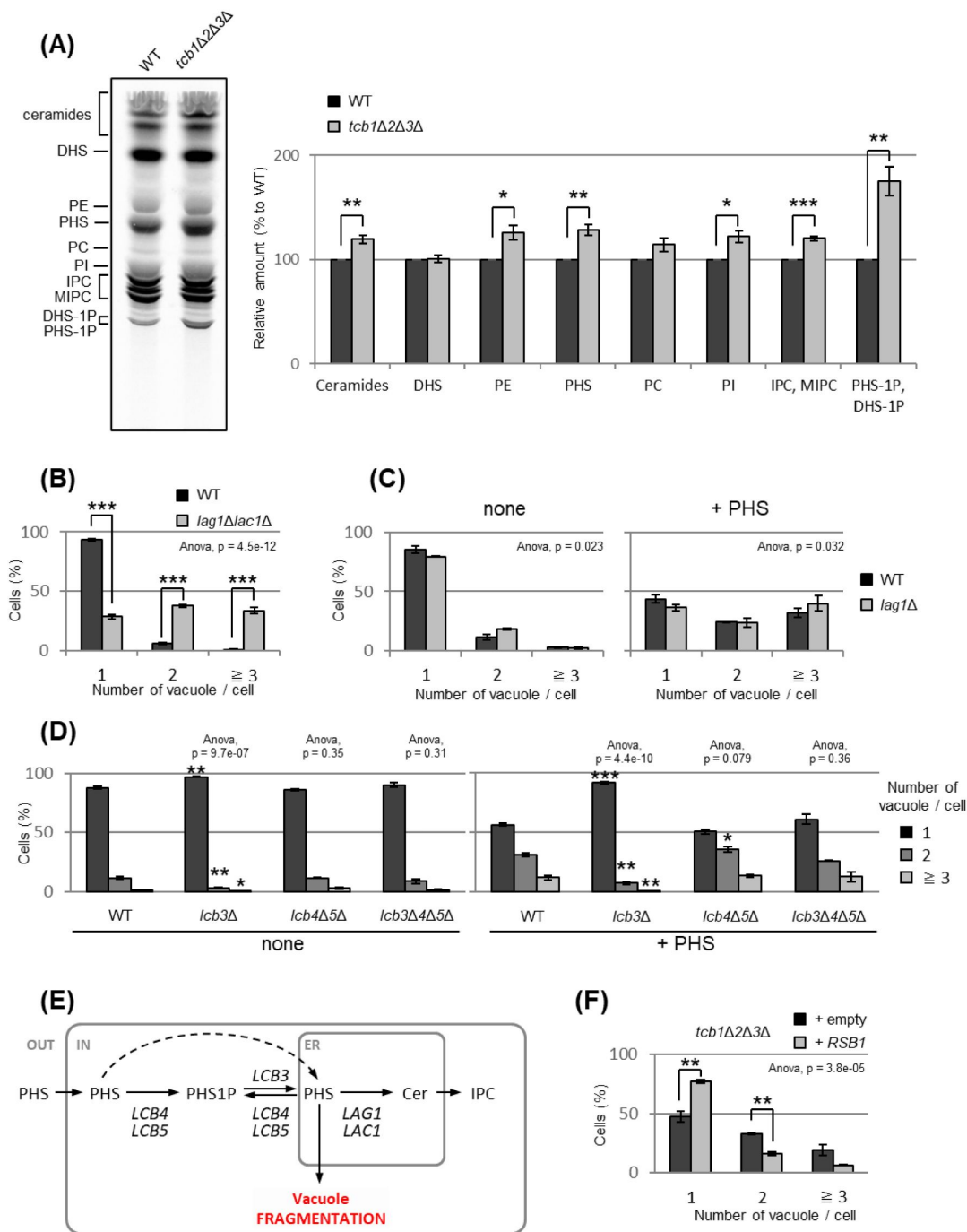


Figure 3.

Accumulated PHS in *tcb1Δ2Δ3Δ* causes vacuole fragmentation.

(A) Cells (FKY2577 and FKY2927) were grown at 25°C, and labeled with [3 H]DHS for 3 hours. Labelled lipids were applied to TLC plates using solvent system (Chloroform-methanol-4.2N ammonium hydroxide (9:7:2, v/v/v)). Incorporation of [3 H]DHS into each lipid was quantified and the percentage of the total radioactivity (%) in WT cells was determined. Data represent mean $\pm$ SE of four independent experiments. ** $P < 0.01$ by Student's t-test.

(B-D, F) Cells (FKY5687 and FKY5688 in B; FKY3340 and YKC121-59 in C; FKY36, FKY37, FKY33 and FKY38 in D; FKY2927 in F) were grown overnight at 25 °C in YPD. PHS was added at 160 μ M (C) or 80 μ M (D) for 2 hours. Vacuoles were stained with FM4-64 and imaged by fluorescence microscopy. The number of vacuoles per cell was counted and categorized into one of three groups. The data represent mean $\pm$ SE of three independent experiments, each based on more than 100 cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test compared with WT (B, D) or empty cells (F). Significant differences analysis between the pairwise combination of groups was performed using two-way ANOVA.

(E) Illustration showing intracellular utilization pathway of exogenous PHS.

The nucleus-vacuole junction is required for PHS- or *tcb3Δ*-induced vacuole fragmentation

To characterize further the relationship between PHS and vacuole morphology, we asked whether PHS delivered from the ER to the vacuole induces vacuole fragmentation. We hypothesized that if PHS are transported to the vacuole by either the vesicular transport pathway through the Golgi apparatus or the non-vesicular transport pathway via inter-organellar MCS triggers vacuole fission, then blockage of the transport could rescue the PHS-induced vacuole fragmentation. Accordingly, we used two types of mutant strains, one blocking vesicular transport and the other blocking the NVJ. In a temperature sensitive *sec18-20* mutant, vesicular transport is abolished at the restrictive temperature of 30 °C or higher, but even at the permissive temperature of 25 °C, vesicular transport is partially impaired [56]. As shown in **Fig 4A** (left), under the condition without exogenous PHS, some vacuoles in *sec18-20* mutant cells became fragmented when shifted to the non-permissive temperature of 30 °C. We also observed that vacuoles in *sec18-20* mutant at 30 °C underwent fragmentation after addition of PHS to the same extent as at the permissive temperature of 25 °C (**Fig 4A**, right), suggesting that vesicle-mediated transport is not required for PHS-induced vacuolar fragmentation. To test the role of NVJ-mediated transport for PHS-induced vacuolar fragmentation we employed a quadruple ΔNVJ mutant (*nvj1Δ nvj2Δ nvj3Δ mdm1Δ*) that was used in a previous study in which complete loss of the NVJ was observed [57], but thus has a different background to other strains of this study. When PHS was added to the ΔNVJ mutant, we observed a significant suppression of vacuole fragmentation compared to WT (**Fig 4B**). Finally, to investigate the requirement for NVJ in tricalbin deletion-induced vacuolar fragmentation, we constructed the *tcb3Δ nvj1Δ* and *tcb3Δ nvj1Δ nvj2Δ nvj3Δ mdm1Δ* mutants. *TCB3* single disruption sufficiently induced vacuolar fragmentation (**Fig 1B**), whereas as expected, the fragmentation was partially suppressed by loss of only *NVJ1* and completely suppressed by loss of all NVJ factors (*NVJ1*, *NVJ2*, *NVJ3*, and *MDM1*) (**Fig 4C**). Taken together, we conclude from these findings that accumulated PHS in tricalbin deleted cells triggers vacuole fission via non-vesicular transport of PHS at the NVJ.

Discussion

In the present study, we found that the accumulation of PHS triggers the fission of vacuoles. Our results suggest that MCSs are involved in this process in two steps. First, the intracellular amount of PHS is modulated by tricalbin-tethered MCSs between the ER and PM or Golgi (**Fig 4D** blue arrow). Second, the accumulated PHS in the tricalbin-deleted cells induces vacuole fission via most likely the NVJ (**Fig 4D** red arrow). Thus, we propose that MCSs regulate vacuole morphology via sphingolipid metabolism. Fundamental questions that arise from our data concern the accumulation of PHS in the tricalbin deletion strain and the mechanism of PHS-induced vacuole fission. Possible mechanisms for elevated PHS levels in tricalbin-deleted cells are the following. MCS deficiency between ER and PM has been shown to reduce the activity of Sac1p, a PtdIns4P phosphatase [36]. Sac1p disruption strain decreases the levels of complex sphingolipids such as IPC and MIPC, while it increases the levels of their precursors, ceramide, LCB, and LCB-1P [58]. This is probably due to the loss of function of Sac1p, which reduces the level of PtdIns used for IPC synthesis, thereby impairing IPC synthesis, and resulting in the accumulation of precursors such as the substrate ceramide. This model is also consistent with the results of accumulated PtdIns4P and reduced IPC synthesis in Δ tether cells [47]. As tricalbins are required for ceramide nonvesicular transport from the ER to the Golgi [37], it is also possible that impaired ceramide nonvesicular transport due to tricalbin deficiency causes ceramide and its precursor, LCB, to accumulate in the ER. Another possibility is that MCS facilitate PHS diffusion between the ER and the PM, which might be coordinated with the LCB export from the PM by Rsb1p. The loss of tricalbins partially disrupts the ER-PM tether, possibly resulting low efficiency of PHS ejection by Rsb1p. This is supported by the result that overexpression of Rsb1p suppressed vacuolar fragmentation in tricalbin-deleted cells (**Fig 3F**).

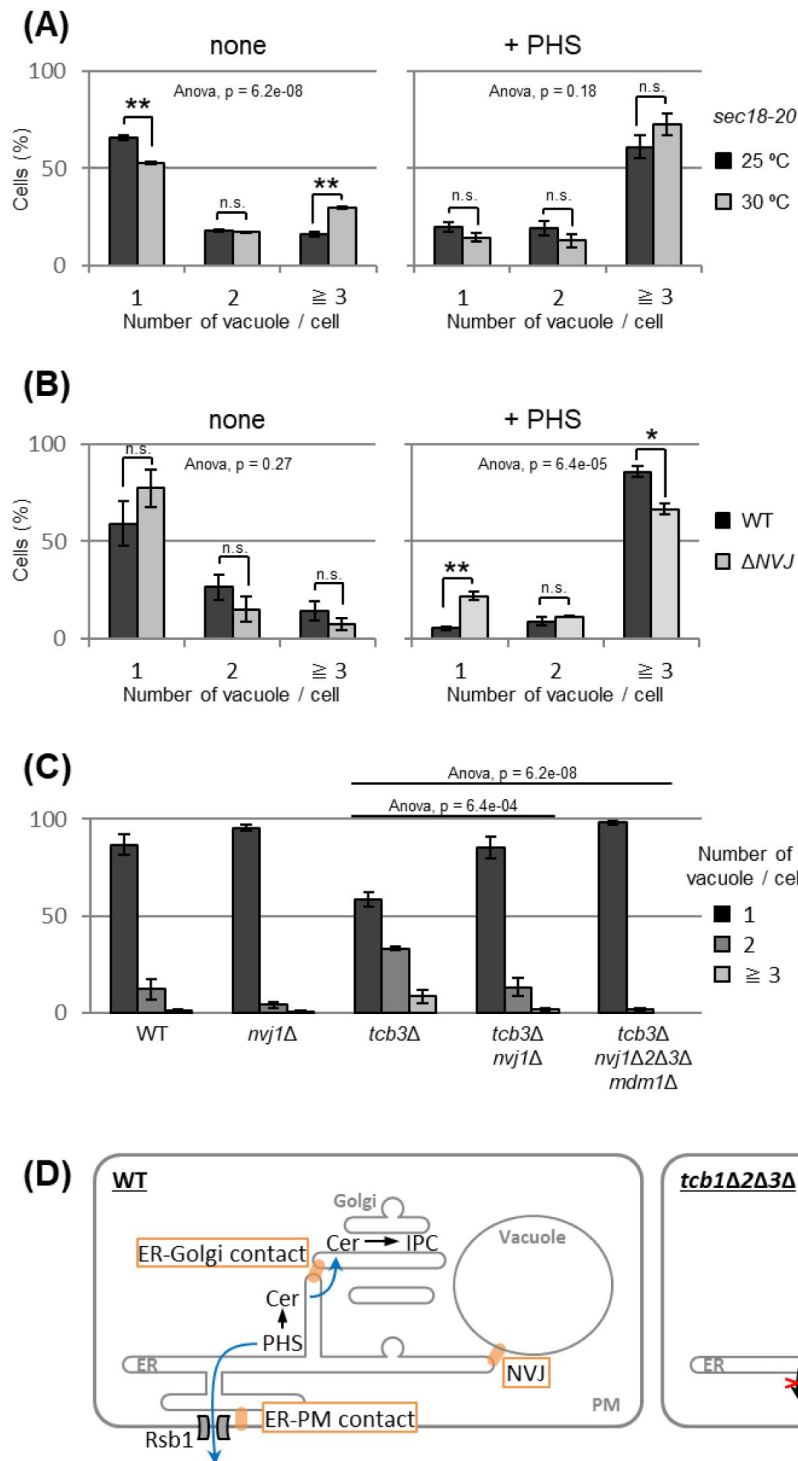


Figure 4.

NVJ is required for PHS-induced vacuole fragmentation

(A-C) Cells (FKY2929 in A; FKY3868 and FKY5560 in B; FKY6187, FKY6189, FKY6190, FKY6188 and FKY6409 in C) were grown overnight at 25 °C in YPD. PHS was added at 40 μ M for 2 hours at 30 °C (A) and 25 °C (A, B). Vacuoles were stained with FM4-64 and imaged by fluorescence microscopy. The number of vacuoles per cell was counted and categorized into one of three groups. The data represent mean $\pm$ SE of three independent experiments, each based on more than 100 cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test. Significant differences analysis between the pairwise combination of groups was performed using two-way ANOVA. (D) Membrane contact sites regulate vacuole morphology via sphingolipid metabolism. See the main text for details.

Because both PHS- and tricalbin deletion-induced vacuolar fragmentations were partially suppressed by the lack of NVJ (**Fig 4B** [4](#), [4C](#)), it is suggested that transport of PHS into vacuoles via the NVJ is involved in triggering vacuolar fragmentation. Recently, it has been reported that sphingoid bases are transferred between ER and vacuole via the NVJ, and that Mdm1p, a key tethering protein for the formation of the NVJ, may play an additional role in LCB transfer [[35](#) [4](#)]. Perhaps, Mdm1p may be responsible for pulling out and passing LCBs. However, we cannot rule out the possibility that the repression of vacuolar fragmentation in the absence of NVJ is not due to inhibition of PHS transfer, but rather to changes in the lipid composition of the vacuolar membrane caused by the lack of supply of other substances capable of triggering vacuolar fragmentation other than PHS, like sterols and lipids such as PtdIns[[3](#) [4](#), [5](#) [4](#)]P2 and its precursors. Further analysis in this regard is warranted.

How accumulated PHS triggers vacuolar fragmentation remains undetermined. Sphingosine, as a bioactive lipid, has been reported to exert effects on enzyme activity in humans and yeast [[59](#) [4](#)] [[60](#) [4](#)] [[61](#) [4](#)], and it is possible that PHS may induce vacuolar fragmentation through established signaling pathways. Yabuki et al [[61](#) [4](#)] reported that LCB accumulation activates a signaling pathway that includes major yeast regulatory kinases such as Pkh1/2p, Pkc1p and TORC1, which may be a candidate to promote vacuolar fragmentation. Fab1p, a target of TORC1, is responsible for the production of PtdIns[[3](#) [4](#), [5](#) [4](#)]P2, which is a well-established inducer of vacuolar fragmentation. Fab1p exhibits co-localization with the TORC1-activating EGO complex, and its activity is controlled by Ivy1p [[62](#) [4](#)] and TORC1 [[63](#) [4](#)]. PtdIns[[3](#) [4](#), [5](#) [4](#)]P2 was shown to regulate vacuole fission employing Vps1p and Atg18p as executioners [[64](#) [4](#)] while also promoting TORC1 activity in a positive feedback loop [[65](#) [4](#)]. In this context, we found that PHS-induced vacuolar fragmentation can be suppressed by the loss of Fab1p and its regulatory binding partner Vac14p (**Fig S2A** [4](#)). This observation suggests that PHS-induced vacuolar fragmentation requires PI metabolism. On the other hand, it was shown that membrane division is mediated by certain proteins that contain amphipathic helices (AHs) and interact with lipid cofactors such as PtdIns[[4](#) [4](#), [5](#) [4](#)]P2, PA, and cardiolipin [[66](#) [4](#)]. Thus, PHS may possess a similar regulatory function as a lipid cofactor for the activity of fission-inducing proteins.

If PHS-induced vacuole fragmentation is not due to signaling, another possible model is that PHS accumulation in the vacuolar membrane causes physical changes in the membrane structure that result in membrane fragmentation. The accumulation of sphingosine in the of GARP mutant *vps53Δ*, in which retrograde transport from the endosome to the Golgi is blocked, caused vacuolar fragmentation [[67](#) [4](#)], and thus also supports this model. Sphingosine stabilizes (rigidifies) the gel domains in the membrane, leading to a structural defect between the phase separation of “more rigid” and “less rigid” domains [[68](#) [4](#)]. This structural defect may result in high membrane permeability. Sphingosine also forms small and unstable channels (compared to the channels formed by ceramide) in the membrane [[69](#) [4](#)]. Sphingosine channels are not large enough to release proteins, but are believed to induce a permeability transition. Other studies have suggested that sphingosine induces non-lamellar structures by interacting with negatively charged lipids such as PA [[70](#) [4](#)]. Finally, PHS alone or in concert with negatively charged lipids such as PtdIns[[3](#) [4](#), [5](#) [4](#)]P2 may be actively involved in the fission process by inducing structural changes in the vacuolar membrane.

Besides PHS-induced vacuolar fission, it is generally well-known that vacuolar division can be triggered as an acute response to osmotic shock. We made an interesting observation under hyperosmotic conditions (0.2 M NaCl), wherein the loss of NVJ led to complete suppression of vacuolar division (**Fig S2B** [4](#)). Although the involvement of PHS transport in this process remains unclear, our finding suggests a significant role for NVJ in vacuolar fission as a hyperosmotic response. Collectively, we propose that NVJ plays a general regulatory role in vacuolar morphology.

Yeast strains

All strains of *S. cerevisiae* used for this work are listed in [Supplementary Table 1](#).

Plasmids

All plasmids used for this work are listed in [Supplementary Table 2](#).

Culture conditions

Yeast cells were grown either in rich YPD medium (2% glucose, 1% yeast extract, 2% peptone) or in synthetic minimal SD medium (2% glucose, 0.15% yeast nitrogen base, 0.5% ammonium sulfate, bases as nutritional requirements) and supplemented with the appropriate amino acids.

FM4-64 stain and Fluorescence Microscopy

Yeast cells were cultured in YPD medium at 25°C for 15 hours to achieve $OD_{600} = 0.5$. The cells were collected and suspended in the same medium to achieve $OD_{600}=20$. 20 mM FM4-64 (N-(3-Triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium) dissolved in DMSO was added (a final concentration of 20 μ M) while shielded from light. The cells were incubated with FM4-64 for 15 minutes at 25°C and then the cells were washed twice with YPD medium. Cells were suspended to achieve $OD_{600}=10$ in the same YPD medium or YPD containing reagents (such as rapamycin or PHS), and incubated at 25°C for 2 hours under light shielding to label the vacuoles. After incubation, cells were collected and observed with a fluorescence microscope.

Western Blotting

To analyze Sch9 phosphorylation, protein extracts from cells expressing SCH9-5HA were treated with 2-nitro-5-thiocyanobenzoic acid overnight, resolved by SDS-PAGE, immunoblotted, and visualized using rat anti-HA monoclonal antibody (3F10; Roche) and anti-rat IgG antibody (A9037; Sigma-Aldrich) produced in goat. Bands were quantified using ImageJ to determine the relative amounts of phosphorylated Sch9.

Lipid Labelling with [³H]DHS

In vivo labelling of lipids with [³H]DHS was carried out as described [71]. Radiolabeled lipids were extracted with chloroform-methanol-water (10:10:3, vol/vol/vol), and analyzed by thin-layer chromatography (TLC) using a solvent system (Chloroform-methanol-4.2N ammonium hydroxide (9:7:2, vol/vol/vol)). Radiolabeled lipids were visualized and quantified on an FLA-7000 system.

Protein complex modelling

The modeled Tcb1p, Tcb2p, and Tcb3p structures were obtained from AlphaFold2 Protein Structure Database (<https://alphafold.ebi.ac.uk/>). Based on the modeled structures, the amino acids of TM regions were predicted as 79-171 for Tcb1p, 79-162 for Tcb2p, and 189-268 for Tcb3p. The protein complex structures of the TM regions in Tcb1p, Tcb2p, and Tcb3p were modelled by AlphaFold2 program [72] worked on the AlphaFold Colab web space (<https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb#scrollTo=pc5-mbsX9PZC>). The figures were drawn using PyMOL software provided by Schrödinger, Inc. (<https://pymol.org/2/>).

Data and Materials Availability Statement

The data and materials that support the findings of this study are included in the article and its Supplemental Information, or are available from the corresponding authors on request.

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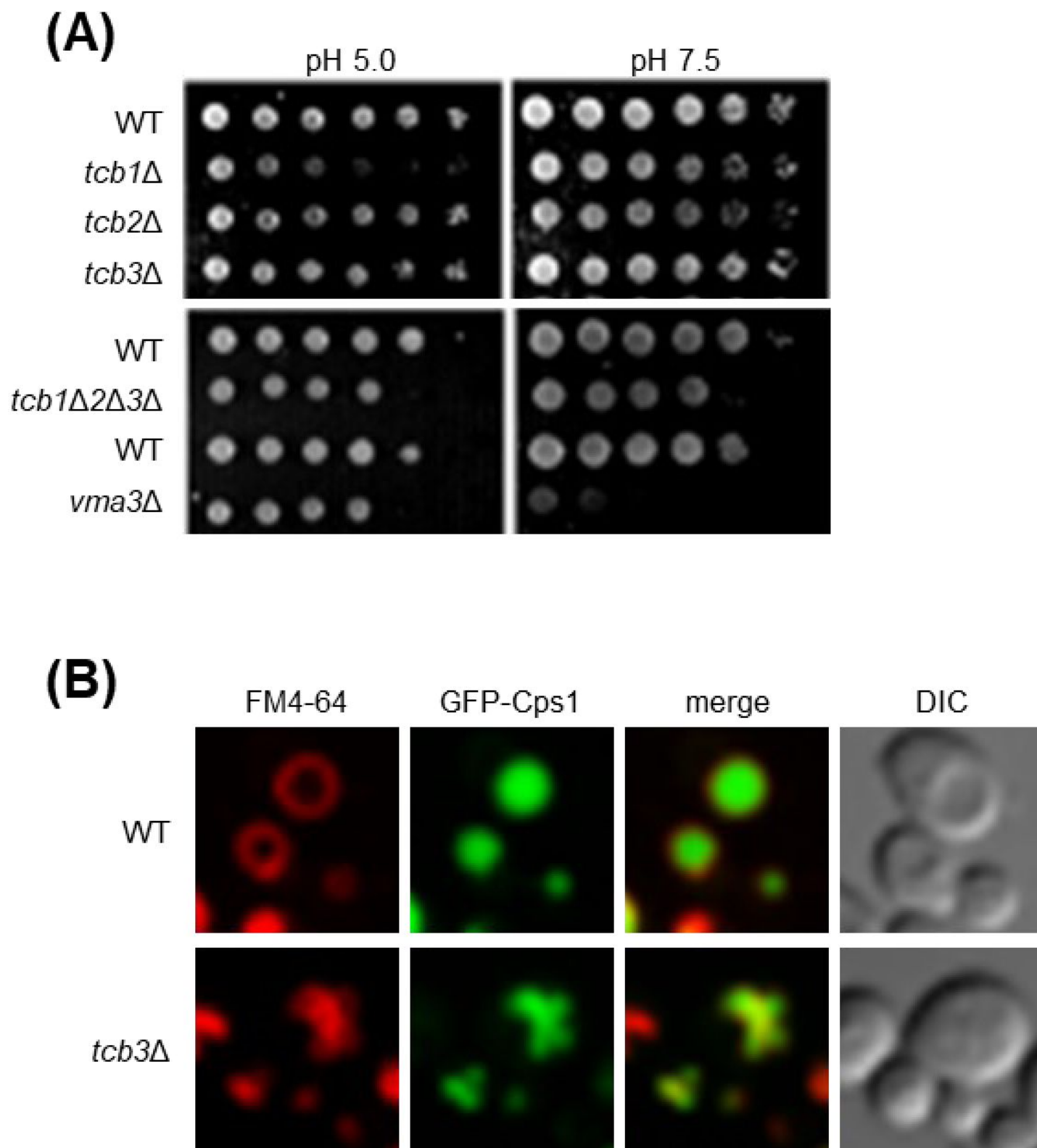
Author Contributions

K.N., A.I. and K.F. designed research; K.H., K. N., A.I., S.Y., A.N. and S.F. performed research; A.I. analyzed data; and A.I., P.S. and K.F. wrote the paper.

Competing Interest Statement

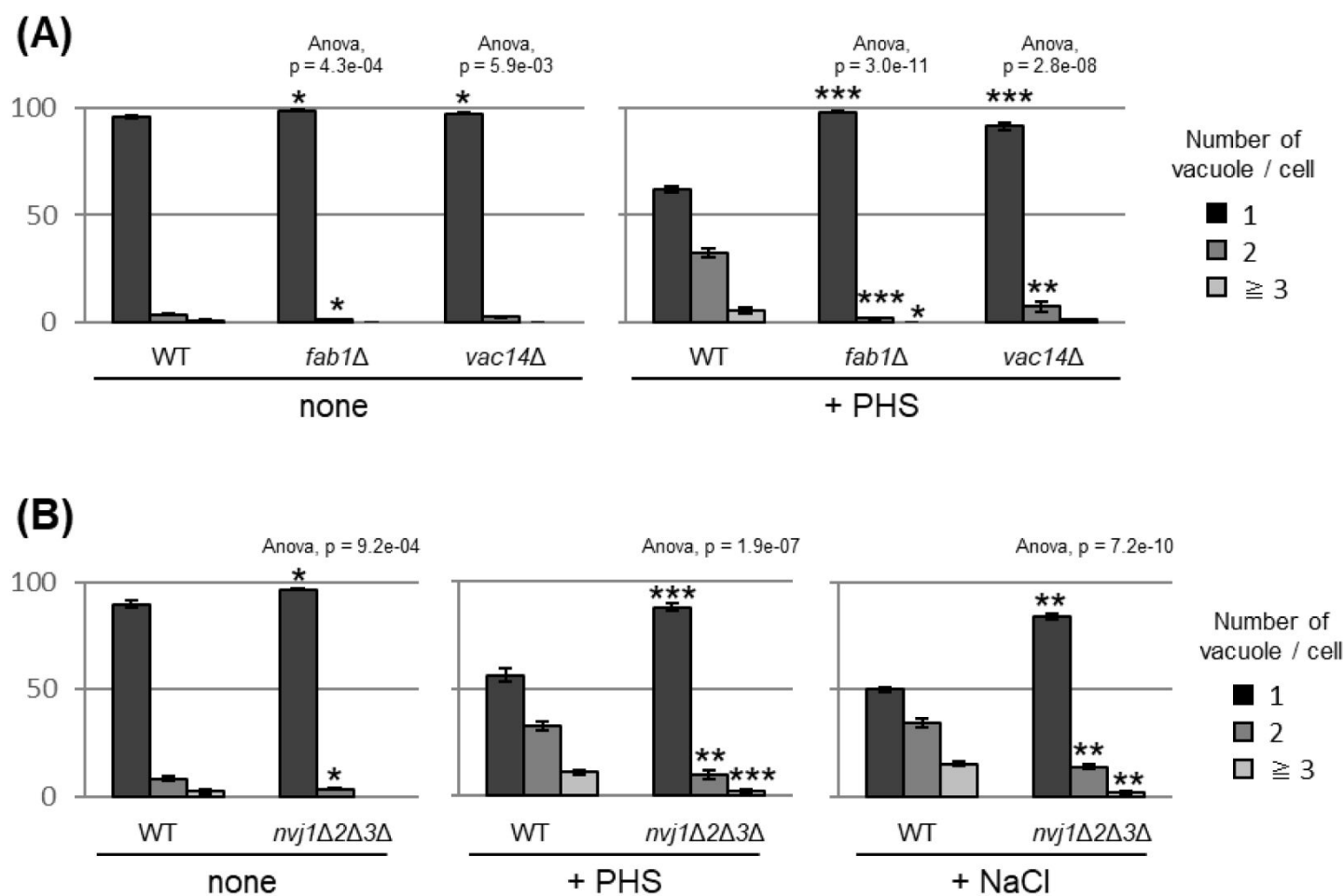
The authors declare no competing interest.

Supplementary information



Supplementary Figure 1

(A) Cells (FKY2577, FKY2909, FKY3819, FKY2924, FKY2927, FKY3340 and YKC112-01) were adjusted to $OD_{600} = 1.0$ and fivefold serial dilutions were then spotted on YPD plates of indicated pH, then incubated at 25 °C. (B) GFP-Cps1p fusion protein was transformed into WT (FKY2577) and *tcb3*Δ (FKY2924) strains and analyzed its subcellular localization with respect to FM4-64-stained vacuoles using by fluorescent microscopy.



Supplementary Figure 2

(A, B) Cells (FKY3340, YKC145-21 and YKC149-61 in A; FKY6187 and FKY6140 in B) were grown overnight at 25 °C in YPD. PHS was added at 80 μ M for 2 hours, or cells were incubated with 0.2 M of NaCl for 2 hours. Vacuoles were stained with FM4-64 and imaged by fluorescence microscopy. The number of vacuoles per cell was counted and categorized into one of three groups. The data represent mean $\pm$ SE of three independent experiments, each based on more than 100 cells. * p < 0.05, ** p < 0.01 and *** p < 0.001 by Student's t -test. Significant differences analysis between the pairwise combination of groups was performed using two-way ANOVA.

Strain	Number	MAT	Genotype	Source
WT (RH)	FKY2577	a	<i>ura3 his3 leu2 trp1 lys2 bar1-1</i>	Ikeda A et al. iScience. 2020;23(10):101603.
<i>tcb1</i> Δ2 Δ3 Δ	FKY2927	a	RH except for <i>tcb1</i> Δ::TRP1 <i>tcb2</i> Δ::HIS3 <i>tcb3</i> Δ::LEU2	Ikeda A et al. iScience. 2020;23(10):101603.
<i>tcb1</i> Δ	FKY2909	a	RH except for <i>tcb1</i> Δ::TRP1	Ikeda A et al. iScience. 2020;23(10):101603.
<i>tcb2</i> Δ	FKY3819	alpha	RH except for <i>tcb2</i> Δ::HIS3	Ikeda A et al. iScience. 2020;23(10):101603.
<i>tcb3</i> Δ	FKY2924	a	RH except for <i>tcb3</i> Δ::LEU2	Ikeda A et al. iScience. 2020;23(10):101603.
<i>tcb1</i> Δ2 Δ	FKY3023	a	RH except for <i>tcb1</i> Δ::TRP1 <i>tcb2</i> Δ::HIS3	Ikeda A et al. iScience. 2020;23(10):101603.
<i>tcb1</i> Δ3 Δ	FKY3820	alpha	RH except for <i>tcb1</i> Δ::TRP1 <i>tcb3</i> Δ::LEU2	Ikeda A et al. iScience. 2020;23(10):101603.
<i>tcb2</i> Δ3 Δ	FKY3008	a	RH except for <i>tcb2</i> Δ::HIS3 <i>tcb3</i> Δ::LEU2	Ikeda A et al. iScience. 2020;23(10):101603.
TCB3 (Full)-GBP	FKY3903	a	RH except for <i>TCB3</i> -GBP Δ::KanMX	This study
TCB3 (TM-SMP)-GBP	FKY3904	a	RH except for <i>TCB3</i> (1F-489R)-GBP::KanMX	This study
TCB3 (TM)-GBP	FKY3905	a	RH except for <i>TCB3</i> (1F-259R)-GBP::KanMX	This study
<i>tcb1</i> Δ2 Δ <i>TCB3</i> (TM)-GBP	FKY4754	a	RH except for <i>tcb1</i> Δ::TRP1 <i>tcb2</i> Δ::HIS3 <i>TCB3</i> (1F-259R)::KanMX	This study
WT	FKY36 / RH4423	alpha	RH except for <i>HIS3</i> + <i>TRP1</i> + <i>LYS2</i> + <i>pep4-3</i>	Funato K et al. J Biol Chem. 2003;278(9):7325-7334.
<i>lcb3</i> Δ	FKY37 / RH4427	alpha	RH except for <i>lcb3</i> Δ::KanMX <i>HIS3</i> + <i>LEU2</i> + <i>TRP1</i> + <i>LYS2</i> + <i>pep4-3</i>	Funato K et al. J Biol Chem. 2003;278(9):7325-7334.
<i>lcb4</i> Δ5 Δ	FKY33 / RH4402	alpha	RH except for <i>lcb4</i> Δ::URA3 <i>lcb5</i> Δ::LEU2 <i>HIS3</i> + <i>TRP1</i> + <i>LYS2</i> + <i>pep4-3</i>	Funato K et al. J Biol Chem. 2003;278(9):7325-7334.
<i>lcb3</i> Δ4 Δ5 Δ	FKY38 / RH4464	alpha	RH except for <i>lcb3</i> Δ::kanMX <i>lcb4</i> Δ::URA3 <i>lcb5</i> Δ::LEU2 <i>HIS3</i> + <i>TRP1</i> + <i>LYS2</i> + <i>pep4-3</i>	Funato K et al. J Biol Chem. 2003;278(9):7325-7334.
<i>sec18-20</i>	FKY2929	alpha	RH except for <i>sec18-20</i> <i>LYS2</i> +	Ikeda A et al. iScience. 2020;23(10):101603.
WT (RH)	FKY6187	alpha	<i>ura3 his3 leu2 trp1 lys2 bar1-1</i>	This study
<i>nvj1</i> Δ	FKY6189	a	RH except for <i>nvj1</i> Δ::HIS3	This study
<i>tcb3</i> Δ	FKY6190	alpha	RH except for <i>tcb3</i> Δ::LEU2	This study
<i>tcb3</i> Δ <i>nvj1</i> Δ	FKY6188	a	RH except for <i>tcb3</i> Δ::LEU2 <i>nvj1</i> Δ::HIS3	This study
<i>tcb3</i> Δ <i>nvj1</i> Δ2 Δ3 Δ <i>mdm1</i> Δ	FKY6409	a	RH except for <i>nvj1</i> Δ::URA3 <i>nvj2</i> Δ::KanMX <i>nvj3</i> Δ::HIS3 <i>mdm1</i> Δ::KanMX <i>tcb3</i> Δ::LEU2	This study
<i>nvj1</i> Δ2 Δ3 Δ	FKY6140	a	RH except for <i>nvj1</i> Δ::URA3 <i>nvj2</i> Δ::KanMX <i>nvj3</i> Δ::HIS3	This study
WT (W303)	FKY5687 / RL1	a	<i>his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100 GAL</i> + <i>bar-</i>	Lucena R et al. Curr Biol. 2018;28(2):196-210.
<i>lag1</i> Δ <i>lac1</i> Δ	FKY5688 / RL40	a	W303 except for <i>lag1</i> Δ::KanMX6 <i>lac1</i> Δ::HIS	Lucena R et al. Curr Biol. 2018;28(2):196-210.
WT (BY4742)	FKY3340	alpha	<i>his3</i> Δ1 <i>leu2</i> Δ0 <i>lys2</i> Δ0 <i>ura3</i> Δ0	Open biosystem, inc
<i>lag1</i> Δ	YKC121-59	alpha	BY4742 except for <i>lag1</i> Δ::KanMx	Open biosystem, inc
<i>vma3</i> Δ	YKC112-01	alpha	BY4742 except for <i>vma3</i> Δ::KanMx	Open biosystem, inc
<i>fab1</i> Δ	YKC145-21	alpha	BY4742 except for <i>fab1</i> Δ::KanMx	Open biosystem, inc
<i>vac14</i> Δ	YKC149-61	alpha	BY4742 except for <i>vac14</i> Δ::KanMx	Open biosystem, inc
WT (SEY6210)	FKY3868	alpha	<i>ura3-52 leu2-3,112 his3-Δ100 trp1-Δ901 lys2-801 suc2-Δ9</i>	Yabuki Y et al. Genetics. 2019;212(1):175-186.
ΔNVJ	FKY5560	alpha	SEY6210 except for <i>nvj1</i> Δ::TRP1 <i>nvj2</i> Δ::HIS3 <i>nvj3</i> Δ::NatMX <i>mdm1</i> Δ::KanMX	Henne WM et al. J Cell Biol. 2015;210(4):541-551.

Supplementary Table 1

Yeast strains used in this study, Related to All Figures.

Plasmid	Number	Relevant information	Source
pRS426-TDH3	FKP51	2μ, yeast <i>TDH3</i> promoter, empty, <i>URA3</i>	Mumberg D et al., Gene. 1995;156(1):119-122
pRS426-TDH3/RSB1	FKP1065	2μ, yeast <i>TDH3</i> promoter, <i>RSB1</i> , <i>URA3</i>	This study
pRS316/PTDH3-EGFP-CPS1-TCMK1	FKP621	CEN, yeast <i>TDH3</i> promoter, GFP- <i>CPS1</i> <i>URA3</i>	Howard Riezman

Supplementary Table 2

Plasmids used in this study, Related to All Figures.

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Reviewer #1 (Public Review):

The manuscript investigates the role of membrane contact sites (MCSs) and sphingolipid metabolism in regulating vacuolar morphology in the yeast *Saccharomyces cerevisiae*. The authors show that tricalbin (1-3) deletion leads to vacuolar fragmentation and the accumulation of the sphingolipid phytosphingosine (PHS). They propose that PHS triggers vacuole division through MCSs and the nuclear-vacuolar junction (NVJ). The study presents some solid data and proposes potential mechanisms underlying vacuolar fragmentation driven by this pathway. However, there are some concerns regarding the strength and interpretation of their lipid data, and the robustness of some conclusions. The manuscript would benefit from addressing these concerns and providing more conclusive evidence to support the proposed conclusions. Overall, the study provides valuable insights into the connection between MCSs, lipid metabolism, and vacuole dynamics, but further clarification will be highly valuable to strengthen the conclusions.

Reviewer #2 (Public Review):

This manuscript investigates the mechanism behind the accumulation of phytosphingosine (PHS) and its role in triggering vacuole fission. The study proposes that membrane contact sites (MCSs) are involved in two steps of this process. First, tricalbin-tethered MCSs between the endoplasmic reticulum (ER) and the plasma membrane (PM) or Golgi modulate the

intracellular amount of PHS. Second, the accumulated PHS induces vacuole fission, most likely via the nuclear-vacuolar junction (NVJ). The authors suggest that MCSs regulate vacuole morphology through sphingolipid metabolism.

While some of the results in the manuscript are interesting the overall logic is hard to follow. In my assessment of the manuscript, my primary concern lies in its broad conclusions which, in my opinion, exceed the available data and raise doubts. Here are some instances where this comes into play for this manuscript:

2.) Major points for revision

1.) The rationale to start investigating a vacuolar fission phenotype in the beginning is very weak. It is basically based on a negative genetic interaction with NVJ1. Based on this vacuolar fragmentation is quantified. The binning for the quantifications is already problematic as, in my experience, WT cells often harbor one to three vacuoles. How are quantifications looking when 1-3 vacuoles are counted as "normal" and more than 3 vacuoles as "fragmented"? The observed changes seem to be relatively small and the various combinations of TCB mutants do not yield a clear picture.

2.) The analysis of the structural requirements of the Tcb3 protein is interesting but does not seem to add any additional value to this study. While it was used to quantify the mild vacuolar fragmentation phenotype it does not reoccur in any following analysis. Is the *tcb3Δ* sufficient to yield the lipid phenotype that is later proposed to cause the vacuolar fragmentation phenotype?

3.) The quantified lipid data also has several problems. i) The quantified effects are very small. The relative change in lipid levels does not allow any conclusion regarding the phenotypes. What is the change in absolute PHS in the cell. This would be important to know for judging the proposed effects. ii) It seems as if the lipid data is contradictory to the previous study from the lab regarding the role of tricalbins in ceramide transfer. Previously it was shown that ceramides remain unchanged and IPC levels were reduced. This was the rationale for proposing the tricalbins as ceramide transfer proteins between the ER and the mid-Golgi. What could be an explanation for this discrepancy? Does the measurement of PHS after labelling the cells with DHS just reflect differences in the activity of the Sur2 hydroxylase or does it reflect different steady state levels.

4.) Determining the vacuole fragmentation phenotype of a *lag1Δlac1Δ* double mutant does not allow the conclusion that elevated PHS levels are responsible for the observed phenotype. This just shows that *lag1Δlac1Δ* cells have fragmented vacuoles. Can the observed phenotype be rescued by treating the cells with myriocin? What is the growth rate of a *LAG1 LAC1* double deletion as this strain has been previously reported to be very sick. Similarly, what is the growth phenotype of the various *LCB3 LCB4* and *LCB5* deletions and its combinations.

5.) The model in Figure 3 E proposes that treatment with PHS accumulates PHS in the endoplasmic reticulum. How do the authors know where exogenously added PHS ends up in the cell? It would also be important to determine the steady state levels of sphingolipids after treatment with PHS. Or in other words, how much PHS is taken up by the cells when 40 μM PHS is added?

6.) Previous studies have observed that myriocin treatment itself results in vacuolar fragmentation (e.g. Hepowit et al. *bioRxiv* 2022, Fröhlich et al. *eLife* 2015). Why does both, depletion and accumulation of PHS lead to vacuolar fragmentation?

7.) The experiments regarding the NVJ genes are not conclusive. While the authors mention that a *NVJ1/2/3 MDM1* mutant was shown to result in a complete loss of the NVJ the observed effects cannot be simply correlated. It is also not clear why PHS would be transported towards the vacuole. In the cited study (Girik et al.) the authors show PHS transport from the vacuole towards the ER. Here the authors claim that PHS is transported via the NVJ towards the vacuole. Also, the origin of the rationale of this study is the negative genetic interaction of *tcb1/2/3Δ* with *nvj1Δ*. This interaction appears to result in a strong growth defect according to the Developmental Cell paper. What are the phenotypes of the mutants used here? Does the additional deletion of NVJ genes or MDM1 results in stronger growth phenotypes?

8.) As a consequence of the above points, several results are over-interpreted in the discussion. Most important, it is not clear that indeed the accumulation of PHS causes the observed phenotypes.

Reviewer #3 (Public Review):

In this manuscript, the authors investigated the effects of deletion of the ER-plasma membrane/Golgi tethering proteins tricalbins (Tcb1-3) on vacuolar morphology to demonstrate the role of membrane contact sites (MCSs) in regulating vacuolar morphology in *Saccharomyces cerevisiae*. Their data show that tricalbin deletion causes vacuolar fragmentation possibly in parallel with TORC1 pathway. In addition, their data reveal that levels of various lipids including ceramides, long-chain base (LCB)-1P and phytosphingosine (PHS) are increased in tricalbin-deleted cells. The authors find that exogenously added PHS can induce vacuole fragmentation and by performing analyses of genes involved in sphingolipid metabolism, they conclude that vacuolar fragmentation in tricalbin-deleted cells is due to the accumulated PHS in these cells. Importantly, exogenous PHS- or tricalbin deletion-induced vacuole fragmentation was suppressed by loss of the nucleus vacuole junction (NVJ), suggesting the possibility that PHS transported from the ER to vacuoles via the NVJ triggers vacuole fission.

This work provides valuable insights into the relationship between MCS-mediated sphingolipid metabolism and vacuole morphology. The conclusions of this paper are mostly supported by their results, but there is concern about physiological roles of tricalbins and PHS in regulating vacuole morphology under known vacuole fission-inducing conditions. That is, in this paper it is not addressed whether the functions of tricalbins and PHS levels are controlled in response to osmotic shock, nutrient status, or ER stress.

There is another weakness in their claim that the transmembrane domain of Tcb3 contributes to the formation of the tricalbin complex which is sufficient for tethering ER to the plasma membrane and the Golgi complex. Their claim is based only on the structural simulation, but not on biochemical experiments such as co-immunoprecipitation and pull-down.