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Articles

Vacuole-Specific Lipid Release for Tracking Intracellular Lipid Metabolism and Transport in *Saccharomyces cerevisiae*

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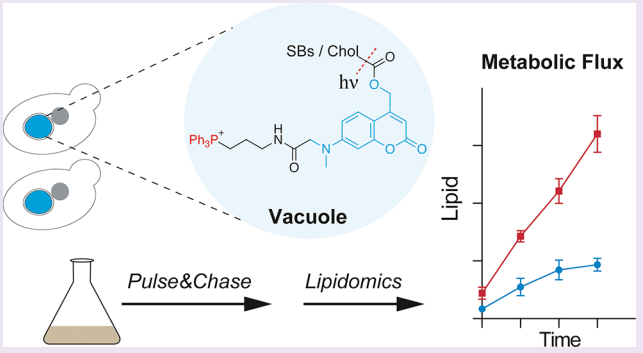
ABSTRACT: Lipid metabolism is spatiotemporally regulated within cells, yet intervention into lipid functions at subcellular resolution remains difficult. Here, we report a method that enables site-specific release of sphingolipids and cholesterol inside the vacuole in *Saccharomyces cerevisiae*. Using this approach, we monitored real-time sphingolipid metabolic flux out of the vacuole by mass spectrometry and found that the endoplasmic reticulum-vacuole-tethering protein Mdm1 facilitated the metabolism of sphingoid bases into ceramides. In addition, we showed that cholesterol, once delivered into yeast using our method, could restore cell proliferation induced by ergosterol deprivation, overcoming the previously described sterol-uptake barrier under aerobic conditions. Together, these data define a new way to study intracellular lipid metabolism and transport from the vacuole in yeast.



INTRODUCTION

Lipids are heterogeneously distributed in eukaryotic cells with their composition varying among the specific subcellular compartments.[1] This heterogeneity is achieved even though most lipid species are first synthesized in one organelle and transported to other organelles for use or further modification. Although most enzymes responsible for lipid metabolism have been identified, how lipids are transported within cells is much less known, and many lipid transportation routes are still waiting to be established. Importantly, new methods are needed to be able to quantify the flux of lipids between intracellular compartments.

In recent years, a number of lipid transfer proteins (LTPs) have been found to facilitate non-vesicular lipid transport through contact sites between organelles.[2] In many cases, structural information and in vitro lipid transfer assays need to be established to validate the roles of LTPs in lipid transport. This can be a challenging task because many LTPs are transmembrane proteins and lack detailed structural information. Mass spectrometry (MS)-based lipidomics techniques can contribute to our understanding of transport by providing quantitative analysis of many chemically diverse lipid species. Designed to carry out lipidome-wide scans, it has been particularly useful when combined with genetic perturbation in yeast or mammalian cells.[3−6] Nevertheless, the results of gene deletion do not always translate into lipid profile changes. For example, simply removing one LTP may not generate changes in lipid profiles if a lipid transportation route is maintained by several proteins with overlapping functions, as seems to be often the case. Clearly, it is desirable to develop new approaches that overcome gene redundancy issues and do not rely on in vitro protein−lipid reconstruction.

Previously, we have developed a series of coumarin (Cou)-based photocleavable (caged) lipid probes targeted to mitochondria and lysosomes, respectively.[7,8] Upon illumination, these probes quickly decomposed, thereby releasing the corresponding native lipid molecules inside the targeted organelle. Using isotope-labeled sphingosine as a lipid precursor, we detected its metabolic products by MS with high sensitivity and showed that sphingolipid metabolic patterns depend highly on subcellular localization.[7] While this strategy is firmly established in mammalian cells, it was unclear whether the same concept is applicable to yeast, an extensively used model organism for studying lipid homeostasis owing to its simplicity, ease of genetic manipulation, and similar organization to metazoans on cellular and subcellular levels.

To explore their use in yeast, we applied mitochondrial-targeted caged probes onto *Saccharomyces cerevisiae* (*S. cerevisiae*) and found surprisingly that these probes accumulated inside the vacuole instead of mitochondria. This unexpected finding led us to synthesize a series of caged lipids, including sphinganine (Sa), phytosphingosine (PHS), and cholesterol,

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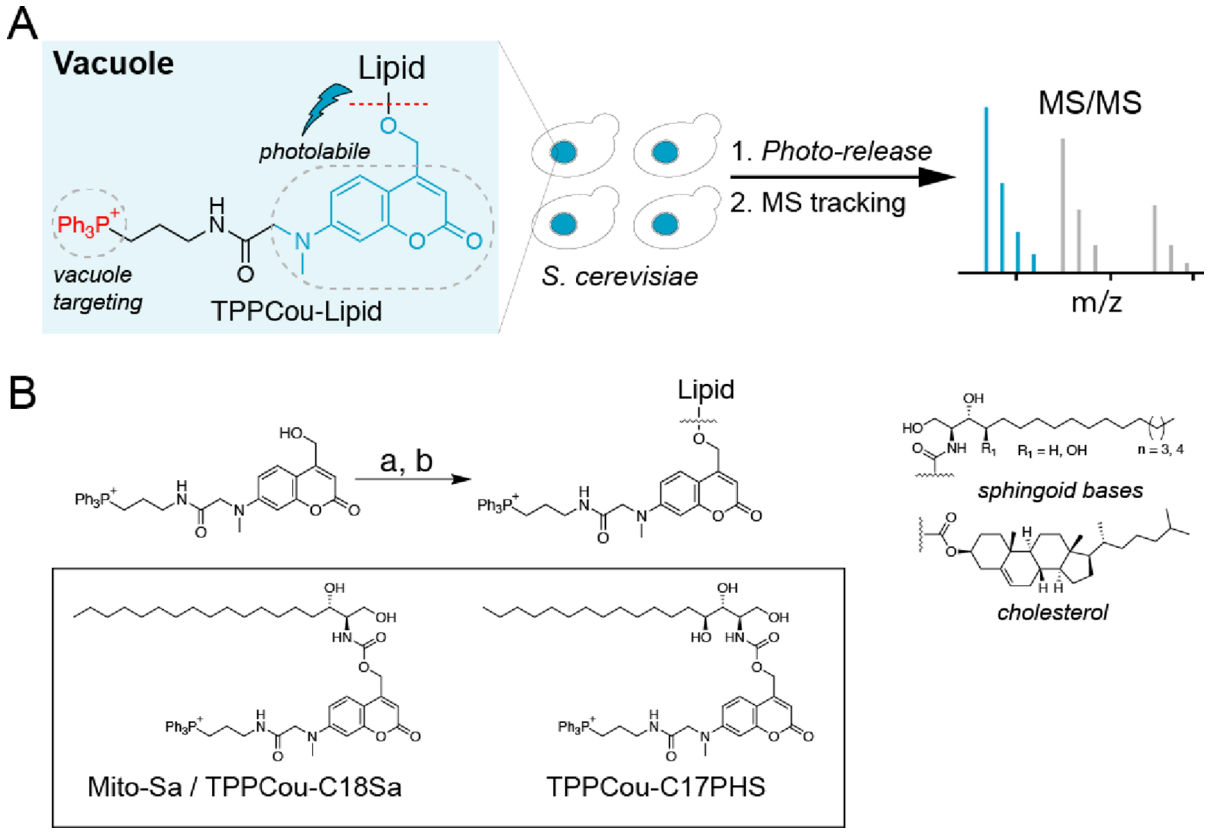

Figure 1. Schematic illustration of MS-based metabolic tracking of vacuole-targeted photocleavable (TPPCou) lipid probes. (B) Synthesis of TPPCou-caged lipids and chemical structures of TPPCou-caged sphingoid bases. For long-chain sphingoid bases (LCBs), (a) bis-(4-nitrophenyl) carbonate, diisopropylethylamine, dimethylformamide, r.t., 3 h; (b) LCB, 60 °C 3 h, 40−60%. For TPPCou-Chol, cholesteryl chloroformate, DCM, DMAP, 18 h, 60%.

which were applied to investigate vacuole-derived lipid metabolism (Figure 1A). Using MS as a readout, we characterized the metabolic products of vacuolar sphingoid bases, explored potential effectors on the sphingolipid metabolic pathway, and provided clear evidence that an endoplasmic reticulum (ER)-anchored protein, which also contacts the vacuole surface, Mdm1, facilitated sphingolipid turnover. Additionally, we successfully delivered cholesterol into *S. cerevisiae* using the same strategy. We showed that cholesterol can restore cell proliferation induced by ergosterol deprivation, and that deletion of *NCR1*, the orthologue of NPC1, which is important for cholesterol transport in mammals,[9,10] did not strongly affect vacuole-derived cholesterol utilization in yeast cells. Collectively, our approach, which combines synthesis, imaging, lipidomics, and genetic modification, defined a new way of studying intracellular lipid transport and metabolism in *S. cerevisiae*.

MATERIALS AND METHODS

Chemicals and Reagents. All internal lipid standards, C17PHS (PHS), and C17Sa (sphinganine) were purchased from Avanti Polar Lipids (Alabaster, AL). Yeast media components: glucose monohydrate, Bacto Peptone, Bacto yeast extract, and Bacto agar were purchased from BD Biosciences (Sparks, MD); yeast synthetic drop-out amino acid supplements were from Sigma (St. Louis, MO). Plasmids pBP73G-Mdm1-green fluorescent protein (GFP) and pBP73G-Nvj1-GFP were created as previously described.[11]

Yeast Strains and Culture. Baker's yeast *S. cerevisiae* were grown in standard rich medium supplemented with adenine and uracil or in synthetic defined medium.[12] Yeast strains were constructed using standard techniques.[13] Yeast strains used in the present study are listed in the Table S1 (Supporting Information).

Fluorescence Microscopy and Image Analysis. Cells were grown in YPUAD or selective synthetically defined (SD) medium to the exponential phase, washed, and resuspended in low-fluorescence medium (LFM)SD medium lacking folate and riboflavin. Cells were put onto Concanavalin-A pretreated glass slides and incubated at room temperature for 5 min. The samples were imaged immediately with the LSM700 Zeiss confocal microscope using a 100× oil immersion objective. Quantification of fluorescence intensity was carried out using ImageJ software. Pearson's correlation coefficients were calculated using EzColocalization ImageJ plugin.

Labeling with Synthetic Chemical Probes. Yeast cells were grown in YPUAD or SD medium to the logarithmic phase, labeled with triphenylphosphonium (TPP)-Cou probes for 20 min at 30 °C, centrifuged at 800*g*, resuspended in LFM medium, and processed for imaging as described above.

Chemical Synthesis, Photouncaging, and Lipidomics. The detailed description of ultraviolet (UV) uncaging, sphingoid base and lipidome analysis, as well synthesis of the caged probes can be found in the Methods Section in the Supporting Information.

RESULTS AND DISCUSSION

Results. *TPPCou-Caged Sphingoid Bases Accumulate in the Yeast Vacuole.* The TPP cation is a well-known mitochondrial targeting motif and has been used to deliver a wide range of small molecules into the mitochondrial matrix in mammalian cells.[14] In the past, we have used TPP-modified Cou as a photocleavable protecting group to prepare caged sphingolipids targeting to the mitochondria in mammalian cells. Unlike in HeLa cells, the TPP-coupling strategy has not

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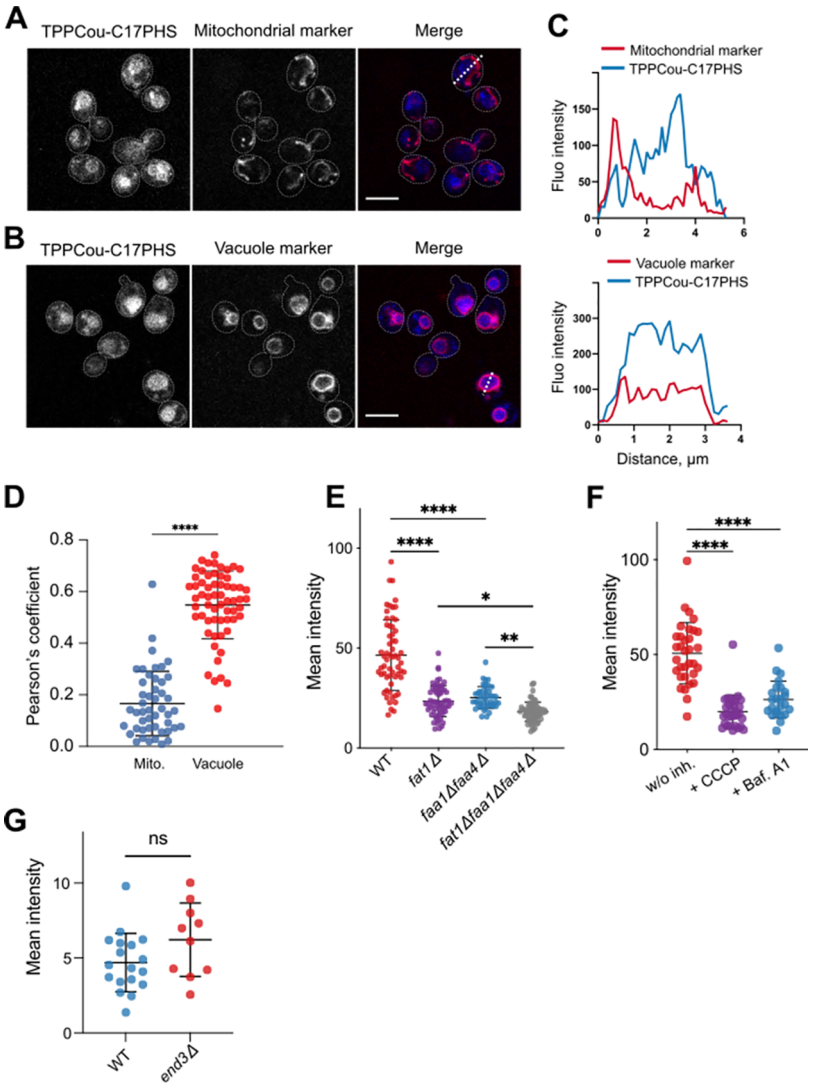

Figure 2. TPPCou-C17PHS accumulated in the yeast vacuole. (A,B) Representative fluorescence images of yeast cells stably expressing mCherry-tagged (red) mitochondrial marker protein Mdh1 (A) or vacuolar protein Vph1 (B) stained with 10 μM TPPCou-C17PHS (blue). Bars, 5 μm, (C) intensity plots of white dotted lines shown on (A,B) for the indicated fluorescent channels. (D) Quantification of colocalization of TPPCou-C17PHS with a mitochondrial marker (Mdh1-mCherry) and vacuole marker (Vph1-mCherry), respectively. (E−G) Quantification of mean intracellular fluorescence intensity of TPPCou-C17PHS. Cells were treated with 20 μM TPPCou-C17PHS for 15 min in the presence of indicated conditions. *p < 0.05, **p < 0.01, ****p < 0.0001, ns., not significant, Student's t-test.

been widely used in yeast, yet several studies in the pathogenic yeast *Candida albicans* showed that TPP-conjugated antifungal compounds inhibited multidrug resistance efflux pumps and interfered with mitochondrial functions.[15,16] In another study, TPP was coupled to a fatty-acid anion-stimulated respiration and thus was presumably targeted to mitochondria in *S. cerevisiae*, but no visual localization studies supporting mitochondrial localization were provided.[17]

Therefore, we examined the localization of TPP- Cou-caged compounds, which are easily seen using a fluorescence microscope because the Cou is also a fluorophore in addition to being a photocleavable protecting group. Because it was unclear whether these caged probes would accumulate in yeast mitochondria, we incubated the caged Sa (Mito-Sa, Supporting Information, Figure S1A) in *S. cerevisiae* expressing a fluorescent mitochondrial marker, Mdh1-mCherry. To our surprise, while we recorded a strong fluorescence signal in the Cou channel, the staining pattern did not overlap with the Mdh1-mCherry signal, but rather looked like the vacuole (Supporting Information, Figure S1B). This unexpected finding prompted us to examine colocalization with a vacuole marker, Vph1-mCherry, which consistently overlapped well with the Cou fluorescence (Supporting Information, Figure S1C). Because Mito-Sa localized to the mitochondria in HeLa cells, but to the vacuole in yeast cells, we renamed it to TPPCou-C18Sa in order to avoid any confusion. Because PHS is the major form of LCBs in yeast,[18] we synthesized and purified TPPCou-C17PHS, to distinguish it from the native C18PHS (Figure 1B), and investigated its localization in *S. cerevisiae*. Using fluorescence microscopy, we have found, consistently, that TPPCou-C17PHS accumulates inside the vacuole, but not the mitochondria (Figure 2A−D). To learn which factors affect the vacuole staining, we treated the cells with diethylaminocoumarin-caged PHS (Cou-PHS) without the TPP cation and TPPCou alone, respectively (Supporting Information, Figure S2A−C), but both failed to generate any meaningful fluorescence signals, indicating that both the TPP cation and a lipid chain are compulsory for effective transport of caged probes

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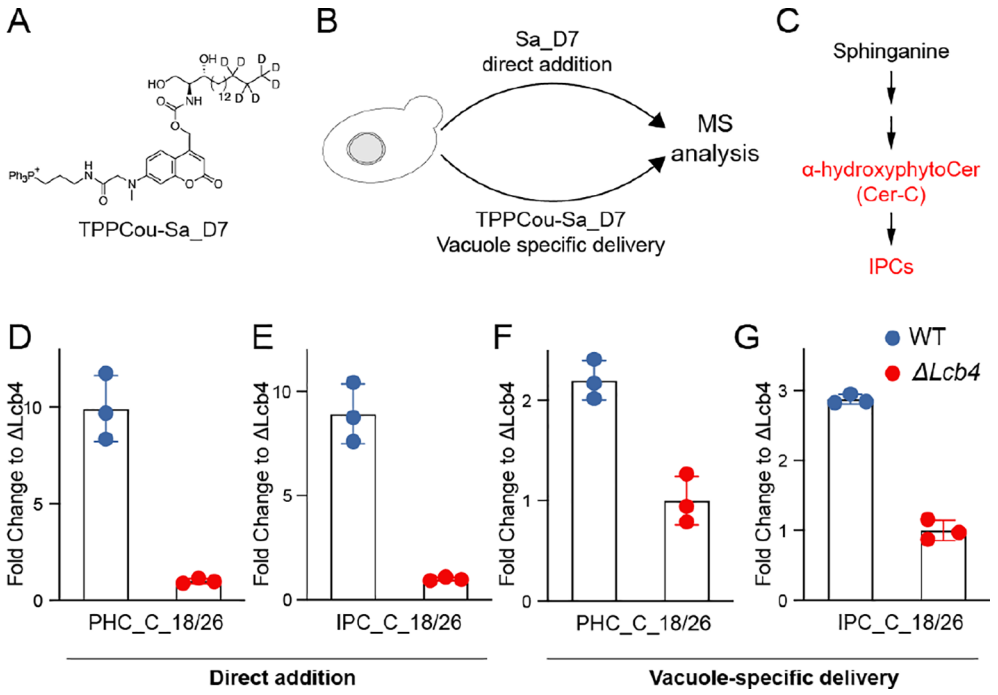

Figure 3. Comparison of D7- Sa conversion to PHC and IPC. (A) Chemical structure of deuterated TPPCou-Sa. (B) Schematic illustration of the experimental design. (C) Simplified metabolic routes of Sa. (D−G) Quantification of PHC_C_18/26 and IPC_C_18/26 using two D7- Sa delivery methods as indicated. Error bars represent SD, $n = 3$.

into the vacuole. It is possible that TPPCou was pumped out by yeast efflux pumps[19] because the TPP cation could be delivered into yeast cell only by applying pulsed electric fields.[20]

Due to the dependence on the sphingoid base, we postulated that proteins involved in long-chain base uptake may play a role in the internalization of TPPCou-C17PHS. Indeed, we found a significant decrease of fluorescence signals in the absence of fatty acyl-CoA synthetases (*faa1*, *faa4*) and/or transporter (*fat1*), which have been shown to affect uptake of exogenous sphingoid bases[21] (Figure 2E). Next, we treated the cells with TPPCou-C17PHS in the presence of CCCP and bafilomycin A1, respectively. Either disrupting the proton gradient with CCCP or blocking vacuolar V-ATPases with bafilomycin significantly reduced the fluorescence intensities of TPPCou-C17PHS, suggesting that the vacuole staining is dependent on the pH gradient across the vacuolar membrane (Figure 3F). Furthermore, we investigated whether the uptake of TPPCou-C17PHS was via endocytosis. Using cells that lack *end3*, essential for endocytosis,[22] we found that the fluorescence intensities from *end3*Δ cells are even slightly higher than the ones from the wildtype (WT) cells (Figure 2G), suggesting that the transport does not rely on endocytosis. Notably, the average fluorescence intensities under both conditions (Figure 2G) are relatively lower compared to other experiments (Figure 2E,F), likely caused by the differences among the yeast strain backgrounds. Together with the imaging data of TPPCou-C18Sa (Supporting Information, Figure S1), we showed that the TPPCou-caged lipids accumulated inside the yeast vacuole, and that elements crucial for the import and localization include the proton gradient across the vacuolar membrane and proteins required for sphingoid base and fatty acid import.

Metabolism of Vacuole-Released Sphingoid Bases is Less Dependent on LCB4. In *S. cerevisiae*, the levels of LCB are tightly regulated by coordinated action of metabolic enzymes.[23] We have previously reported that Lcb4 kinase is essential for incorporating exogenous LCBs into complex sphingolipids,[24] and its activity accounts for 95% of long-chain base kinase activity.[25] Lcb4 has been localized to the Golgi, late endosomes,[26] but more direct localizations without protein tagging found it on the cortical ER juxtaposed to the plasma membrane.[27] Given the localization of Lcb4 outside of the vacuole, it is therefore interesting to know whether the conversion of vacuole-released sphingoid bases into dihydroceramides is also subject to regulation by Lcb4. To this end, we prepared a caged, deuterated C18 Sa probe (TPPCou-Sa-D7, Figure 3A) and analyzed its metabolic products after photoreleasing in WT and mutant cells lacking LCB4 (*lcb4*Δ), respectively. In parallel, we used exogenously added C18 Sa (Sa-D7) as a control (Figure 3B). The downstream metabolites of the deuterated Sa include phytoceramides (PHCs) and inositol phosphorylceramide (IPC), which can be detected by MS (Figure 3C). Indeed, we observed that conversion of the sphingoid base into PHC and IPC was drastically reduced in *lcb4*Δ cells (Figure 3D,E), similar to that in our previous study.[24] Decreased PHC and IPC synthesis was also found in the vacuole-released Sa-D7 experiments, but to a much lesser extent (Figure 3F,G). These results suggest that a large part of the Lcb4-dependence for complex sphingolipid production from exogenous sphingoid bases may be related to cellular uptake. However, this cannot be the entire dependence because some dependence was also seen in vitro after reconstitution.[24] In contrast, our data here show that a large part of the recycling pathway of sphingoid bases from the vacuole into complex sphingolipids does not depend on LCB4, consistent with the previously described Lcb4 localization in proximity to the plasma membrane.[27]

Metabolism of Vacuole-Released C17PHS. The lysosome/vacuole serves as a primary site to break down complex lipid molecules into basic building blocks, which are later transported to other sites such as ER for lipid synthesis.[28] These processes of

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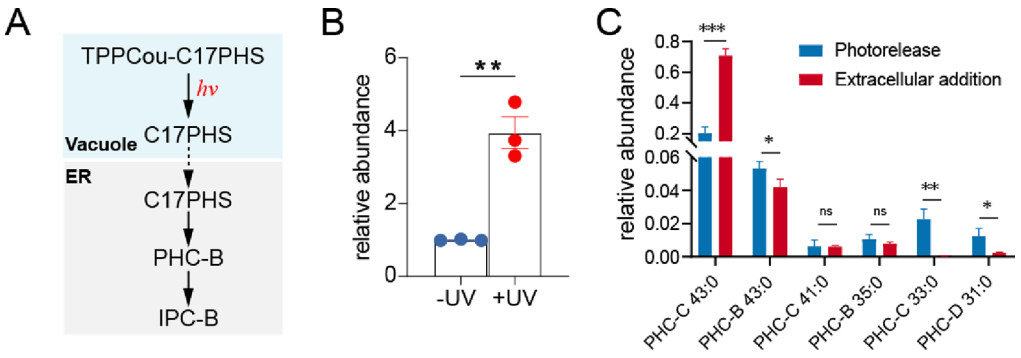

Figure 4. Metabolism of vacuole-released C17PHS. (A) Metabolic scheme of C17PHS after the vacuole-specific uncaging. After transport from the vacuole to the ER, PHS is converted into PHC-B, which is transported further to the Golgi, where PHC-B is metabolized into IPC-B. (B) Analysis of C17PHS levels before (−UV) and after (+UV) UV uncaging in cells labeled with 5 μM TPPCou-C17PHS. (C) Comparison of ceramide species derived from TPPCou-C17PHS (after photorelease) or extracellularly added C17PHS. Error bars represent SEM, $n = 3$ *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant, Student's t-test.

storage and recycling are of fundamental importance and have been linked to a number of diseases, but many aspects, particularly on the sphingolipid transport, remain elusive.[29] Previously, we have applied the local uncaging methods in HeLa cells to show that sphingolipid metabolism and turnover have distinct properties depending on the subcellular localization of the release,[7] though the mechanism of lipid transport was not defined. Here, we applied the same principle to examine vacuole-released sphingolipid metabolism, with a particular focus on lipid transport.

The vacuole maintains a low pH environment and hosts a range of enzymes to disassemble lipid molecules. To assess the stability of caged probes after import into the vacuole, cells were treated with TPPCou-C17PHS, collected before or after UV illumination, and analyzed by MS (Figure 4B). We could detect some C17PHS even without illumination, likely due to undesired enzymatic activities in the vacuole. Nevertheless, the majority remained intact, judging from the UV light-released C17PHS, which is about four times higher than the control. After learning that the majority of the caged probe was intact, we analyzed its metabolic products after uncaging and compared to the ones delivered by extracellular addition of C17PHS. PHS can be transported from the vacuole to the nuclear ER, where it is converted into various ceramides and other complex sphingolipids (Figure 4A, Supporting Information Figure S3). Our analysis showed that exogenously added C17PHS was predominantly converted into PHC-C (PHC-C_C43). In photo-released cells, however, a significantly higher proportion of C17PHS was used for the synthesis of PHC-B (PHC-B_C43) and shorter-chain ceramides (Figure 4C). Although the amount of C17PHS being delivered into intracellular space was different under the two experimental conditions, these results clearly revealed subcellular dependence of sphingolipid metabolism, in accordance with our previous findings in mammalian cells.

MDM1 Facilitated Sphingolipid Turnover. Lipids can be transported within cells by two types of pathways, vesicular and non-vesicular, but for most lipids it is thought that majority of traffic is carried out by non-vesicular trafficking, most likely involving LTPs and membrane contact sites (MCS), areas where membranes from two organelles are found in close apposition.[30] Nucleus-vacuole junctions (NVJs) are an example of MCS between the vacuole and the nuclear ER wrapped around the nucleus forming a contiguous membrane.[31] A number of imaging-based studies indicated the universal presence of MCS, but functional studies are still lagging behind.[32] In recent years, we have identified Mdm1 as a tethering protein that localized to the ER but formed contacts with the vacuole and lipid droplets in yeast.[33,34] *MDM1* is highly conserved in metazoans and plays essential roles in regulating lipid homeostasis.[35,36] We have found that mutations in yeast *MDM1* perturb sphingolipid metabolism, but whether this tether is involved in interorganelle lipid trafficking remained elusive.[11] In support of a potential lipid-trafficking model, recent structural predictions using the machine-learning algorithm Alphafold2 indicate that Mdm1p and its orthologs contain a putative novel lipid transport region formed from two of its domains, the PXA and PXC, which fold intramolecularly into a bidomain module with a large hydrophobic cavity.[37] Motivated by these observations, we next examined whether Mdm1 may influence ER-vacuole lipid trafficking.

We used the vacuole-specific uncaging method to examine whether sphingolipid turnover is influenced by Mdm1p over time. Accordingly, we developed a pipeline in which C17PHS was first released inside the vacuole, and its metabolic products were measured by MS at different time points. PHC-B_C43 and IPC-B_C43 are two of the major metabolic products derived from C17PHS and gave lowest signal-to-noise ratios, hence were selected in our analysis. Our data showed that the accumulation of both PHC-B_C43 and IPC-B_C43 was significantly higher in cells, in which *MDM1* was overexpressed (Figure 5A,B). However, when we overexpressed *NVJ1*, another established nuclear ER-vacuole junction (NVJ) tether,[31] we did not observe any significant difference when compared to the control cells (Figure 5C,D). This striking contrast between *MDM1* and *NVJ1* overexpression, which both promote increased MCS formation, indicates that *MDM1* promoted the turnover of vacuolar sphingolipids by additional means than its role in MCS formation. Intriguingly, when we attempted to use *mdm1*Δ or *nvj1*Δ knockout strains in the "pulse-chase" experiments, we detected high levels and variable quantities of endogenous C17 sphingolipids. Because those sphingolipids share the same chemical structures with the ones converted from the C17PHS after uncaging, it was difficult to deduce any conclusion from those experiments. The reason for the appearance of odd-chain sphingolipids in these mutants is unknown. Because neither *MDM1* nor *NVJ1* is essential for cell growth, cells do not seem to rely on a single component/pathway to transport lipids between the vacuole and the ER. The functional redundancy and/or quick metabolic adaptation could also mean that overexpression rather than gene deletion is more suitable to capture differences

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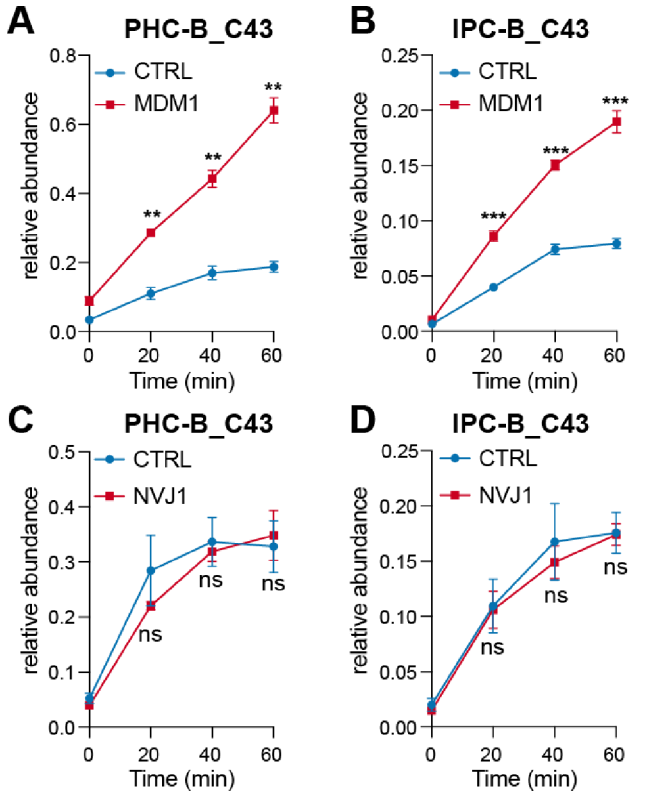

Figure 5. *MDM1* facilitated sphingolipid turnover. Lipid analysis of sphingolipids derived from vacuole-released C17PHS after UV uncaging in cells overexpressing empty vector, Mdm1 (A,B) or Nvj1 (C,D). Data represents the average of three independent experiments. Error bars represent SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant, Student's *t*-test.

in the metabolic tracking studies using our method. In addition, our lipidomics analysis indicated that major lipid profiles were not significantly shifted after the photorelease (Supporting Information, Figure S4), most likely because the LCBs delivered to the cells are tracers and only comprise a very small portion of the endogenous lipid pool.

Cholesterol Delivered to Yeast Using TPPCou as Cargo. Cholesterol is a fundamentally important molecule to human health, yet its physiological roles are still not clearly defined and some are highly debated.[38] This is partially because cholesterol is so abundant and essential in mammalian cells that any experimental means for reducing cholesterol levels faces the risk of jeopardizing numerous cellular functions. In contrast, WT yeast does not produce cholesterol and use another sterol, ergosterol, for maintaining cellular activities. Having engineered a yeast strain that effectively produces cholesterol, we demonstrated previously that functions of ergosterol can be partially replaced by cholesterol, which makes yeast an attractive model organism for studying cholesterol transport and metabolism.[39] However, while mammalian cells import cholesterol through receptor-mediated endocytosis, *S. cerevisiae* does not take up sterols under aerobic conditions termed aerobic sterol exclusion.[40]

To gain insights into cholesterol transport, we prepared TPPCou-caged cholesterol (TPPCou-Chol, Figure 6A) and inspected whether it can be effectively delivered to the vacuole in WT yeast cells. As hoped for, the probe was successfully delivered into the vacuole, like the TPPCou-C17PHS (Figure 6B,C). In addition, we measured the cholesterol uptake under various conditions and found that cholesterol was indeed delivered to the cells using TPPCou-Chol, although there was only minimal additional effect due to UV illumination (Figure 6D). These results suggest that most TPPCou-Chol was cleaved by enzymes prior to UV exposure, which also partially explains why the fluorescence signals from TPPCou-Chol were weaker than the ones from TPPCou-caged LCBs (Figure 6B,C). Despite lacking optical control on the TPPCou-Chol, the data marked the successful vacuole-targeted delivery of cholesterol under aerobic conditions, enabling us to further investigate cholesterol transport in *S. cerevisiae*. First, we blocked endogenous ergosterol biosynthesis using fenpropimorph,[41] then treated cells with TPPCou-Chol in increasing amounts, and monitored cell growth. The growth curves indicated that TPPCou-Chol effectively rescued the deficiency of ergosterol in a dose-dependent manner (Figure 6E), while free cholesterol-treated cells failed to alleviate any cell growth suppressed by fenpropimorph (Figure 6F). Using fenpropimorph, we also performed another set of experiments on *ncr1* knockout cells. We observed similar growth curves positively correlated with the amount of TPPCou-Chol, but did not see any significant difference between WT and n*cr1* knockout cells (Figure 6G,H). Our results suggest that Ncr1p is not essential for transporting cholesterol from the lysosomes/vacuoles, in agreement with suggestions from previous findings.[10,42] Overall, our data showed that the vacuole-specific uncaging approach is not limited to sphingoid bases and has the potential for broader application to study lipid recycling. Furthermore, we have established a novel protocol to introduce sterols into aerobically grown yeast, overcoming the sterol exclusion mechanism, which should permit future studies on the function of sterols in cell biology.

Discussion. In eukaryotic cells, distinct lipid distribution and metabolism are associated with subcellular compartments, but related tools to directly address these issues are very limited. Recently, we have developed a local uncaging strategy that offers a direct experimental means to study organelle-specific lipid metabolism. Here, we introduced this concept in the yeast system and we have extended its application to intracellular lipid transport. Together with MS, we provide quantitative information of sphingolipid metabolism, its dependence on sphingosine kinase, and unveiled the active role of Mdm1p in facilitating the conversion of LCBs into ceramides. In addition, by releasing cholesterol in the vacuole, we showed that cholesterol can effectively rescue the deficiency of ergosterol, that cholesterol transport was not substantially hindered by the removal of *NCR1*, and that it is possible using this method to introduce sterols into aerobically grown yeast.

The fluorescence-imaging data shows that TPPCou-caged lipids accumulated in the vacuole, but it is still unclear why they are enriched inside vacuole instead of mitochondria. The TPP cation is known as a mitochondrial targeting signal and has been extensively used in HeLa cells, yet we did not find any targeting to mitochondria in yeast. Previously, antifungal TPP-coupled compounds could be targeted to mitochondria in the pathogenic yeast *C. albicans*.[15,16] Another study demonstrated that TPP-conjugated with a fatty acid stimulated yeast mitochondrial respiration,[17] but the localization of the compound was not addressed and its effects on mitochondria could have been indirect. It has been known that cationic chemotherapeutic drugs are trapped in lysosomes, organelles analogous to the yeast vacuole.[43] It is also known that the budding yeast expresses multiple H+-drug antiporters and at least one of which, Vba4p, is

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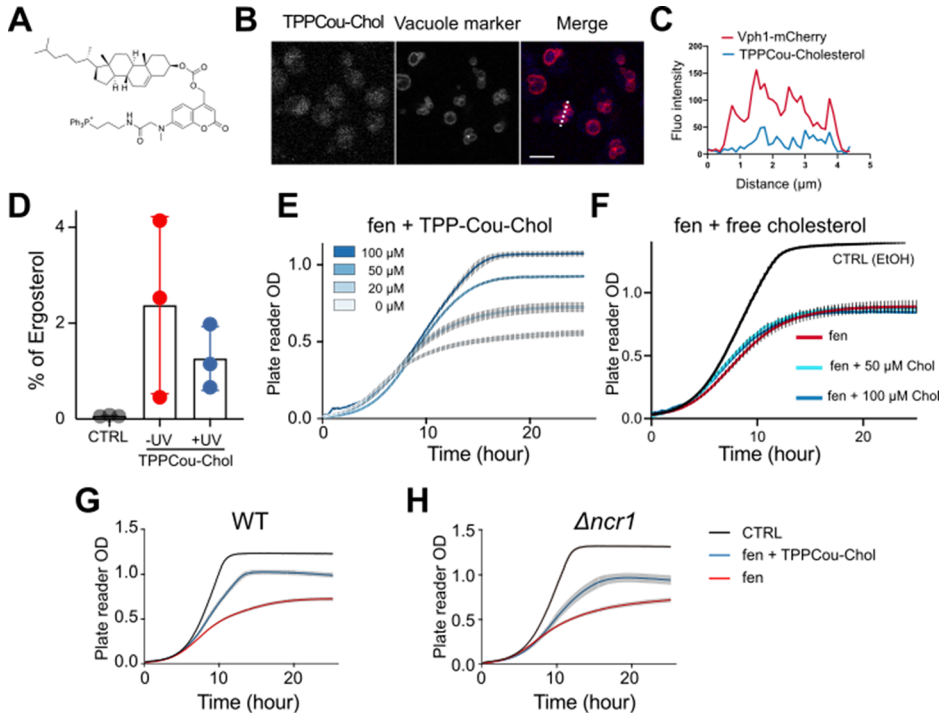

Figure 6. Vacuole-released cholesterol rescues growth of ergosterol-depleted yeast cells. (A) Chemical structure of TPPCou-Chol. (B) Representative confocal images of yeast cells stably expressing mCherry-tagged (red) vacuolar marker protein Vph1 stained with 50 μM TPPCou-Chol (blue) for 20 min at 30 °C. Bars, 5 μm. (C) Intensity profile of the white dotted line in (B) in TPPCou-cholesterol and Vph1-mCherry channel, respectively. (D) GC−MS analysis of vacuole-released cholesterol in yeast cells treated with 50 μM TPP-Cou-cholesterol or ethanol (CTRL) and subjected to UV uncaging (+UV). Cholesterol amounts are shown as percentage of total ergosterol levels. Data represents the average of three independent experiments. Error bars represent *SEM*. (E) Growth curves of cells treated with TPPCou-Chol in the presence of fenpropimorph (8 μM). (F) Growth curves of cells treated with free cholesterol in the presence of fenpropimorph (8 μM), or ethanol (CTRL) as indicated. (G,H) Growth curves of WT or *ncr1*Δ mutant cells. Cells were grown in the presence of fenpropimorph (8 μM), TPPCou-Chol or control (ethanol). Cells were incubated in a plate reader, and the growth was recorded by taking regular optical density measurements at 30 °C. Individual values represent the average of four biological replicates. Error bars represent SEM.

found in the vacuolar membrane.[44] The activity of H+-antiporters depends on the vacuolar pH gradient, and experiments using CCCP and bafilomycin indicate that the pH gradient is essential to localize the TPP-Cou probes inside the vacuoles. Although CCCP is commonly used as an uncoupler of mitochondrial potential, it has also been shown to equilibrate the yeast vacuolar pH with that of the extracellular medium.[45] It is difficult to rule out the possibility that some of the TPPCou-based probes went into mitochondria, but the amount accumulated in mitochondria is under the detection threshold and thus must be limited. We explored the uptake mechanism of TPPCou-caged lipids using knockout strains, and our results show that probe uptake involves the participation of acyl-CoA synthases but is independent of endocytosis. Because the vacuole is a recycling hub for breaking down complex lipids into building blocks, it is not a surprise that we observed partial hydrolysis of TPPCou-C17PHS prior to UV illumination, and that TPPCou-Chol was completely decomposed. As the probes are stable in acidic solvents during the purification steps, consistent with our previous results,[7] the cleavage is unlikely caused by the low pH of the vacuoles but should have occurred in the vacuole, not before delivery there, because incubation with TPPCou alone did not lead to vacuolar labeling. On the other hand, the successful delivery of LCBs and cholesterol also suggests a possible broader application of using TPPCou as cargo to transport other lipid molecules to the vacuole in *S. cerevisiae*.

The major lipid-trafficking routes are organized by non-vesicular lipid transport, particularly through MCS with the involvement of a large group of LTPs. Several imaging-based studies revealed the precise localization of these proteins, but functional studies often lag behind. Herein, we released PHS in vacuole and monitored its metabolic products over time to establish a system, which can directly measure the influence of LTPs on lipid movement and metabolism. Our results showed that overexpressing Mdm1, an ER-vacuole tethering protein, facilitated formation of ceramide and IPC species, unlike another nuclear ER-vacuole junction protein, Nvj1. Because ceramide synthases are localized to the nuclear ER, active lipid transport from vacuole to the ER is a prerequisite before the metabolic conversion. We have previously shown that *mdm1* mutants suppress cell survival in myriocin treated plating assays,[11] but details were lacking because suppressing sphingolipid biosynthesis by myriocin can have profound effects on numerous aspects of cellular activities. In our current study, we measured the real-time metabolic flux of sphingolipids originating from the vacuole, thus providing a direct link between Mdm1p and the metabolic turnover of sphingoid bases. Our recent studies also found that Mdm1p directly interacts with fatty acids via its hydrophobic N-terminal region and promotes lipid droplet formation.[33,34] It is unclear for now whether Mdm1p directly binds LCBs, but even if the direct interaction occurs, it is likely that the mechanisms are somewhat different because the head group of sphingoid bases is much more polar and hydrophilic than fatty acids. However, it is

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notable that recent structural predictions using Alphafold2 suggest that Mdm1p and its orthologs encode a putative LTP-like module composed of its PXA and PXC domains.[37] An intriguing model is that this LTP-like module is capable of transporting lipids including C17PHS at interorganelle contacts.

We also demonstrated that the same principle can be used to study cholesterol transport in yeast using growth as a functional readout, highlighting the flexibility and compatibility of our approach for studying local lipid metabolism. As cholesterol plays essential roles in maintaining numerous cellular functions, switching the system to *S. cerevisiae* should offer more freedom to modulate its levels and to introduce analogs.

In metabolic flux studies, our method should be able to scan multiple lipid transfer protein candidates without the requirement of detailed information on protein structures. Arguably, our design relies on biochemical conversion and hence enzymatic activities, but this requirement should be matched without difficulty thanks to the abundance of numerous lipid metabolic enzymes (Supporting Information, Figure S3). In future applications, we can use lipid molecules bearing a clickable motif and a diazirine, which allows crosslinking to proteins in close proximity after exposing to an orthogonal UV light to photouncage, as has been previously demonstrated.[46] In this case, after photouncaging, crosslinking followed by "click" chemistry with a fluorophore should enable us to visualize lipid localization over time without relying on enzymatic activities, though it will lack the information of lipid species.

In conclusion, we presented here a novel technique of delivering lipid precursors specifically to the vacuole in *S. cerevisiae*, which enabled us to visualize and track lipid recycling from the vacuole. It has also provided a novel method to bypass the sterol exclusion barrier of aerobically grown yeast. Together with MS-based lipidomics, we have found that Mdm1p plays an active role in mediating sphingolipid metabolism. Collectively, our approach provides a new framework of analyzing lipid transporters without prior structural information or in vitro reconstitution.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.2c00120.

Localization of TPPCou-C18 Sa; Lack of uptake of TPPCou or Cou-PHS; sphingolipid biosynthetic pathway in yeast; unchanged amounts of the major classes of glycerophospholipids; description of the materials and methods; and NMR data of the synthesized and purified compounds (PDF)

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Author Contributions

S.F., V.G., and H.R. conceived the idea and designed the experiments. S.F. synthesized caged compounds. H.H. and W.M.H. created the plasmids. V.G. and S.F. performed experiments and analyzed the results. W.M.H. and H.R. supervised the study. S.F. and H.R. wrote the manuscript with the help of all other authors. V.G. and S.F. contributed equally.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PHS	phytosphingosine
PHC	phytoceramide
LCB	long-chain base
Sa	sphinganine
IPC	inositolphosphorylceramide
TPP	triphenylphosphonium
Cou	coumarin

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