A Dynamic Interface between Vacuoles and Mitochondria in Yeast

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SUMMARY

Cellular life depends on continuous transport of lipids and small molecules between mitochondria and the endomembrane system. Recently, endoplasmic reticulum-mitochondrial encounter structure (ERMES) was identified as an important yet nonessential contact for such transport. Using a high-content screen in yeast, we found a contact site, marked by Vam6/Vps39, between vacuoles (the yeast lysosomal compartment) and mitochondria, named vCLAMP (vacuole and mitochondria patch). vCLAMP is enriched with ion and amino-acid transporters and has a role in lipid relay between the endomembrane system and mitochondria. Critically, we show that mitochondria are dependent on having one of two contact sites, ERMES or vCLAMP. The absence of one causes expansion of the other, and elimination of both is lethal. Identification of vCLAMP adds to our ability to understand the complexity of interorganellar crosstalk.

INTRODUCTION

Mitochondria generate the majority of cellular energy and house enzymes required for the synthesis, breakdown, and interconversion of various species of amino acids, lipids, iron/sulfur clusters, and other small molecules. Due to their diverse functions and essential roles in cellular metabolism, mitochondria serve as hubs for signaling in events such as growth, differentiation, or cell death. Loss of optimal mitochondrial activity is therefore, not surprisingly, implicated in a growing number of human diseases as well as in aging (Bratic and Larsson, 2013; Costa and Scorrano, 2012; Griffiths, 2012; Vives-Bauza and Przedborski, 2011; Yu et al., 2012).

The central tasks of mitochondria in cells necessitate constant communication and transport of small molecules with other organelles. However, mitochondria are not connected to the endomembrane system via the vesicular pathway. Instead, mitochondria have been shown to communicate with the endomembrane system by virtue of a membrane contact site (MCS) where membranes of mitochondria come into close proximity to membranes of the endoplasmic reticulum (ER). This MCS, also termed mitochondria-associated-membranes (Achleitner et al., 1999; Vance, 1990), enables ions and lipids to be rapidly transported in a nonvesicular manner (Elbaz and Schuldiner, 2011; Levine and Loewen, 2006; Tatsuta et al., 2014). Understanding the molecular machineries creating and regulating this MCS has been the arena of intense investigations in the past decade.

In yeast, the molecular identity of the ER-mitochondria tethering complex was recently uncovered and is mediated by a four-protein complex termed the ER-mitochondria encounter structure (ERMES) (Kornmann et al., 2009). One of its hypothesized functions was to enable phospholipid transport (Kopec et al., 2010; Kornmann and Walter, 2010) required for building mitochondrial membranes as well as for the three-step biosynthetic pathway of aminoglycerophospholipids. Therefore, it was of great surprise when the loss of ERMES subunits had very little effect on cellular levels of aminoglycerophospholipids (Kornmann et al., 2009; Nguyen et al., 2012; Voss et al., 2012). Hence, it became clear that alternate routes of phospholipid transport must exist in the cell, and uncovering them should shed light on novel modes of communication between mitochondria and the endomembrane system. We report here our findings of an MCS between mitochondria and vacuoles (the yeast lysosomal compartment) and its functional significance in lipid transport between the endomembrane system and mitochondria.

RESULTS

A High-Content Screen Uncovers that Vam6/Vps39 Influences the Number of MCSs between the ER and Mitochondria

We set out to uncover alternate paths for communication between mitochondria and the endomembrane system using a systematic genetic screening approach. We reasoned that the extent of ERMES mediated contact sites must be regulated so that loss of a parallel pathway would result in an increase in ERMES mediated contacts. Therefore, we screened for genetic backgrounds yielding a pronounced increase in the amount of ERMES foci per cell (Figure 1A). Using the synthetic genetic array (SGA) methodology (Cohen and Schuldiner, 2011; Tong and Boone, 2006; Tong et al., 2001), we created a library in which each strain harbors a green fluorescent protein (GFP)-tagged ERMES subunit (Mdm34-GFP) on the background of a single gene mutation (Breslow et al., 2008; Giaever et al., 2011; Yu et al., 2012). vCLAMP is enriched with ion and amino-acid transporters and has a role in lipid relay between the endomembrane system and mitochondria.Critical,..
covering the entire yeast genome. Following the automated imaging of all strains generated (Breker et al., 2013), we manually examined the resulting images to uncover strains with the phenotype of choice (Figure 1A). The screen resulted in identification of over 100 different genetic backgrounds in which the Mdm34-GFP signal was altered (Table S1 available online). Strains that altered Mdm34-GFP morphology included deletion strains of ERMES components themselves that caused loss of the junction altogether, reduction in proteasomal subunits that caused an increase in protein levels, and loss of a large repertoire of mitochondrial proteins that caused a decrease in the intensity of foci. We focused on the four mutant backgrounds that displayed elevated number of foci per cell: Δdnm1, Δfis1 (Figure S1A), Δvam6/vps39 (hereinafter referred to as vps39), and Δvam7 (Figures 1B; Figures S1B and S1C).

Dnm1 and Fis1 are essential components of the mitochondrial fission machinery (Bleazard et al., 1999; Mozdy et al., 2000). Interestingly, it has recently been shown that mitochondrial/ER contact sites mark the sites for fission to occur (Friedman et al., 2011; Murley et al., 2013). We therefore reasoned that the increase in ERMES foci on these backgrounds is most probably a result of an indirect effect on mitochondrial morphology or due to crosstalk with the ERMES complex.

Vps39 and Vam7 have been previously characterized as components of the vacuolar fusion process (Price et al., 2000; Stroupe et al., 2006). These hits were surprising because no apparent direct link was reported between vacuolar morphology and ER-mitochondria connections or function. It has, however, previously been reported that the absence of Vps39 leads to impaired respiration capacity (Merz and Westermann, 2009). Therefore, we decided to examine the connection between the absence of Vps39 to the increase in ERMES foci.

**Vps39 Localizes to Contact Sites between Mitochondria and Vacuoles Termed vCLAMPs**

To see if Vps39 has any spatial connection to mitochondria, we tagged Vps39 with an N-terminal GFP (hereinafter referred to as GFP-Vps39), since tagging on the N terminus was previously shown to retain the function of the protein (Angers and Merz, 2009). Following GFP-Vps39 localization by fluorescence microscopy, we found that it was highly enriched in defined patches on the vacuolar membrane, which colocalized with a mitochondrial marker (a matrix-targeted blue fluorescent protein; MTS-BFP) (Figure 2A). We therefore attempted to uncover whether such an overlap between vacuoles and mitochondria implies an undescribed MCS.

Electron microscopy demonstrated that indeed there exists a tight interface between the vacuolar membrane and the...
mitochondrial outer membrane. Similarly to what was observed by fluorescence microscopy, this MCS was labeled by GFP-Vps39. The membranes of the two organelles maintained a separate identity and were not fused, as would be expected, Vps39. The membranes of the two organelles maintained a significant manner (false discovery rate [FDR] = 0.05; Table S3). We found that, in all cases, the combination of both mutations was lethal (Figure S4A), indicating that both mdm34 and S1B). Therefore, we wanted to explore the effect of deleting ERMES on the vCLAMP. Loss of ERMES (caused by deletion of mdm34 [Figures 3A and S3A] or any of the other three complex members [Figure 3C]) caused a dramatic expansion of the vCLAMP interface. In fact, in this genetic background, vCLAMP completely surrounded mitochondria, despite the fact that the cells retained normal morphology (Figure S3C). Electron microscopy demonstrated that the contact site expanded to the entire circumference of each mitochondrion (Figures 3B and S3B). This finding demonstrates that ERMES and vCLAMP are tightly coregulated, which would ensure that mitochondria does not detach from the endomembrane system and, as a consequence, no reduction in endomembrane/mitochondrial transport occurs in the absence of one MCS.

The tight interplay between ERMES and vCLAMP would indeed ensure that, in the absence of one structure, the increase in the other would serve as a “backup” path for small-molecule transport. This predicts that disturbing both MCSs should, in fact, cause a dramatic cellular phenotype. To obtain double mutants, we crossed a vps39 strain with a strain harboring a deletion in each of the ERMES subunits. Diploid yeast were sporulated, and the haploid progeny from single meiosis events were dissected. We found that, in all cases, the combination of both mutations was lethal (Figure S4A), indicating that both MCSs cannot be simultaneously disturbed. Thus, as would be expected, mitochondria must be in contact with at least one organelle in the endomembrane system to maintain proper cellular functions.

Loss of Both Mitochondrial Contact Sites Causes Defects in Phospholipid Transport

Phospholipid transport between the endomembrane system and mitochondria is essential for the three-step enzymatic pathway generating aminoglycerophospholipids (see model in Figure 4H). In this pathway, phosphatidylserine (PtdSer) that is synthesized in the ER is transported to mitochondria to be converted to phosphatidylethanolamine (PtdEtn) by the inner mitochondrial membrane enzyme, PtdSer decarboxylase 1 (Psd1). Some of the PtdEtn is then transported back to the ER, where it can serve as a source for generation of phosphatidylcholine (PtdCho) (Birner and Daum, 2003; Daum et al., 1996). Although ~5% of PtdEtn is created in the late endomembrane system by the exact cellular localization. Five of the eight vacuolar proteins were not homogenously distributed over the vacuolar membrane but rather localized to specific patches (Figure S2B). Further colocalization with a mitochondrial marker showed that GFP-Mnr2 and GFP-Pho91 partially colocalized with mitochondria. (Figures S2C and S2D). This could suggest that the vCLAMP, marked by GFP-Vps39, could serve as a hub for interorganelle transport of small molecules.

The Two Contact Sites of Mitochondria with the Endomembrane System Are in Dynamic Equilibrium and Serve as Mutual Backup Pathways

Our original hypothesis for uncovering the vCLAMP was that optimal communication of mitochondria and the endomembrane system requires coregulation with ERMES. Indeed, our screen showed that loss of vCLAMP caused an increase in the number of ERMES mediated contact sites (Figure 1B and Figures S1A and S1B). Therefore, we wanted to explore the effect of deleting ERMES on the vCLAMP. Loss of ERMES (caused by deletion of mdm34 [Figures 3A and S3A] or any of the other three complex members [Figure 3C]) caused a dramatic expansion of the vCLAMP interface. In fact, in this genetic background, vCLAMP completely surrounded mitochondria, despite the fact that the cells retained normal morphology (Figure S3C). Electron microscopy demonstrated that the contact site expanded to the entire circumference of each mitochondrion (Figures 3B and S3B). This finding demonstrates that ERMES and vCLAMP are tightly coregulated, which would ensure that mitochondria does not detach from the endomembrane system and, as a consequence, no reduction in endomembrane/mitochondrial transport occurs in the absence of one MCS.

Defects in Phospholipid Transport

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A Dynamic Mitochondria/Vacuole Interface

Figure 2. Vps39 Marks a Contact Site between Mitochondria and Vacuoles

(A) GFP-tagged Vps39 is enriched in defined patches on the vacuolar membrane that colocalize with mitochondria. Scale bars, 5 μm.

(B) Immunoelectron microscopy analysis of a GFP-Vps39 strain, using antibodies against GFP, demonstrates that Vps39 accumulates specifically at the mitochondria-vacuole MCS that we termed vCLAMP. Distribution analysis of gold particles (n = 400): 64% vCLAMP; 15% vacuole; 10% mitochondria; 7% cytosol; 4% nucleus. V, vacuole; M, mitochondria.

Developmental Cell
paralogous enzyme Psd2 (Trotter and Voelker, 1995), the mitochondrial Psd1 is the major enzyme, and so a large lipid flux between the ER and mitochondria should exist. If indeed vCLAMP and ERMES are coregulated to sustain constant contact area between the endomembrane system and mitochondria, then this would explain the lack of phenotype on PtdEtn levels caused by losing ERMES alone (Kornmann et al., 2009; Nguyen et al., 2012; Voss et al., 2012). It would also predict that concomitant loss of both MCSs would result in a more dramatic decrease in the phospholipids that require such transport events. To measure this effect, we created a conditional strain that is deleted for vps39 and expresses MDM34 under the control of a repressible GalS promoter. We eliminated additional pathways for de novo PtdEtn synthesis by growing cells in synthetic media depleted for ethanolamine, so as to limit the Kennedy pathway (Daum et al., 1998), and by deleting the late endomembrane localized PtdSer decarboxylase, Psd2 (Trotter and Voelker, 1995). Under these conditions, the only existing pathway for de novo PtdEtn synthesis involves the mitochondrial Psd1 and the obligation of transporting ER synthesized PtdSer into mitochondria.

The phospholipid composition of the triple mutant compared to control cells was examined after growth for 24 hr in glucose (to repress GalSp-MDM34 expression), at which point the cells are still alive (Figure S4B) but mitochondrial shape is already affected (Figure S4C), indicating a depletion in Mdm34 levels. Thin-layer chromatography (TLC) of phospholipids extracted from whole cells (Figure 4A, independent quadruplicate quantified in Figure 4B) or enriched mitochondrial samples (Figure 4C, independent triplicate quantified in Figure 4D) demonstrated that the mutant has up to 40% decrease in the amounts of PtdEtn and cardiolipin (CL) (CL observed only in the enriched mitochondrial samples) concomitantly with near doubling in phosphatidylinositol (PtdIns) levels. Specifically, the decrease in PtdEtn could only be seen in this condition, whereas in the single mutants (Δvps39 or ΔGalSp-MDM34 in glucose; data not shown) or after short-term repression of GalSp-MDM34 in the triple mutant (Figures S4D and S4E), this reduction was not seen. This demonstrates that only the concomitant loss of both MCSs causes a halt in shuttling of PtdSer. More generally, PtdEtn, CL, and PtdIns are all synthesized from a common precursor, phosphatidic acid (PA); however, PtdEtn and CL are synthesized in mitochondria, whereas PtdIns is synthesized in the ER. Thus, accumulation of PtdIns and reduction of CL most probably also reflect a defect in PA transport from ER to mitochondria (Figure 4H).

In order to monitor the direct conversion of PtdSer to PtdEtn and, consequently, to PtdCho, we metabolically labeled the cells using 3H-serine. The radioactive serine was incorporated in the ER to PtdSer, and, following phospholipid extraction and separation by TLC, we could follow its fate in control versus mutant cells. In the mutant cells, where vCLAMP is absent and ERMES is gradually depleted, there is a massive accumulation of PtdSer

Figure 3. vCLAMP and ERMES Are in Dynamic Equilibrium
(A) In a strain deleted for mdm34, mitochondria (visualized by MTS-RFP) become large and spherical, and the GFP-Vps39 marked vCLAMP expands to surround mitochondria. Scale bars, 5 μm.
(B) GFP-Vps39 Δmdm34
(C) Deletion of each of the ERMES subunits yields a similar effect on vCLAMP dynamics.

See also Figure S3.
Figure 4. The vCLAMP Serves as a Route for Phospholipid Transport

(A) TLC of phospholipids extracted from whole cells of control (WT) and a mutant disrupted for both ERMES and vCLAMP. PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

(B) Quantitation of four biologically independent repeats of whole cell extracts. Bars represent means ± SD. The disruption of both ERMES and vCLAMP causes a reduction in PE levels, although PtdIns1 is present in mitochondria.

(C) TLC of phospholipids levels from mitochondrial (Mito) enriched fractions of control (WT) and a mutant disrupted for both ERMES and vCLAMP. Abbreviations for lipids are described in (A).

(D) Quantitation of three biologically independent repeats of mitochondrial enriched fractions. Bars represent means ± SD.

(E) TLC of phospholipids extracted from whole cells following a 2 hr pulse of 3H-serine to control (WT) and a mutant disrupted for both ERMES and vCLAMP. Abbreviations for lipids are described in (A).

(F) Quantitation of four biologically independent repeats. Bars represent means ± SD. Upon disruption of both vCLAMP and ERMES cells, phospholipid transport both into and out of mitochondria is impaired. As a result, high levels of de novo synthesized PtdSer accumulate in the ER, while very low levels of PtdCho are generated.

(G) Autoradiograph of TLC of phospholipids extracted from whole cells of control (WT) and a mutant strain (Dvps39, GalSp-MDM34, Δpsd2) after 24 hr growth in glucose-containing media. Cells were labeled with 3H-serine for indicated time points, followed by 1 mM cold L-serine addition and phospholipid extraction.

(H) Phospholipid biosynthesis pathways involve enzymes in both the ER and mitochondria, thus necessitating transport of substrates between both compartments. Etn, ethanolamine; Cho, choline; CDP-DAG, cytidine diphosphate diacylglycerol; DAG, diacylglycerol.

(I) A schematic model representing the tight coregulation of ERMES and vCLAMP. Loss of one causes an enlargement of the other, and loss of both is lethal. See also Figure S4.
(PtdSer content mutant/wild-type = 1.75), suggesting that as both contacts between mitochondria and the endomembrane system are gradually collapsing, more and more PtdSer, which cannot be transported to mitochondria, is accumulating in the ER (Figure 4E and independent quadruplicate quantified in Figure 4F, see also Figure 4G). Quantification of the various lipid species demonstrates a large difference in the amounts of de novo synthesized PtdCho. While PtdCho accumulates in control cells, it is hardly synthesized in the mutant. This difference probably reflects the inability of PtdEtn that was synthesized in the mitochondria to be transported back to the ER for further processing. Taken together, the results of steady-state and de novo phospholipid amounts demonstrate the role of both ERMES and vCLAMP in phospholipid transport. More importantly, they highlight how well coordinated these back-up pathways are, as only elimination of both yields a dramatic change in cellular phospholipid levels.

DISCUSSION

Functional cooperation between mitochondria and other organelles is essential. Since mitochondria is not connected to the endomembrane system through vesicular trafficking, the ERMES-mediated mitochondrial MCS serves as an important interorganellar interaction, allowing for fast and direct transport of phospholipids and Ca\(^{2+}\). We show here that, in yeast, mitochondria maintain a second MCS, vCLAMP, linking it to the endomembrane system through the vacuole. Since vacuoles are linked to the ER—both directly, through the nuclear vacuolar junction (NVJ), and indirectly, through vesicular traffic—this MCS can serve as a bypass for flow of information and nutrients between the ER and mitochondria. Indeed, we show that ERMES and vCLAMP are coregulated and that vCLAMP serves as a backup for ERMES, as only in the case where both are missing do yeast suffer from dramatic alternations in phospholipid levels and death.

Loss of both mitochondrial MCSs is lethal, and although a dramatic reduction in PtdEtn is observed when there is concomitant loss of ERMES and vCLAMP, it is possible that there exist additional essential functions for the two MCSs. In yeast, where the major ion stores (such as calcium, iron, and copper) reside in vacuoles, an additional essential function may be ion transport. Condition-specific direct traffic between endosomes and mitochondria in reticulocytes have been shown to facilitate iron transfer (Sheftel et al., 2007). Recently, it has been reported that mitochondria and melanosomes, pigment-synthesizing lysosome-like organelles, establish physical contacts. The proximities observed between both organelles correlated with melanosome biogenesis and maturation (Daniele et al., 2014). Hence, the need for sustaining a contact with later endomembrane organelles may be conserved from yeast to higher eukaryotes. More broadly, a better understanding of vCLAMP resident proteins must be obtained before the full extent of crosstalk between mitochondria and the endomembrane system can be determined.

Previously described MCSs have been shown to be dynamic and respond to the needs of the cell. For example, the NVJ enlarges upon entry to stationary growth phase (Pan et al., 2000), the formation of new plasma membrane-ER MCSs is induced upon Ca\(^{2+}\) store depletion (Wu et al., 2006), and a late endosome/lysosome-ER MCS forms solely under conditions of increased cholesterol levels (Du et al., 2011). vCLAMP is dynamic too, in response to both loss of ERMES and changes in carbon source (see Hönscher et al., 2014). It is this dynamic and condition-specific nature that can most probably account for the fact that the vCLAMP has remained elusive for so long.

The dynamic nature of vCLAMP and its apparent regulation through metabolic cues (see Hönscher et al., 2014) marks yet another link between intracellular organelle positioning, morphology, and degree of contact as determinants in the cell’s adaptation to changing metabolic requirements (Liesa and Shirihai, 2013) and cell fate (Cordás et al., 2006). To summarize, we demonstrate an MCS between vacuoles and mitochondria. This contact site, which we term vCLAMP, is in dynamic equilibrium with the ERMES mediated junction between mitochondria and the ER and works in parallel to enable the shuttling of small molecules (such as lipids) between the endomembrane system and mitochondria (model in Figure 4I). More generally, identification of the vCLAMP demonstrates the true complexity of interorganellar crosstalk: not only must an interface between two organelles be formed, but bypass systems must also be in place, and their size should be coregulated to serve the changing needs of the cell. Future efforts should be directed to elucidate the nature of the vCLAMP tether. Our proteomic analysis did not yield a definite answer as to how Vps39 is tethered to the mitochondria; whether through an adaptor protein or a yet-to-be-discovered mitochondrial partner. Future studies aimed at understanding the coregulation of the vCLAMP and ERMES should provide novel insights into what the cells sense to ensure optimal MCS surface area. Having visual markers and the genetic capacity to alter junction size should now give a platform for in depth investigations of this new cellular organization module.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

Strains created in this study are listed in a table in the Supplemental Experimental Procedures. All yeast strains in this study are based on the BY4741 laboratory strains (Brachmann et al., 1998). Genetic manipulations were performed using the Li-acetate, polyethylene glycol, single-stranded DNA method for transforming yeast strains (Gietz and Woods, 2006) using integration plasmids described elsewhere (Janke et al., 2004; Longtine et al., 1998).

For mitochondria staining, we used either an MTS-red fluorescent protein (RFP) plasmid (kindly provided by Jodi Nunnari) or an MTS-BFP plasmid (kindly provided by Christian Ungermann and Benedikt Westermann).

SGA and High-Content Screening

The SGA technique was used to efficiently introduce an ERMES marker (GFP-tagged Mdm34) into systematic yeast deletion and hypomorphic allele libraries (Breslow et al., 2008; Giaever et al., 2002). SGA was performed as described elsewhere (Cohen and Schuldiner, 2011; Tong and Boone, 2006; Tong et al., 2001). Microscopic screening was performed using an automated microscopy setup as described elsewhere (Cohen and Schuldiner, 2011). Further information is present in the Supplemental Experimental Procedures.

Manual Fluorescence Microscopy

Imaging was performed using an Olympus IX71 microscope controlled by the DeltaVision SoftWoRx 3.5.1 software with a 60× or 100× oil lens. Images were captured with a Photometrics CoolSnap HQ camera with excitation at 490/20 nm and emission at 528/38 nm (for GFP); excitation at 555/28 nm and emission at 617/73 nm (for mCherry/RFP); or excitation at 402 nm and emission at
Developmental Cell
A Dynamic Mitochondria/Vacuole Interface

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Electron Microscopy
For immunoelectron microscopy, cells were fixed in 4% paraformaldehyde with 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH, 7.4) for 1 hr at room temperature and kept at 4 °C during 1–2 days. The samples were soaked overnight in 2.3 M sucrose and rapidly frozen in liquid nitrogen. Frozen ultrathin (70–90 nm) sections were cut with a diamond knife at –120 °C on a Leica EM UC6 ultramicrotome. The sections were collected on 200-mesh formvar-coated nickel grids. Sections were blocked by a blocking solution containing 1% BSA, 0.1% glycine, 0.1% gelatin, and 1% Tween-20. Immunolabeling was performed using rabbit polyclonal anti-GFP antibody (ab6556, 1:100; Abcam) during 1.5–2 hr at room temperature followed by goat anti-rabbit immunoglobulin G coupled to 10 nm (or 15 nm) gold particles (1:20 dilution) for 30 min at room temperature. Contrasting and embedding were performed as described elsewhere (Tokuyasu, 1986). The embedded sections were scanned and digitally viewed on a Tecnai Spirit transmission electron microscope (FEI) at 120 kV using a CCD Eagle camera with TIA software (FEI).

Phospholipid Analysis
Phospholipids were extracted (Bligh and Dyer, 1959) from mitochondria-enriched fractions (Daum et al., 1982) or from whole-cell homogenates (Folch et al., 1957) and analyzed by TLC using chloroform:methanol:acetic acid:water (25:15:4:2, v/v/v) as the developing solvent. Lipids were visualized using copper sulfate (6.25 mM in 9.4 ml phosphoric acid) and acid:water (50:20:10:15:5, v/v/v/v/v) as the developing solvent. [3H]-labeled lipids were visualized using a phosphoric acid treatment. [3H]-labeled lipids were visualized using a phosphorimaging screen (Fuji) and quantified using ImageGauge Ver 4.0 software (FUJIFILM).

For measuring de novo phospholipid synthesis, we metabolically labeled cells with [1H-serine as follows: cells were grown to early logarithmic phase and pelleted, and 15 optical density units were resuspended in 25 ml synthetic media (S-dextrose) supplemented with 0.5 mM ethanolamine and 1 µM/ml myriocin. [1H-serine (1 mCi/ml) was then added to a final concentration of 2 µCi/ml. Cells continued growth at 30 °C until pulse was stopped by the addition of 1 mM cold L-serine at various time points (as indicated in the figures). Phospholipids were extracted (Folch et al., 1957) and separated by TLC using chloroform:methanol:acetic acid:water (50:20:10:15, v/v/v/v) as the developing solvent. [1H]-labeled lipids were visualized using a phosphorimaging screen (Fuji) and quantified using ImageGauge Ver 4.0 software (FUJIFILM).

Interaction Proteomics
Pull-down of GFP-Vps39 was performed from mitochondria-enriched preparations followed by liquid chromatography-tandem mass spectrometry analysis. Detailed information is present in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.06.007.

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Developmental Cell

A Dynamic Mitochondria/Vacuole Interface


