Published in final edited form as:

Methods Mol Biol. 2019 ; 1949: 213–235. doi:10.1007/978-1-4939-9136-5_16.

Determining the Lipid-Binding Specificity of SMP Domains: An ERMES Subunit as a Case Study

Andrew P. AhYoung and Pascal F. Egea

Abstract

Membrane contact sites between the endoplasmic reticulum (ER) and mitochondria function as a central hub for the exchange of phospholipids and calcium. The yeast Endoplasmic Reticulum–Mitochondrion Encounter Structure (ERMES) complex is composed of five subunits that tether the ER and mitochondria. Three ERMES subunits (i.e., Mdm12, Mmm1, and Mdm34) contain the synaptotagmin-like mitochondrial lipid-binding protein (SMP) domain. The SMP domain belongs to the tubular lipid-binding protein (TULIP) superfamily, which consists of ubiquitous lipid scavenging and transfer proteins. Herein, we describe the methods for expression and purification of recombinant Mdm12, a bona fide SMP-containing protein, together with the subsequent identification of its bound phospholipids by high-performance thin-layer chromatography (HPTLC) and the characterization of its lipid exchange and transfer functions using lipid displacement and liposome flotation *in vitro* assays with liposomes as model biological membranes. These methods can be applied to the study and characterization of novel lipid-binding and lipid-transfer proteins.

Keywords

ERMES; Mdm12; SMP domain; Lipid-transfer protein; Phospholipid; Membrane contact sites; Liposome; HPTLC; Lipid displacement; Liposome flotation

1 Introduction

Membrane contact sites (MCSs) are regions of close apposition between two organelles [1, 2] and involve the ER and a second organelle, such as the mitochondrion or the plasma membrane (PM). Contact sites between the ER and mitochondria, also known as mitochondria-associated membranes (MAMs), are highly conserved from yeast to humans [3, 4], where they function in the non-vesicular exchange of cellular signals, such as calcium and lipids [5]. In humans, dysfunctional MAMs are associated with a broad range of diseases, including Alzheimer's, Parkinson's, obesity, and type 2 diabetes [6, 7]. Organelle-tethering protein complexes are required for the establishment and maintenance of MCSs. For example, the Endoplasmic Reticulum–Mitochondrion Encounter Structure (ERMES) complex physically bridges the ER and mitochondria in yeast [8, 9]. ERMES is composed of five subunits—the ER-anchored Mmm1, the outer-mitochondrial membrane proteins Mdm10, Mdm34, and Gem1, and the cytosolic protein Mdm12 (Fig. 1a).

A unique feature of ERMES is the presence of the lipid-binding module—SMP domain—in Mdm12, Mmm1, and Mdm34. The SMP domain functions as a scaffold that mediates the

assembly of ERMES, and a likely conduit for the transport of lipids between the ER and mitochondria [3, 4] (Fig. 1a). The tubular shape structure of the SMP domain is a common feature shared by members of the tubular lipid-binding protein (TULIP) superfamily [10–12]. SMP TULIP proteins belong to the broad functional class of lipid-transfer proteins. While TULIP proteins carry out a wide range of functions, SMP domains are exclusively associated with membrane contact sites [13–15]. Initially, no functional ortholog for any ERMES subunit had been identified in metazoans. However, the human ER-anchored protein PDZD8 was recently identified as an SMP-containing protein that is functionally orthologous to the ERMES subunit Mmm1 [16]. The SMP fold was first described in the crystal structure of the human Extended synaptotagmin-2 (E-SYT2) [17]. This was followed by several crystal structures of fungal Mdm12 [18–20] and Mmm1 [21] SMP domains; all revealed an elongated tube-shaped domain composed of two α helices capping an antiparallel β -barrel composed of five twisted strands (Fig. 1b). Notably, nearly all structures identified a bound phospholipid, whereby the two fatty acyl chains of the lipid are buried in the central hydrophobic cavity of the SMP domain, and the polar head group is exposed to the surface highlighting the presence of a lateral opening or seam (Fig. 1b, c). The recombinant proteins used to solve these crystal structures were overexpressed in bacteria and co-purified with tightly associated bacterial phospholipids that were clearly resolved in electron density maps. These observations validated the lipid-binding nature of the SMP domain, and supported a role for SMP-containing proteins in phospholipid binding and transfer between organelles at MCSs.

We describe herein a general approach for characterizing phospholipid binding and transfer by the SMP-containing protein Mdm12 (Fig. 2). We first detail the overexpression and purification of yeast Mdm12 overexpressed in bacteria or yeast, and the subsequent identification of co-purified phospholipids by HPTLC [22]. Next, we describe an in vitro lipid displacement assay using a fluorescently labeled phospholipid probe, based on previous work with E-SYT2 [17], to study the phospholipid-binding specificity of Mdm12 [23]. Finally, we describe liposome-based assays [24–27] to study the process of SMP-mediated phospholipid exchange between membranes (i.e., phospholipid extraction from donor membrane and transfer to acceptor membrane). Similar approaches have been used to characterize lipid-binding properties and functions of several lipid-transfer proteins, including the SMP domain of E-SYT2 [17, 28] at ER-PM contact sites, the UPS1-Mdm35 shuttle between outer and inner mitochondrial membranes [29, 30], and the oxysterol-binding proteins Osh6/Osh7 [31] and OSBP [32] at ER-PM and ER-Golgi junctions, respectively.

2 Materials

2.1 Protein Purification from Bacteria and Yeast

All stock solutions and purification buffers are prepared using ultrapure water (18.2 M Ω .cm at 25 °C) sterilized by autoclaving. To prevent microbial growth buffers are supplemented at a final concentration of 0.01% sodium azide (using a 10% (w/v) stock solution).

1. Bacterial cell pellets corresponding to 6 L of *E. coli* C43(DE3) cells expressing histidine-tagged yeast Mdm12 (plasmid pCDF-*Sce* Mdm12) grown in 2 $\times$ LB

medium supplemented with streptomycin [23]. Cells were grown at 37 °C until cultures reached an OD₆₀₀ of 0.4–0.6; after lowering the temperature to 25 °C, protein expression was induced by adding IPTG at 0.8 mM (final concentration) and growing cells for 5 h at 25 °C.

2. Yeast cell pellets corresponding to 9 L of yeast cells constitutively expressing histidine-tagged yeast Mdm12 (plasmid pY23gal10gpd-*Sce* Mdm12) grown in complete synthetic selective medium with glucose and minus histidine for 24 h at 28 °C [23]. Plasmids are available upon request.
3. Emulsiflex C3 cell homogenizer (Avestin).
4. Bead Beater with a 350 mL capacity stainless steel chamber (BioSpec).
5. 0.5 mm sterilized glass beads (BioSpec). Sterilize beads by autoclaving and store at –20 °C.
6. HisPur Cobalt and Nickel tube (BioRad).
7. EconoPac 10DG Desalting Prepacked resins (ThermoFisher).
8. Econo-Column glass chromatographic tube for gravity flow chromatography (BioRad).
9. Thrombin (EM Millipore) resuspended at 0.5 units/μL in a storage buffer composed of 20 mM HEPES, pH 7, 150 mM NaCl, and 40% (v/v) glycerol. Thrombin aliquots are stored at –20 °C.
10. CaptoQ HiTrap columns 1 mL and 5 mL (GE Healthcare).
11. HisTrap column 1 mL (GE Healthcare).
12. Superdex 200 Increase GL10/300 column (GE Healthcare).
13. ÄKTA Purifier chromatographic workstation (GE Healthcare).
14. Amicon Ultra-4 and Ultra-15 centrifugal filter units with 30 kDa molecular weight cutoff (EMD Millipore).
15. Tabletop refrigerated centrifuge for protein concentration by ultrafiltration.
16. AccuRuler RGB protein ladder (BioPioneer).
17. Tabletop eppendorf centrifuge and eppendorf 1.5 mL micro-centrifuge tubes.
18. Lysis buffer: 500 mM NaCl, 40 mM Tris–HCl, pH 7.8, 10% (v/v) glycerol, 0.2 mM Phenyl methylenesulfonyl fluoride (PMSF), 2.8 mM β-mercaptoethanol (βME). Add 1 tablet of protease inhibitor cocktail (Pierce) per 100 mL of buffer (for purification of protein expressed in yeast).
19. Co-IMAC Buffer A: 12.5 mM imidazole–HCl, pH 8, 500 mM NaCl, 20 mM Tris–HCl, pH 7.8, 10% (v/v) glycerol, 0.2 mM PMSF, 1.4 mM βME. To prepare this buffer and other IMAC buffers (*see below*), a stock solution of imidazole–HCl 2 M, pH 8 and a stock solution of Tris–HCl 2 M, pH 7.8, are used to reach

the indicated concentrations. Following mixing of all components, the pH is not adjusted.

20. Co-IMAC Buffer B: 125 mM imidazole-HCl, pH 8, 500 mM NaCl, 20 mM Tris-HCl, pH 7.8, 10% (v/v) glycerol, 0.2 mM PMSF, 1.4 mM β ME.
21. Ni-IMAC Buffer A: 25 mM imidazole-HCl, pH 8, 500 mM NaCl, 20 mM Tris-HCl, pH 7.8, 10% (v/v) glycerol, 0.2 mM PMSF, 2.8 mM β ME.
22. Ni-IMAC Buffer B: 250 mM imidazole-HCl, pH 8, 500 mM NaCl, 20 mM Tris-HCl, pH 7.8, 10% (v/v) glycerol, 0.2 mM PMSF, 2.8 mM β ME.
23. PD10D buffer: 100 mM NaCl, 20 mM Tris-HCl, pH 7.8, 2% (v/v) glycerol, 0.2 mM PMSF, 1.4 mM β ME.
24. AIE buffer A: 25 mM NaCl, 20 mM Tris-HCl, pH 7.8, 2% (v/v) glycerol, 0.2 mM PMSF, 1.4 mM β ME.
25. AIE buffer B: 1 M NaCl, 20 mM Tris-HCl, pH 7.8, 2% (v/v) glycerol, 0.2 mM PMSF, 1.4 mM β ME.
26. Size exclusion chromatography (SEC) buffer: 150 mM NaCl, 20 mM Tris-HCl, pH 7.8, 2% (v/v) glycerol, 0.2 mM PMSF, 0.15 mM Tris(2-carboxyethyl)phosphine (TCEP).
27. X-cell SureLock mini-cell vertical protein electrophoresis system (Life Technologies).
28. 4–20% ExpressPlus SDS-PAGE gels (Genscript) and Tris-MOPS-SDS running buffer (Genscript).
29. 4 $\times$ LDS sample buffer (Life Technologies).
30. Coomassie blue gel staining solution: 0.25% (w/v) filtered solution of Coomassie brilliant blue (R250) dissolved in a mixture of water/ethanol/glacial acetic acid (50:40:10, v/v/v).
31. Destaining solution: Water/ethanol/glacial acetic acid (50:40:10, v/v/v).

2.2 Lipid Extraction and Analysis by High-Performance Thin-Layer Chromatography (HPTLC)

All solvents are of HPLC grade

1. Lipid extraction organic reagent: Chloroform/methanol (1:2, v/v).
2. Lipid extraction acidic aqueous reagent: 0.5% (v/v) acetic acid in 500 mM NaCl.
3. Phospholipids (Avanti Polar Lipids) dried under a nitrogen gas stream and resuspended in methanol at a final concentration of 10 mM: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE); 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS); 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC); L- α -phosphatidic acid (PA); L- α -phosphatidylglycerol (PG); liver L- α -

phosphatidylinositol (PI); cardiolipin (CL); Yeast total lipid extract; Yeast polar lipid extract; *E. coli* total lipid extract.

4. Glass Pasteur pipette.
5. Compressed nitrogen gas tank.
6. HPTLC Silica gel 60 glass plates 10 × 10 cm (Merck EMD Millipore).
7. Glass tanks for HPTLC (two distinct tanks).
8. Oven.
9. Tabletop eppendorf centrifuge and 1.5 mL microcentrifuge tubes.
10. Lyophilizer.
11. Chemical hood.
12. Regular Imaging scanner.
13. Phase A: Dichloromethane/ethyl-acetate/acetone (80:16:4, v/v/v).
14. Phase B: Chloroform/acetone/isopropanol/ethyl-acetate/ethanol/methanol/water/acetic acid (30:6:6:6:16:28:6:2, v/v/v/v/v/v/v).
15. Charring reagent: 10% (w/v) solution of CuSO₄ in an 8% aqueous solution of phosphoric acid.

2.3 Lipid-Displacement Assay

2.3.1 NBD-PE Loading and Displacement Assay

1. 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE, Avanti Polar Lipids). Prepare a stock solution at 2.5 mM in methanol.
2. Slide-A-lyzer mini-dialyzing units with 20 kDa molecular weight cutoff (ThermoFisher).
3. Displacement buffer: 250 mM NaCl, 10 mM HEPES-NaOH, pH 7.5, 0.2 mM Lauryl-Dimethyl-Amine-*N*-Oxide (LDAO, Anatrace).
4. Phospholipids standard at 10 mM in methanol (see Subheading 2.2).

2.3.2 BN-PAGE Analysis of Displacement

1. 4–16% Native-PAGE Bis-Tris gel (Life Technologies).
2. 4 × Native-PAGE sample buffer (Life Technologies).
3. NativeMark unstained protein standards (Life Technologies).
4. 20 × cathode buffer additive solution (1% G-250 Coomassie blue solution, Life Technologies).
5. Fluorescence Imaging Scanner Typhoon 9400 Variable Mode Imager (GE Healthcare).

6. 20 × stock BN-PAGE buffer: Weigh 41.8 g of Bis-Tris (Fisher) and 35.8 g of Tricine (Fisher). Dissolve in sterile deionized water and adjust to a final volume of 200 mL. Filter but do not adjust the pH.
7. Destaining solution and Coomassie blue gel staining solution (as described in Subheading 2.1).

2.4 Liposome-Based Assays

2.4.1 Liposome Exchange Assay

1. Mini Extruder with 1 mL syringes set and 400 nm pore size polycarbonate membranes (Avanti Polar Lipids).
2. Nickel IMAC-NTA spin columns (Qiagen).
3. Liposome reconstitution/extrusion and exchange buffer: 150 mM NaCl, 20 mM Tris-HCl, pH 7.8.
4. Ni-spinIMAC buffer A: 25 mM imidazole-HCl, pH 8, 300 mM NaCl, 20 mM Tris-HCl, pH 7.8.
5. Ni-spinIMAC buffer B: 500 mM imidazole-HCl, pH 8, 300 mM NaCl, 20 mM Tris-HCl, pH 7.8.

2.4.2 Liposome Flotation Assay

1. Ultracentrifugation polycarbonate tubes 11 mm diameter × 32 mm height (Beckman-Coulter).
2. TLS55 swinging bucket ultracentrifuge rotor (Beckman-Coulter).
3. Optima TLX refrigerated ultracentrifuge (Beckman-Coulter).
4. Flotation buffer: 150 mM NaCl, 20 mM Tris-HCl, pH 7.8, 2% (v/v) glycerol.
5. Sucrose stock solutions: 10, 20, and 60% (w/v) sucrose in flotation buffer.
6. Nycodenz (Accurate Chemical) stock solutions: 30 and 80% (w/v) Nycodenz in flotation buffer.
7. 100-nm diameter liposomes composed of DOPC/cholesterol/Rhodamine-DHPE at 54:45:1 molar ratio. Concentration of Rh-DHPE 0.5 mM (FormuMax Scientific Inc.).
8. 100-nm diameter plain pure DOPC liposomes (FormuMax Scientific Inc.).
9. 1420 multi label counter/plate reader (absorbance and fluorescence) measurements (PerkinElmer) or similar.
10. Half-area black flat bottom 96-well assay polystyrene plates (Corning).

3 Methods

3.1 Mdm12 Protein Purification from Heterologous Expression in *E. coli*

3.1.1 Cell Extract Preparation

1. Bacterial cell pellets (6 L culture) are resuspended in 100 mL of lysis buffer.
2. Resuspended cells are disrupted by passing them through an Expression in *E. coli* Emulsiflex C3 pressurized at 15,000 psi. Four to five passes are sufficient to lyse cells.
3. Centrifuge the cell lysate at $25,000 \times g$ for 1 h at 4 °C.
4. Recover the supernatant (soluble extract).

3.1.2 Cobalt Affinity

1. Use 10 mL of HisPur Cobalt resin to pour a gravity-packed column (final bed volume 5 mL).
2. Apply soluble extract from Subheading 3.1.1, step 4.
3. Wash the column with 300 mL of Co-IMAC buffer A and then elute Mdm12 with 35 mL of Co-IMAC buffer B.
4. Concentrate the protein pool by ultrafiltration to a final volume of 3 mL on an Ultra-15 centrifugal filtration membranes.

3.1.3 Desalting and Histidine Tag Cleavage

1. Equilibrate a PD10 desalting column in PD10D buffer.
2. Load concentrated and desalt/elute using 4 mL of PD10D buffer.
3. If the histidine purification tag needs to be cleaved (i.e., crystallization, liposome binding assay), add 25 μ L of thrombin and incubate for 12 h at room temperature before proceeding to the next step (see Note 1).

3.1.4 AIEX Chromatography

1. Dilute the cleaved (or not) desalted protein with 8 mL of AIEX buffer A.
2. Equilibrate a 5 mL Capto Q HiTrap column in AIEX buffer A.
3. Run the column at 1 mL/min flow rate and apply a gradient ranging from 0 to 100% AIEX buffer B in 100 mL while collecting 2 mL fractions (Fig. 3a).
4. Analyze fractions by SDS-PAGE. Electrophoresis fractions are prepared in 1.5 mL eppendorf tubes by adding 10 μ L of 4 $\times$ LDS buffer to 30 μ L of each of the column fractions to be analyzed. Samples are heat-denatured for 5–10 min in a boiling water bath before being briefly centrifuged ($3000 \times g$ for 30 s) to bring down the condensate.

5. Install a 4–20% ExpressPlus SDS-PAGE gel in a X-cell Sure-Lock mini-cell gel apparatus after gently removing its 15-well comb. The upper and lower buffer chambers are filled with 1×Tris-MOPS-SDS running buffer.
6. Load samples on the gel: 7 µL of AccuRuler RGB protein ladder or 20 µL of denatured protein sample fractions.
7. Gel is run at a constant voltage of 150 V and stained then destained using Coomassie blue gel staining and destaining solutions, respectively.
8. Concentrate the pooled protein fractions by ultrafiltration on an Ultra-4 centrifugal filtration membranes.

3.1.5 SEC Chromatography

1. Equilibrate a Superdex 200 increase 10/300 column in SEC buffer.
2. Inject 500 µL of concentrated protein.
3. Run the column at 0.5 mL/min while collecting 0.5 mL fractions (Fig. 3a).
4. Analyze fractions by SDS-PAGE.
5. Concentrate eluted protein to a final concentration of 18 mg/mL on an Ultra-4 centrifugal filter unit.
6. Final yields are around ~10 mg/L of culture. Mdm12 is essentially monomeric (Fig. 3b) and the amounts of protein obtained are sufficient to perform the lipid identification by HPTLC (but also by MS) described in Subheading 3.3. Yeast Mdm12 overexpressed and purified from bacterial cultures is also suited for crystallization trials [19].

3.2 Mdm12 Purification from Homologous Expression in *S. cerevisiae*

3.2.1 Cell Extracts Preparation

1. Yeast cell pellets (9 L culture) are resuspended in a final volume of 225 mL of lysis buffer and transferred in the beadbeater stainless steel chamber.
2. Add 225 mL of cold and dry-autoclaved glass beads. Assemble the bead beater. Fill the cooling jacket with ice.
3. Disrupt cells with 45 s of homogenizing followed by 2 min 15 s of pausing/cooling. Repeat this four times (*see* Note 2).
4. Decant beads and recover the lysate.
5. Wash beads thoroughly with 75 mL of lysis buffer and add this wash to the lysate.
6. Centrifuge the cell lysate at $25,000 \times g$ for 90 min at 4 °C.
7. Recover the supernatant (soluble extract).

3.2.2 Nickel Affinity Chromatography—For protein purified from yeast cells, batch binding is necessary.

1. Add 30 mL of washed HisPur Nickel resin (final bed volume 15 mL) to the 300 mL of soluble extract from Subheading 3.2.1, step 2.
2. Agitate gently at 4 °C for 1.5 h.
3. Pour the resin-extract into a chromatographic tube and let resin settle by gravity while enabling the extract to flow-through. We pass the whole volume of flow-through a second time.
4. Wash column with 500 mL of Ni-IMAC buffer A, then elute Mdm12 with 75 mL of Ni-IMAC buffer B.
5. Concentrate the protein pool by ultrafiltration to a final volume of 1 mL on an Ultra-15 centrifugal filter unit.

3.2.3 AIEX Chromatography

1. Dilute the protein with 9 mL of AIEX buffer A.
2. Equilibrate a 1 mL Capto Q HiTrap column in AIEX buffer A.
3. Run the column at 0.5 mL/min flow rate and apply a gradient ranging from 0 to 100% AIEX buffer B in 20 mL.
4. Collect 0.5 mL fractions.
5. Analyze fractions by SDS-PAGE.
6. Concentrate the pooled protein fractions by ultrafiltration on an Ultra-4 centrifugal filter unit.

3.2.4 Ni Affinity Chromatography

1. Concentrated protein is immediately injected onto a 1 mL HisTrap column equilibrated in Ni-IMAC buffer A. Column is run at 0.5 mL/min while collecting 0.5 mL fractions. The column is washed with 10 mL of Ni-IMAC buffer A and protein eluted with 10 mL of Ni-IMAC buffer B.
2. Analyze fractions by SDS-PAGE.
3. Concentrate the pooled protein fractions by ultrafiltration to a final volume of 500 µL on an Ultra-4 centrifugal filter unit.

3.2.5 SEC Chromatography

1. Equilibrate a Superdex 200 increase 10/300 column in SEC buffer.
2. Inject 500 µL of concentrated protein. Run the column at 0.5 mL/min while collecting 0.5 mL fractions.
3. Analyze fractions by SDS-PAGE.

4. Concentrate eluted protein to a final concentration of 10 mg/mL on an Ultra-4 centrifugal filter unit (*see* Note 1).
5. Final yields are around ~0.2 mg/L of culture. Mdm12 is essentially monomeric (Fig. 3b) and the amounts of protein obtained are sufficient to perform the lipid identification by HPTLC (but also by MS) described in Subheading 3.3.

3.3 Lipid Extraction and Identification

In this section we describe a general procedure for the extraction and characterization of phospholipids bound to lipid-transfer proteins (in this case the SMP domain of Mdm12) purified using the protocols described in Subheadings 3.1 and 3.2. Bound lipids are extracted using a modified procedure of the Bligh and Dyer lipid extraction method [33, 34] and then separated by HPTLC [35, 36]. HPTLC is a simple, rapid, and inexpensive method to separate and identify lipids; furthermore depending upon the protocol used to reveal the plate (charring reagent) one can achieve excellent sensitivity (5–15 ng of phospholipids can be detected). Although the protocol described herein is suited for phospholipids, it can be modified (i.e., choice of separation phases) depending on the class of lipids that needs to be resolved and identified by HPTLC [22].

3.3.1 Lipid Extraction—A volume of 50 μ L of Mdm12 purified from bacterial or yeast cell, as described in Subheadings 3.1 and 3.2, are concentrated to ~10 mg/mL (~0.33 mM) and used to extract an amount of bound phospholipids sufficient for their detection and identification by HPTLC.

1. Pipet 50 μ L of purified Mdm12 in a 1.5 mL Eppendorf tube.
2. Add 3.75 volumes (187.5 μ L) of lipid extraction organic reagent. Vortex vigorously for 1 min.
3. Add 1.25 volumes (62.5 μ L) of chloroform. Vortex vigorously for 1 min.
4. Add 1.25 volumes (62.5 μ L) of lipid extraction acidic aqueous reagent. Vortex vigorously for 1 min.
5. Centrifuge in a tabletop centrifuge at $14,000 \times g$ at room temperature for 15 min.
6. Recover the bottom (organic) layer, preferably with a glass Pasteur pipette, and transfer it in an Eppendorf tube.
7. Lyophilize the organic layer and resuspend the extracted lipids in 30 μ L of methanol. This sample can be used for analysis by HPTLC (*see* Subheading 3.3.2) or MS.

3.3.2 HPTLC Separation and Identification of Lipids

1. Fill the two tanks with the respective solvent mixtures (phase A or phase B) to saturate each chamber. This should be done for at least 1 h before performing the actual separation in order to ensure good saturation. Use enough phase so as the level of solvent is just about 2–3 mm below the loading line as indicated in **step 2** and shown in Fig. 4.

2. Using a pencil, draw a faint line 1.5 cm from the bottom edge of the silica plate to visualize the positions where samples will be applied. Draw a second line 0.5 cm from the top edge to mark the upper limit of solvent migration (migration front). On the bottom line, mark eight equidistant spots to indicate where to load each sample (i.e., lipid standard or lipid extract). We recommend loading no more than eight samples per plate. Do not scratch the silica surface. Wear gloves and avoid touching the surface of the silica.
3. Wet the silica plate with methanol and allow it to dry. Bake the plate in the oven at 110 °C for 15 min. This removes any traces of water and organic contaminants adsorbed on the silica. Allow plate to cool.
4. Apply each sample (30 µL of extracted lipid from Subheading 3.3.1 or lipid standard dissolved in methanol) slightly above the positions marked with the pencil.
5. Transfer silica plate in the glass tank saturated with phase A and allow phase to develop throughout the plate. Track the flow of solvent by capillary action from the bottom to the top of plate.
6. Once the solvent reaches the top, immediately remove plate, place it on a flat surface, and let it dry completely in fume hood (this is critical to ensure the phospholipids do not continue to migrate).
7. Transfer silica plate in the glass tank saturated with phase B and allow phase to develop throughout the plate as described in the previous step.
8. Upon completion, dry the silica plate completely and then spray with the charring reagent.
9. Gently tap the silica-side with a paper towel and wipe the back of plate to remove excess charring reagent.
10. Heat the plate 145 °C for at least 5 min, or until the desired signal intensity is achieved (this can take up to 8 min).
11. Once developed, remove plate from oven and allow to cool. The plate can be imaged with a scanner to quantify band intensities. It is recommended to scan the plate immediately after charring.
12. The following HPTLC experiment indicated that Mdm12 purified from bacteria was bound to PE and PG, whereas Mdm12 purified from yeast was bound to PC (Fig. 4). PE and PG phospholipids are abundant in bacterial membranes, while PC is abundant in yeast membranes but absent in bacterial membranes.

3.4 Lipid Displacement Assay

Phospholipids can be labeled with fluorescent dyes on their polar head group or their fatty acid group(s) (see Note 3). We used PE labeled with NBD (NBD-PE) on its polar head group to perform the assay described in this section. Mdm12 is first preloaded with NBD-PE, and then incubated with different phospholipids to determine which phospholipid is capable of displacing NBD-PE (see Fig. 5). Preloading and displacement are performed in

the presence of the detergent LDAO used at 0.2 mM (~twice its critical micellar concentration) to ensure the presence of mixed micelles (detergent/NBD-PE or detergent/competitor phospholipid) that optimize the reactions (see Note 4).

3.4.1 Fluorescent-Phospholipid Preloading

1. Dilute 100 μ L of purified Mdm12 (18 mg/mL, see Subheadings 3.1 and 3.2) ten times by adding 900 μ L of displacement buffer (final concentration 1.8 mg/mL, ~55 μ M).
2. Incubate 200 μ L of diluted Mdm12 (at 1.8 mg/mL) with 10 μ L of NBD-PE stock solution (2.5 mM) for 2 h on ice: this corresponds to a 2.1 fold molar excess of NBD-PE over Mdm12.
3. Excess of unbound NBD-PE is removed by dialyzing the reaction mix in a Slide-A-lyzer mini-dialyzing unit against 1 L of displacement buffer for 1 h at 4 °C.
4. Efficiency of preloading is assessed by BN-PAGE as described in Subheading 3.4.3 where two samples are run: Mdm12 before incubation with NBD-PE and Mdm12 preloaded with NBD-PE after dialysis (Fig. 5a) (see Note 5).

3.4.2 Displacement Assay

1. Each displacement reaction has a total volume of 20 μ L. Mix 10 μ L of Mdm12 preloaded with NBD-PE (1.7 mg/mL of protein) with 9 μ L of displacement buffer and 1 μ L of 10 mM competitor phospholipid dissolved in methanol (seven different phospholipids are tested: PG, PA, PC, PE, PS, PI, and CL). A reference control reaction—lipid-less/methanol only—must also be performed using 1 μ L of pure methanol only instead of competitor PL dissolved in methanol.
2. Incubate on ice for 1 h.
3. Analyze each reaction mix by BN-PAGE as described in Sub-heading 3.4.3. Under these conditions the protein-to-lipid ratio is about 1:18 (~28 μ M:0.5 mM) and displacement/competition occurs in the presence of the detergent LDAO (as in the case of the preloading) that solubilizes the competitor PL.

3.4.3 Analysis of Lipid Displacement by Blue-Native PAGE—Following the loading of Mdm12 SMP domain with the NBD-PE fluorescent lipidic probe described in Subheading 3.4.1 and the lipid displacement/competition reaction described in Subheading 3.4.2, results can be analyzed by native polyacrylamide gel electrophoresis (N-PAGE). Protein and protein complexes can be analyzed by clear native PAGE (CN-PAGE) [37] or blue native PAGE (BN-PAGE) [38]. In the case of yeast Mdm12, BN-PAGE works particularly well.

1. Protein samples from BN-PAGE analysis are prepared by adding 15 μ L of each displacement assay reaction from Subheading 3.4.2, **step 3** to 5 μ L of 4 Native PAGE sample buffer. 2. Assemble a 4–16% Native-PAGE Bis-Tris gel in a X-cell Sure-Lock mini-Cell electrophoresis tank. Remove the 10-well comb carefully.

2. Fill up the lower (outside/anode) chamber with exactly 600 mL of anode buffer prepared with 30 mL of 20 stock BN-PAGE and 570 mL of cold sterile deionized water.
3. Load samples 10 μ L (~6.5 μ g of protein per lane). Load 5 μ L for NativeMark unstained protein standards.
4. Fill up the upper (inside/cathode) chamber with exactly 200 mL of cathode buffer consisting of 10 mL of 20 stock BN-PAGE and 1 mL of 20 cathode buffer additive solution diluted to a final volume of 200 mL with cold sterile deionized water.
5. Run the gel at 4 °C (cold room) at a constant voltage of 150 V for 60 min then set up to a constant voltage to 250 V for the remainder of the run (30–90 min, usually ~50–60 min). Observe the migration front materialized by a sharp and intensely colored blue band.
6. Detect and quantify the fluorescence signal (excitation wavelength at 460 nm, emission wavelength at 535 nm) due to the presence of NBD-PE bound to Mdm12 using a Typhoon 9400 Variable Mode Imager.
7. Following the NBD-PE fluorescence scan, stain and fix the gel with Coomassie blue gel-staining solution. Destain as required with gel-destaining solution. Detect and quantify the total Mdm12 protein signal using a Typhoon 9400 Variable Mode Imager (*see Note 6*).
8. Percentages of displacement are determined by comparing the remaining fluorescence in the presence of a given competitor phospholipid to the control reaction (NBD-PE-preloaded protein incubated with methanol only). Fluorescence signal from NBD-PE is normalized to the total protein signal extrapolated from the Coomassie blue stained gel (Fig. 5b) (*see Note 7*). Based on our assay, Mdm12 seems to preferentially bind PG, PC, and PE over PI and PS.

3.5 Liposome-Based Assays

These series of assays uses liposomes as systems to mimic biological membranes (*see Note 8*). Interaction between the SMP domains and biological membranes can be detected and quantified using liposome flotation assays [25, 26]. Using these assays, we can demonstrate that ERMES SMP domains (1) can interact with biological membranes and (2) can extract and transfer glycerophospholipids from and between biological membranes. Liposomes can be prepared with lipid extracts from different sources such as total lipid from *E. coli* or yeast (*S. cerevisiae*) or with purified lipids to control their exact compositions [24].

3.5.1 Liposome Exchange Assay—Mdm12 is incubated with liposomes; its content in phospholipids is analyzed by HPTLC to assess its ability to extract phospholipids from biological membranes and its binding specificity (Fig. 6).

1. Yeast total lipid extract (7.5 mg) or yeast polar lipid extract (5 mg) are dissolved in 1 mL of chloroform and gently dried under a stream of nitrogen gas to form a lipid film.
2. The lipid films are resuspended in 800 μ L of liposome exchange buffer.
3. Liposome (400-nm diameter in size) are formed by extrusion through a polycarbonate membrane (400-nm pore size) to reach a total lipid concentration of ~9.4 mg/mL (equivalent to ~2.3 mM in PC) and 6.25 mg/mL (equivalent to ~2.4 mM in PC), respectively; we assume that PC accounts for about 29% in weight of the total amount of lipids present in these commercial extracts (Avanti Polar Lipids specifications) (*see* Note 9).
4. Incubate 75 μ L of Mdm12-His (purified from *E. coli* and concentrated at 15 mg/mL, ~0.5 mM) with 75 μ L of liposome suspension at room temperature for 1 h (*see* Note 1). Control reactions consisting of liposomes incubated with buffer only (no protein) or protein only (no liposomes) are performed and analyzed in parallel.
5. Mdm12-His is then purified on Ni IMAC-NTA spin columns (50 μ L resin bed). After binding, each column is washed three times with 500 μ L of Ni-spinIMAC buffer A to remove unbound lipids and liposomes.
6. Columns are spun at $100 \times g$ for 1 min.
7. Mdm12-His is then eluted with 400 μ L of Ni-spinIMAC buffer B (Fig. 6b) (*see* Note 10).
8. Total lipids present in the final elution are extracted as described in Subheading 3.3.1 and analyzed by HPTLC as described in Subheading 3.3.2. Yeast total polar lipid extract contains about 31% PC, 26% PI, 12% PE, 8% PS, 2% PA, and 1% PG. Upon incubation with liposomes, Mdm12 exchanges nearly all of its endogenously bound PLs (PG and PE in *E. coli*) with PC and also PI extracted from the liposomes (Fig. 6b). This agrees with the results of the lipid displacement assay.

3.5.2 Liposome Flotation Assay—Liposomes labeled with a fluorescent phospholipid (NBD-PE or Rhodamine-PE) are incubated with Mdm12. The reaction is then overlaid with a noncontinuous gradient of Nycodenz or sucrose solutions and a layer of buffer on top. During isopycnic density gradient centrifugation, liposomes migrate (float) to the top buffer layer, while the protein will remain in the lower fractions of the gradient (bottom). However, if the protein binds to the liposomes, it will also cosediment in the top buffer layer with the liposomes. Such flotation assay can be used to study the reverse reaction by incubating Mdm12 preloaded with the fluorescent phospholipid NBD-PE (as described in Subheading 3.4.1) and monitoring the transfer of the fluorescent probe into unlabeled liposomes by isopycnic density gradient centrifugation (Fig. 7).

1. Prepare two types of reactions on ice. For the reaction 1 (Mdm12 extracts a fluorescent lipid probe from liposomes), mix 50 μ L of Mdm12 (50 μ M) with 50 μ L of DOPC/cholesterol/Rhodamine-DHPE liposomes (5 mM). For the reaction

2 (Mdm12 transfers the fluorescent lipid probe into liposomes), mix 50 μ L of NBD-PE-preloaded Mdm12 (50 μ M) with 50 μ L of DOPC liposomes (5 mM). Whether the extraction or transfer reactions are studied, two other control reactions must be run in parallel containing only protein (no liposome) or only liposome.

2. Incubate reactions at room temperature for 4 h (*see* Note 3).
3. Perform flotation assays with Nycodenz gradients. Mix 75 μ L of each protein/liposome reaction with 75 μ L of 80% Nycodenz solution and carefully overlaid with 850 μ L of 30% Nycodenz solution in 11 $\times$ 32 mm Beckman ultracentrifuge polycarbonate tubes. The gradient is covered with 200 μ L of flotation buffer (Fig. 7a).
4. Alternatively, perform flotation assays with sucrose gradients. Mix 75 μ L of each protein/liposome reaction with 125 μ L of 60% sucrose solution and carefully overlaid with 425 μ L of 30% sucrose solution then 425 μ L of 10% sucrose solution in 11 $\times$ 32 mm Beckman ultracentrifuge polycarbonate tubes. The gradient is covered with 150 μ L of flotation buffer.
5. Each tube contains a volume of 1200 μ L of solution and is spun at $214,200 \times g$ max ($166,180 \times g$ average) for 2 h 30 min at 4 $^{\circ}$ C in a TLS55 swinging bucket rotor (Fig. 7b) (*see* Note 11).
6. Recover tubes carefully (take a picture if necessary) and immediately withdraw 200 μ L fractions, a total of six fractions, from top to bottom. Liposomes can easily be visualized in the first top fraction (Fig. 7b). Recover 200 μ L fractions for the extraction or transfer reactions and the protein-only or liposome-only reactions.
7. The distributions of protein and NBD-PE along the density gradient are analyzed by SDS-PAGE and fluorescence, respectively (*see* Note 12). For SDS-PAGE, 15 μ L of fraction is mixed with 5 μ L of 4 $\times$ LDS sample buffer and analyzed on 4–20% SDS-PAGE gels (Fig. 7c). Dispense 100 μ L of each of the six 200 μ L fractions withdrawn from a centrifugation tube in the specified 96-well plates and quantify the NBD-PE fluorescence using a fluorimeter plate reader (excitation wavelength at 465 nm and emission wavelength at 535 nm; 0.1 s read per well; each plate is scanned three times at 5 s intervals) (Fig. 7c). Reactions with protein-only or liposome-only are used to subtract any background and verify that flotation buffer, gradient solutions (Nycodenz or sucrose), unloaded Mdm12 protein, and nonfluorescent liposomes do not fluoresce (*see* Note 13).

Under these conditions, we show that Mdm12 transfers NBD-PE into liposomes (Fig. 7b, c); however, the protein does not associate tightly with liposomes.

4 Notes

1. The presence of the tag does not interfere with HPTLC or lipid displacement assays. It is not necessary to remove the histidine tag for Mdm12 purified from

yeast cells or when Mdm12 is used for lipid displacement assay. The histidine tag must not be removed when the protein is used for liposome exchange assay. The histidine tag should be removed when the protein is used for crystallization or liposome binding using a flotation assay.

2. Yeast cell disruption using glass bead generates significant heat. All buffers and materials should be kept ice cold. It is recommended to work in the cold room. In our hands, 4 cycles of grinding in the bead beater are enough to achieve up to 75% cell disruption.
3. All steps using lipids labeled with fluorescent dyes (NBD-PE or Rhodamine-PE) should be performed protected from light (as much as possible).
4. We recommend testing a few detergents to optimize preloading and displacement. We surveyed LDAO, TritonX-100, *n*-dodecyl- β -D-maltoside, CHAPS, lyso-FosCholine-10, and FosCholine-14; all were tested at twice their respective critical micellar concentrations. The optimal detergent is the one that maximizes NBD-PE preloading of Mdm12 without displacing NBD-PE (when testing it on protein preloaded in the absence of detergent). In our hands LDAO gave the best results and did not interfere with BN-PAGE [23].
5. Use methanol—not chloroform—as organic solvent to solubilize phospholipids (including NBD-PE). We recommend controlling the concentration of protein before and after pre-loading with NBD-PE. Under the conditions of incubation described in our protocol, no methanol-induced precipitation of Mdm12 was observed. Mdm12 is a robust protein; nevertheless, the volume of phospholipid dissolved in organic solvent (methanol) should not exceed 2.5% of the total volume of the reaction.
6. Coomassie blue staining and destaining of the BN-PAGE gel and protein band intensity quantification must be performed after scanning of the NBD-PE fluorescence signal.
7. For accuracy purpose, each displacement assay is performed in triplicate.
8. It is also possible to purchase premade liposomes of given compositions (PC, PE, and/or PS), given sizes, and containing fluorescent phospholipids.
9. Upon preparation, liposomes should be kept on ice for no more than a few days and used as soon as possible. Do not freeze/thaw liposomes.
10. Under these conditions, no liposome carryover is observed in the final elution.
11. In our hands, liposome flotation by isopycnic gradient centrifugation works best when using Nycodenz as it tends to give sharper bands and better-defined boundaries. We recommend running each flotation assay in duplicate or triplicate for the purpose of accuracy.
12. Using the protein (and liposome) concentrations described herein, proteins can be detected by Coomassie blue staining after SDS-PAGE. If lower protein

concentrations are used, detection with silver staining or Western blotting can be used instead.

13. For each fraction, fluorescence is plotted as a percentage of the total fluorescence contained in each assay. We find this is the most consistent way to visualize extraction or exchange. Since we use a 96-well plate format and each tube contains 1200 μL , it is possible to withdraw $12 \times 100 \mu\text{L}$ fractions (instead of 6) and measure/quantify their fluorescence.

Acknowledgments

This work was supported by the Stein-Oppenheimer seed grant program, the Alexander and Renée Kolin Chair Endowment, and grant GM120173 from the National Institutes of Health to P.F.E. A.P.A.Y was a Gates Foundation Millennium Scholar and the recipient of a Dissertation of the Year Fellowship at UCLA.

References

1. Prinz WA (2014) Bridging the gap: membrane contact sites in signaling, metabolism, and organelle dynamics. *J Cell Biol* 205:759–769 [PubMed: 24958771]
2. Phillips MJ, Voeltz GK (2016) Structure and function of ER membrane contact sites with other organelles. *Nat Rev Mol Cell Biol* 17:69–82 [PubMed: 26627931]
3. Kojima R, Endo T, Tamura Y (2016) A phospholipid transfer function of ER-mitochondria encounter structure revealed in vitro. *Sci Rep* 6:30777 [PubMed: 27469264]
4. Tamura Y, Endo T (2017) Role of intra- and inter-mitochondrial membrane contact sites in yeast phospholipid biogenesis. *Adv Exp Med Biol* 997:121–133 [PubMed: 28815526]
5. Helle SC, Kanfer G, Kolar K et al. (2013) Organization and function of membrane contact sites. *Biochim Biophys Acta* 1833:2526–2541 [PubMed: 23380708]
6. Tubbs E, Rieusset J (2017) Metabolic signaling functions of ER-mitochondria contact sites: role in metabolic diseases. *J Mol Endocrinol* 58:R87–R106 [PubMed: 27965371]
7. Liu Y, Zhu X (2017) Endoplasmic reticulum-mitochondria tethering in neurodegenerative diseases. *Transl Neurodegener* 6:21 [PubMed: 28852477]
8. Kornmann B, Currie E, Collins SR et al. (2009) An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science* 325:477–481 [PubMed: 19556461]
9. Stroud DA, Oeljeklaus S, Wiese S et al. (2011) Composition and topology of the endoplasmic reticulum-mitochondria encounter structure. *J Mol Biol* 413:743–750 [PubMed: 21945531]
10. Kopec KO, Alva V, Lupas AN (2010) Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria. *Bioinformatics* 26:1927–1931 [PubMed: 20554689]
11. Kopec KO, Alva V, Lupas AN (2011) Bioinformatics of the TULIP domain superfamily. *Biochem Soc Trans* 39:1033–1038 [PubMed: 21787343]
12. Wong LH, Levine TP (2017) Tubular lipid binding proteins (TULIPs) growing everywhere. *Biochim Biophys Acta* 1864:1439–1449
13. Toulmay A, Prinz WA (2011) Lipid transfer and signaling at organelle contact sites: the tip of the iceberg. *Curr Opin Cell Biol* 23:458–463 [PubMed: 21555211]
14. Toulmay A, Prinz WA (2012) A conserved membrane-binding domain targets proteins to organelle contact sites. *J Cell Sci* 125:49–58 [PubMed: 22250200]
15. Reinisch KM, De Camilli P (2015) SMP-domain proteins at membrane contact sites: Structure and function. *Biochim Biophys Acta* 1861:924–927 [PubMed: 26686281]
16. Hirabayashi Y, Kwon SK, Paek H et al. (2017) ER-mitochondria tethering by PDZD8 regulates Ca^{2+} dynamics in mammalian neurons. *Science* 358:623–630 [PubMed: 29097544]
17. Schauder CM, Wu X, Saheki Y et al. (2014) Structure of a lipid-bound extended synaptotagmin indicates a role in lipid transfer. *Nature* 510:552–555 [PubMed: 24847877]

18. Jeong H, Park J, Lee C (2016) Crystal structure of Mdm12 reveals the architecture and dynamic organization of the ERMES complex. *EMBO Rep* 17:1857–1871 [PubMed: 27821511]
19. AhYoung AP, Lu B, Cascio D et al. (2017) Crystal structure of Mdm12 and combinatorial reconstitution of Mdm12/Mmm1 ERMES complexes for structural studies. *Biochem Biophys Res Commun* 488:129–135 [PubMed: 28479252]
20. Kawano S, Tamura Y, Kojima R et al. (2017) Structure-function insights into direct lipid transfer between membranes by Mmm1-Mdm12 of ERMES. *J Cell Biol* 217:959–974 [PubMed: 29279306]
21. Jeong H, Park J, Jun Y et al. (2017) Crystal structures of Mmm1 and Mdm12-Mmm1 reveal mechanistic insight into phospholipid trafficking at ER-mitochondria contact sites. *Proc Natl Acad Sci U S A* 114:E9502–E9511 [PubMed: 29078410]
22. Fuchs B, Suss R, Teuber K et al. (2011) Lipid analysis by thin-layer chromatography—a review of the current state. *J Chromatogr A* 1218:2754–2774 [PubMed: 21167493]
23. AhYoung AP, Jiang J, Zhang J et al. (2015) Conserved SMP domains of the ERMES complex bind phospholipids and mediate tether assembly. *Proc Natl Acad Sci U S A* 112: E3179–E3188 [PubMed: 26056272]
24. Hofer CT, Herrmann A, Muller P (2010) Use of liposomes for studying interactions of soluble proteins with cellular membranes. *Methods Mol Biol* 606:69–82 [PubMed: 20013390]
25. Busse RA, Scacioc A, Schalk AM et al. (2016) Analyzing protein-phosphoinositide interactions with liposome flotation assays. *Methods Mol Biol* 1376:155–162 [PubMed: 26552682]
26. Busse RA, Scacioc A, Hernandez JM et al. (2013) Qualitative and quantitative characterization of protein-phosphoinositide interactions with liposome-based methods. *Autophagy* 9:770–777 [PubMed: 23445924]
27. Krick R, Busse RA, Scacioc A et al. (2012) Structural and functional characterization of the two phosphoinositide binding sites of PROPPINs, a beta-propeller protein family. *Proc Natl Acad Sci U S A* 109:E2042–E2049 [PubMed: 22753491]
28. Saheki Y, Bian X, Schauder CM et al. (2016) Control of plasma membrane lipid homeostasis by the extended synaptotagmins. *Nat Cell Biol* 18:504–515 [PubMed: 27065097]
29. Connerth M, Tatsuta T, Haag M et al. (2012) Intramitochondrial transport of phosphatidic acid in yeast by a lipid transfer protein. *Science* 338:815–818 [PubMed: 23042293]
30. Watanabe Y, Tamura Y, Kawano S et al. (2015) Structural and mechanistic insights into phospholipid transfer by Ups1-Mdm35 in mitochondria. *Nat Commun* 6:7922 [PubMed: 26235513]
31. Maeda K, Anand K, Chiapparino A et al. (2013) Interactome map uncovers phosphatidylserine transport by oxysterol-binding proteins. *Nature* 501:257–261 [PubMed: 23934110]
32. Mesmin B, Bigay J, Moser von Filseck J et al. (2013) A four-step cycle driven by PI(4)P hydrolysis directs sterol/PI(4)P exchange by the ER-Golgi tether OSBP. *Cell* 155:830–843 [PubMed: 24209621]
33. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917 [PubMed: 13671378]
34. Weerheim AM, Kolb AM, Sturk A et al. (2002) Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography. *Anal Biochem* 302:191–198 [PubMed: 11878797]
35. Churchward MA, Brandman DM, Rogasevskaja T et al. (2008) Copper (II) sulfate charring for high sensitivity on-plate fluorescent detection of lipids and sterols: quantitative analyses of the composition of functional secretory vesicles. *J Chem Biol* 1:79–87 [PubMed: 19568800]
36. Vaden DL, Gohil VM, Gu Z et al. (2005) Separation of yeast phospholipids using one-dimensional thin-layer chromatography. *Anal Biochem* 338:162–164 [PubMed: 15707948]
37. Wittig I, Schagger H (2005) Advantages and limitations of clear-native PAGE. *Proteomics* 5:4338–4346 [PubMed: 16220535]
38. Wittig I, Braun HP, Schagger H (2006) Blue native PAGE. *Nat Protoc* 1:418–428 [PubMed: 17406264]

Fig. 1. SMP domains in the ERMES. (a) Schematic organization of the ERMES. Mdm12, Mmm1 and Mdm34, three of the five subunits of ERMES, contain an SMP domain. Mdm12 is the only cytosolic protein while all others are membrane-bound. (b) Simplified topology of the SMP/TULIP fold emphasizing the presence of a central hydrophobic pocket and conduit in the tubule-shaped domain and a possible model for the sliding of phospholipids (PL) along its lateral seam. Helices $\alpha1$ and $\alpha2$ capping the highly twisted 5-stranded antiparallel β -barrel, a key feature of the TULIP/SMP fold, delineate the lateral seam. Two variable insertions (VI1 and VI2) and the N and C termini are indicated. (c) Crystal structures of different SMP domains bound to phospholipids as seen in human E-SYT2 [17] and the fungal Mdm12 ERMES subunit from *Saccharomyces cerevisiae* (Sce) [18, 19] and *Kluyveromyces lactis* (Kla) [20]. Notice the different positions occupied by the PL molecules along the lateral seam

Fig. 2.
Flowchart summarizing the rational study of the lipid-binding and -transfer properties of the SMP domain of ERMES subunit Mdm12

Fig. 3. Purification of yeast Mdm12. **(a)** IEX and SEC profiles of Mdm12 purified from *E. coli*. Sce-Mdm12 is mostly monomeric (*M) with very little dimer present (**D). **(b)** Homogeneity of yeast Mdm12 (31.2 kDa protein) purified from heterologous source (*E. coli*) as assessed by SDS-PAGE and BN-PAGE as in Subheading 3.4.3 (7.5 μ g of Mdm12 were loaded on each gel)

Fig. 4.

Lipid content identification of Mdm12 by High-Performance Thin-Layer Chromatography (HPTLC). **(a)** Schematic of the HPTLC setup. **(b)** Representative HPTLC analysis of the extracted phospholipids bound to recombinant yeast Mdm12 overexpressed and purified from bacteria (heterologous source) or yeast (endogenous source). Yeast Mdm12 purified from its homologous source contains PC, a phospholipid absent in bacterial membranes. Lipid standards: *PA* phosphatidic acid, *PG* phosphatidylglycerol, *PE* phosphatidylethanolamine, *PS* phosphatidylserine, *PC* phosphatidylcholine, *CL* cardiolipin. Total lipid extracts from *E. coli* and *S. cerevisiae* are also loaded as standards

Fig. 5.

Lipid specificity of Mdm12 using lipid displacement assay. **(a)** Mdm12 preloading with fluorescent PE (NBD-PE). BN-PAGE gel showing the efficient binding of NBD-PE in Mdm12 replacing the endogenous phospholipids (ePL) copurifying with Mdm12. The same gel is colored with Coomassie blue and scanned for NBD-fluorescence, left and right panels, respectively. **(b)** Lipid displacement analysis by BN-PAGE. NBD-preloaded Mdm12 was incubated with seven distinct competitor phospholipids (PA, PS, PE, PC, PG, PI, and CL) and a background control (methanol only, no PL). Samples were run on a BN-PAGE gel stained with Coomassie blue and scanned for NBD-PE fluorescence, top and bottom panels, respectively. Intensity of bands was measured and corrected/normalized against protein amounts to calculate the displacement percentages achieved by each competitor PL

Fig. 6. Lipid transfer/exchange activity of Mdm12. **(a)** Liposome transfer assay. His-tagged Mdm12 incubated with liposomes exchanges phospholipids. Following incubation, the protein is separated from liposomes by spin Ni-NTA IMAC, its phospholipid ligands are then extracted and identified by HPTLC. **(b)** Lipid exchange assay analysis by HPTLC. Mdm12 extracts phospholipids from liposomes made of yeast total lipid extracts. Following transfer, Mdm12 exchanges bacterial PE and PG with PC and PI. A control reaction (liposome without Mdm12) shows the absence of lipid/liposome carryover through the process of spin column purification

Fig. 7.

Lipid transfer/exchange activity of Mdm12. **(a)** Liposome flotation assay. Mdm12 preloaded with fluorescent NBD-PE transfers its phospholipid to acceptor liposomes. Following incubation, the Mdm12/liposome mixture is subjected to isopycnic density gradient centrifugation to separate macromolecules based on their respective densities. **(b)** Isopycnic gradient centrifugation in Nycodenz. Characteristic aspect of tubes shown for three experiments: a Green Fluorescent Protein (GFP) control incubated with liposomes labeled with Rhodamine-PE (purple), Mdm12 incubated with Rhodamine-PE liposomes, and Mdm12 preloaded with NDB-PE (orange) incubated with unlabeled liposomes (white); proteins (P) and liposomes (L) are separated. **(c)** Lipid-transfer assay analysis by SDS-PAGE and fluorescence. SDS-PAGE analysis of the centrifuged fractions from top to bottom of the density gradient. The GFP control protein but also Mdm12 do not tightly associate with liposomes. Fluorescence measurement of the centrifuged fractions from top to bottom of the density gradient showing that NBD-PE gets transferred from Mdm12 to acceptor liposomes. NBD-PE total fluorescence distribution is plotted as a function of the position along the density gradient from the top (F1) toward the bottom (F6) of the tube; NBD-PE-preloaded Mdm12 in the presence (red) or absence (blue) of unlabeled (nonfluorescent) liposomes