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TITLE:
Reconstitution of Msp1 Extraction Activity with Fully Purified Components

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SUMMARY:
Here, we present a detailed protocol for reconstitution of Msp1 extraction activity with fully purified components in defined proteoliposomes.

ABSTRACT:
As the center for oxidative phosphorylation and apoptotic regulation, mitochondria play a vital role in human health. Proper mitochondrial function depends on a robust quality control system to maintain protein homeostasis (proteostasis). Declines in mitochondrial proteostasis have been linked to cancer, aging, neurodegeneration, and many other diseases. Msp1 is a AAA+ ATPase anchored in the outer mitochondrial membrane that maintains proteostasis by removing mislocalized tail-anchored proteins. Using purified components reconstituted into proteoliposomes, we have shown that Msp1 is necessary and sufficient to extract a model tail-anchored protein from a lipid bilayer. Our simplified reconstituted system overcomes several of the technical barriers that have hindered detailed study of membrane protein extraction. Here, we provide detailed methods for the generation of liposomes, membrane protein reconstitution, and the Msp1 extraction assay.

INTRODUCTION:
Proper cellular function depends upon a process called proteostasis, which ensures that functional proteins are at the correct concentration and cellular location[1]. Failures in proteostasis lead to compromised organelle function and are associated with many neurodegenerative diseases[2–4]. Membrane proteins present unique challenges to the proteostasis network as they must be targeted to the correct membrane while avoiding aggregation from the hydrophobic transmembrane domains (TMDs)[5]. Consequently, specialized machinery has evolved to shield the hydrophobic TMD from the cytosol and facilitate targeting and insertion into the proper cellular membrane[6–15].

Mitochondria are the metabolic hub of the cell and are involved in numerous essential cellular processes such as: oxidative phosphorylation, iron-sulfur cluster generation, and apoptotic regulation[16,17]. These endosymbiotic organelles contain two membranes, referred to as the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM). Over 99% of the 1,500 human mitochondrial proteins are encoded in the nuclear genome and need to be translocated across one or two different membranes[18,19]. Proper mitochondrial function thus depends on a robust proteostasis network to correct any errors in protein targeting or translocation.

Our lab focuses on a subset of mitochondrial membrane proteins called tail-anchored (TA) proteins, which have a single transmembrane domain at the very C-terminus[20–24]. TA proteins are involved in a number of essential processes, such as apoptosis, vesicle transport, and protein translocation[25]. The unique topology of TA proteins requires post-translational insertion, which occurs in the endoplasmic reticulum (ER) by the Guided Entry of Tail-anchored (GET) or Endoplasmic reticulum Membrane protein Complex (EMC) pathways or into the OMM by a poorly characterized pathway[20,26–28]. The biophysical properties of the TMD are necessary and sufficient to guide TA proteins to the correct membrane[29]. The recognition of biophysical characteristics rather than a defined sequence motif limits the fidelity of the targeting pathways[5]. Thus, mislocalization of TA proteins is a common stress for the proteostasis networks. Cellular stress, such as inhibition of the GET pathway, causes an increase in protein mislocalization to the OMM and mitochondrial dysfunction unless these proteins are promptly removed[30,31].

A common theme in membrane proteostasis is the use of AAA+ (**A**TPase **A**ssociated with cellular **A**ctivities) proteins to remove old, damaged, or mislocalized proteins from the lipid bilayer[1,32–38]. AAA+ proteins are molecular motors that form hexameric rings and undergo ATP dependent movements to remodel a substrate, often by translocation through a narrow axial pore[39,40]. Although great effort has been devoted to studying the extraction of membrane proteins by AAA+ ATPases, the reconstitutions are complex or involve a mixture of lipids and detergent[41,42], which limits the experimental power to examine the mechanism of substrate extraction from the lipid bilayer.

Msp1 is a highly conserved AAA+ ATPase anchored in the OMM and peroxisomes that plays a critical role in membrane proteostasis by removing mislocalized TA proteins[43–47]. Msp1 was also recently shown to alleviate mitochondrial protein import stress by removing membrane proteins that stall during translocation across the OMM[48]. Loss of Msp1 or the human homolog ATAD1 results in mitochondrial fragmentation, failures in oxidative phosphorylation, seizures, increased injury following stroke, and early death[31,49–56].

We have shown that it is possible to co-reconstitute TA proteins with Msp1 and detect the extraction from the lipid bilayer[57]. This simplified system uses fully purified proteins reconstituted into defined liposomes which mimic the OMM (**Figure 1**)[58,59]. This level of experimental control can address detailed mechanistic questions of substrate extraction that are experimentally intractable with more complex reconstitutions involving other AAA+ proteins. Here, we provide experimental protocols detailing our methods for liposome preparation, membrane protein reconstitution, and the extraction assay. It is our hope that these experimental details will facilitate further study of the essential but poorly understood process of membrane proteostasis.

PROTOCOL:

1. Liposome Preparation

1.1. Combine chloroform stocks of lipids in appropriate ratios to mimic the outer mitochondrial membrane.

1.1.1. Prepare 25 mg of lipid mixture. We use a previously established mixture of lipids that mimic mitochondrial membranes, consisting of a 48:28:10:10:4 molar ratio of chicken egg phosphatidyl choline (PC), chicken egg phosphatidyl ethanolamine (PE), bovine liver phosphatidyl inositol (PI), synthetic 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), and synthetic 1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol (TOCL)[58,59]. Sample calculations are shown in **Table 1**.

1.1.2. Bring all lipid stocks to room temperature before opening as this will limit condensation. As most labs do not have a precise way to measure the concentration of the lipids, any water absorbed by the chloroform stock will change the concentration of the lipid stock and thus the ratio of lipids used in the assay.

1.1.3. As lipid stocks come in glass ampules, transfer the required amount of lipid to a glass vial using a 1 mL syringe. Add 2 mg of dithiothreitol (DTT) to the vial to prevent lipid oxidation. Work quickly as evaporation of chloroform will change the concentration of the lipid.

1.1.4. Transfer any remaining lipid to a separate glass vial and fit with a PFTE septa. Add 2 mg of DTT to the vial, wrap with parafilm and store at -20 °C to prevent lipid oxidation. Try to use the lipids within 3 months of transfer to the vials. To prevent potential contamination of the chloroform stocks by marker runoff, transfer the stickers from the original ampules to the glass vials rather than label with marker.

1.2. Evaporate chloroform under a very gentle stream of nitrogen while spinning the glass vial continuously by hand in a fume hood, essentially acting as a manual rotovap. Spin the vial at a consistent speed (20-40 rpm) by hand to keep the lipids moving. The goal is to evaporate all the chloroform and get an even coating of lipids over the entire glass vial.

CAUTION: Chloroform is neurotoxic, and this step should be performed in the fume hood.

1.2.1. Attach a fresh Pasteur pipette to the nitrogen tube. Do not let any of the lipids splash out of the vial or onto the Pasteur pipette. Aim the tip at the bottom of the vial so the air bounces off the bottom and pushes the lipids up towards the center of vial. Get an even coating over the entire vial while avoiding any accumulation in the corners or by the cap. This whole process takes about 5 minutes.

1.2.2. As the mixture thickens into a “bead” of lipids, guide it into the center of the vial by changing the angle of the vial. Once the bead starts to become smaller, turn up the nitrogen stream slightly to disperse the bead, ensuring that none of the lipids blow out of the vial.

1.3. Remove any remaining chloroform under vacuum.

1.3.1 Put the glass vial on a house vacuum or diaphragm vacuum pump for 1 hour to remove the majority of residual chloroform. These vacuums are generally not strong enough to remove all of the chloroform, but they can tolerate small amounts of solvent better than rotary-vain vacuums.

1.3.2. Put vial on a strong vacuum (<1 mTorr) for 12-16 h to remove all residual chloroform. Be sure to avoid bumping of the vial during this process.

1.4. Resuspend the lipids in 1.25 mL of liposome buffer (50 mM HEPES KOH pH 7.5, 15% glycerol, 1 mM DTT). As we started with 25 mg of lipids, this results in a concentration of 20 mg/mL. Fully resuspend the lipids with no visible chunks. If the lipids pooled in the corner of the vial, this can be a lengthy process.

1.4.1. Vortex the vial vigorously until the sample is milky smooth. We find that if the chloroform evaporation was done properly the night before, this process takes about 5-10 minutes.

1.4.2. To ensure complete resuspension of the lipids, rotate on a wheel at room temperature for 3 hours at ~80 rpm. Remove vial from the wheel once every hour for 1 minute of vortexing to ensure even mixing.

1.5 Transfer lipids carefully to a clean 1.5 mL microcentrifuge tube. Perform 5 freeze-thaw cycles using liquid nitrogen to freeze and a 30 °C heat block to thaw. This step helps to convert multilamellar vesicles to unilamellar vesicles.

1.6 Extrude the lipids.

1.6.1. During the freeze thaw cycles, prepare the mini-extruder. Assemble the mini-extruder with 10 mm filter supports and a polycarbonate membrane of the desired pore size (we use 200 nm). The size of the filter will affect the size of the liposome, which will affect the concentration of proteins required for the reconstitution (**Step 2.3**).

1.6.2 Place the mini-extruder onto a hot plate and bring the extruder temperature up to 60 °C.

1.6.3 Draw up the lipids into a 1 ml gas-tight glass syringe and carefully place into one end of the mini-extruder. Place the empty gas-tight syringe into the other side of the mini-extruder. Allow the lipids to equilibrate to the extruder stand temperature for 5-10 minutes.

1.6.4 Transfer the lipids to the alternate syringe by gently pushing the plunger of the filled syringe. Push the solution from the alternate syringe into the original syringe. Repeat this back-and-forth process 15 times, so that at the 15^{th} pass the lipids end in the alternate syringe. Monitor the volume in each pass to make sure that there are no leaks.

1.7. Prepare single use aliquots of the lipids, flash freeze in liquid nitrogen and store at -80 °C. The liposomes are stable at -80 °C for several months. The reconstitutions require 10 μL of lipid at a time (**Step 2.3.4**), so it is convenient to prepare 10 μL or 20 μL aliquots.

2 Reconstitution of Msp1 and Model TA protein

2.1. Prepare the reconstitution buffer: 50 mM HEPES pH 7.5, 200 mM potassium Acetate, 7 mM magnesium acetate, 2 mM DTT, 10% sucrose, 0.01% sodium azide, 0.2-0.8% Deoxy Big Chaps (DBC). Do not drop the concentration of DBC below the critical micelle concentration (CMC) of 0.12%.

2.1.1. Optimize reconstitution conditions for the new batch of liposomes. The concentration of DBC and biobeads required for optimal reconstitution varies depending on the batch of liposomes used. To limit prep to prep variability, use the same lot of DBC for all experiments. When changing lots of DBC, repeat the optimization process.

2.1.2. Set up a series of reconstitutions with different concentrations of DBC (0.2% - 0.8%) and biobeads (25 mg – 100 mg) each time a new batch of liposomes is prepared. It is important to not drop the DBC below the critical micelle concentration (~0.12%). Once conditions are optimized, we recommend collecting all data using the same liposome prep and reconstitution conditions.

2.1.3 Assay the effectiveness of the various reconstitution conditions by using the extraction assay described in **Step 3**.

2.2. Prepare biobeads at a final concentration of 250 mg/mL.

2.2.1. Weigh out 2.7 g of dried biobeads and resuspend in a 50 mL centrifuge tube of 100% methanol (about 45 mL) to wet the beads. Initially wet the biobeads in methanol to prevent air from being trapped in the pores of the beads. Once in methanol, keep the biobeads wet as any air trapped by the biobeads will alter their ability to absorb detergent.

2.2.2. Remove methanol by washing the beads 8x with about 45 mL of ultrapure water (18.2 m**Ω**), hereafter referred to as ddH_2O. Pellet beads by spinning at 3,200 x g for 1 minute. Decant the liquid and resuspend in ddH_2O.

2.2.3. After washing, resuspend in 10 ml of ddH_2O with 0.02% sodium azide and store at 4 °C. Biobeads can be stored at 4 °C for several months. This stock is 250 mg/mL as it is assumed ~0.2 g is lost during the wash steps.

2.3. Calculate the size of the liposome and the desired number of molecules of TA protein and Msp1 per liposome. This will determine the concentration of Msp1 and TA protein required for the reconstitution.

2.3.1. First, calculate the number of lipid molecules per unilamellar liposome (N_{total}) using the equation $N_{total} = \frac{\left[4\pi\left(\frac{d}{2}\right)^2 + 4\pi\left[\frac{d}{2}-h\right]^2\right]}{a}$ where d is the diameter of the liposome, h is the thickness of the bilayer, and a is the lipid headgroup area.

2.3.1.1. Measure the liposome diameter by DLS. In our example, a value of 70 nm for the liposome diameter (d) was obtained.

2.3.1.2. Use a value of 5 nm for h and 0.71 nm^2 for *a*, which is the headgroup size for phosphatidylcholine. In this particular situation, N_{total} is 37,610.

2.3.2. Next, calculate the molar concentration of lipid M_{Lipid} using the average molecular weight of the lipids in the mixture. In this example, the concentration of lipids is 20 mg/mL (**Step 1.4**) and the average molecular weight of lipids is 810 g/mol (**Table 1**). This results in a value of 0.0247 M for M_{Lipid}.

2.3.3. Next, calculate the molar concentration of liposomes, $M_{Liposome,}$ using the equation $M_{Liposome} = \frac{M_{Lipid}}{N_{Total}}$ where M_{Lipid} is the molar concentration of lipid from **Step 2.3.2**, and N_{total} is the total number of lipids per liposome calculated in **Step 2.3.1**. In this example, the 20 mg/mL stock concentration of liposomes is approximately 660 nM.

2.3.4. Calculate the amount of Msp1 and TA protein required for a 100 µL reconstitution reaction.

NOTE: The final concentration of lipids in the reconstitution is 2 mg/mL, which is a 10x dilution of the liposome stock. This gives a final liposome concentration of 66 nM. The final concentration of Msp1 is 792 nM, which gives an average of 12 total copies (2 functional hexamers) per liposome. The final concentration of TA protein is 660 nM, which gives an average of 10 copies per liposome.

2.4. In a PCR tube, mix together purified Msp1, TA protein, and liposomes in reconstitution buffer. The order of addition is buffer, proteins, and liposomes last. The total volume is 100 µL. Allow the mixture to sit on ice for 10 minutes. Purify the Msp1 and TA protein as previously described[57].

2.4.1. Use the well-characterized model TA protein His-Flag-Sumo-Sec22 as a positive control substrate when first establishing the assay. This construct has a His-tag for easy purification, 3x-Flag tag for detection by western blot, a Sumo domain for increased solubility, and the TMD of the ER-TA protein Sec22 for reconstitution and recognition by Msp1.

2.4.2. Ensure that stock solutions of both Msp1 and the TA protein are approximately 100 µM to minimize the effect of N-Dodecyl β-D-maltoside (DDM) from the protein purification on the reconstitution. Purified Msp1 is in 20 mM HEPES pH 7.5, 100 mM sodium chloride, 0.1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 0.05% DDM whereas the purified TA protein is in 50 mM Tris pH 7.4, 150 mM sodium chloride, 10 mM magnesium chloride, 5 mM β-mercaptoethanol, 10% glycerol, 0.1% DDM. Stock solutions of approximately 100 µM TA protein and Msp1 monomer ensures that these components will make up < 5% of the final reconstitution volume, resulting in a dilution of DDM below the CMC.

2.5. Add the desired amount of biobeads to the sample to remove detergent.

2.5.1. Cut the tip of a p200 pipette tip to about 1/8th inch in diameter so that beads can fit through the tip. Vortex the biobeads tube thoroughly to obtain a uniform mixture and quickly

remove the lid and pipette up the volume before the biobeads settle. Transfer the biobeads to an empty PCR tube.

2.5.2. When the reconstitution has finished its 10-minute incubation on ice, use an uncut pipette tip to remove all of the liquid from the biobeads. Then transfer the 100 µL reconstitution into the tube with the biobeads. This must be done quickly so that the biobeads do not trap air, which will cause the beads to float.

2.6. Allow the reconstitution to rotate on a wheel at ~80 rpm for 16 hours at 4 °C.

2.7. Remove reconstituted material from biobeads. Do a quick spin in a picofuge to pellet the biobeads, and then use an uncut pipet tip to transfer the reconstituted material to a clean PCR tube. Repeat this process 1-2 times until there are no biobeads left in the sample. Keep reconstitution on ice.

2.8. Pre-clear the reconstituted material to remove any proteins which failed to reconstitute into the liposomes.

2.8.1. Prepare Extraction Buffer: 50 mM HEPES pH 7.5, 200 mM potassium acetate, 7 mM magnesium acetate, 2 mM DTT, 100 nM calcium chloride.

2.8.2. Equilibrate the glutathione spin columns with Extraction Buffer according to the manufacturer's directions. This typically involves 3-rounds of washing with 400 µL of buffer and then centrifuging at 700 x g for 2 minutes at room temperature to remove the buffer.

2.8.3. Add 5 µM of each chaperone (GST-SGTA and GST-Calmodulin) to the reconstituted material. These chaperones will bind to the TMD of any proteins which failed to reconstitute into the liposomes. Purification of chaperones was described previously[6,57].

NOTE: These chaperones are commercially available, but we prefer to purify in house to control for cost and quality. Both proteins are in 20 mM Tris pH 7.5, 100 mM sodium chloride, and 0.1 mM TCEP, and have a stock concentration around 160 µM. SGTA recognizes substrates with a highly hydrophobic TMD whereas Calmodulin binds TMDs with moderate hydrophobicity[6,29]. Together, this chaperone cocktail can recognize a broad range of substrates.

2.8.4. Add 100 µL of extraction buffer to the reconstituted material, bringing the volume up to 200 µL. Add this to the equilibrated glutathione spin columns. Note that the glutathione spin columns provide the highest sample recovery when the pre-clearing volume is 200 – 400 µL.

2.8.5. Plug the spin columns and rotate at ~80 rpm at 4 °C for 30 minutes to allow chaperones to bind to resin.

2.8.6. Spin the columns at 700 x g for 2 minutes at room temperature. The flow through is pre-cleared material that is depleted of aggregated proteins. Keep material on ice and proceed directly with extraction assay.

3. Extraction Assay

3.1 Prepare tubes for SDS PAGE analysis. Each reaction will have 4 tubes: INPUT (I), FLOW THROUGH (FT), WASH (W), and ELUTE (E).

3.1.1. Add 45 µL of ddH_2O sample to the INPUT tube, 40 µL of ddH_2O to the FLOW THROUGH tube, and 0 µL to the WASH and ELUTE tubes.

NOTE: The best signal to noise ratio in this assay is obtained when the WASH and ELUTE samples are 5x concentrated relative to the INPUT and FLOW THROUGH samples. Due to the dilutions during the assay, this requires taking 5 µL of sample for the INPUT sample, 10 µL of sample for the FLOW THROUGH sample, and 50 µL of sample for the WASH and ELUTE samples.

3.1.2. Add 16.6 µL of 4x SDS PAGE Loading Buffer to each tube. The total volume of each sample is 50 µL before SDS PAGE Loading Buffer. The final volume is 66.6 µL (50 µL sample + 16.6 µL of 4x SDS PAGE Loading Buffer).

3.2. Assemble the extraction assay.

3.2.1. Prepare the extraction reaction containing 60 µL of pre-cleared proteoliposomes, 5 µM of GST-SGTA, 5 µM of GST-Calmodulin, and 2 mM ATP. Combine all reagents except ATP, which is used to initiate the reaction. Bring to a final volume of 200 µL with Extraction Buffer.

NOTE: As 60 µL of sample are used for each extraction assay, one reconstitution can be used for three different extraction assays. Perform positive and negative controls (+ATP and -ATP) on material from the same reconstitution.

3.2.2. Pre-warm extraction assay in 30 °C heat block for 2 minutes.

3.3. Initiate the extraction assay by adding ATP to final concentration of 2 mM and start timer.

3.3.1. Give a 5 second spin in a picofuge to mix ATP into the reaction. Incubate reaction at 30 °C for 30 minutes.

3.3.2. During the incubation, take 5 µL of the reaction and add to the INPUT tube. The timing of this is flexible.

3.3.3. During this incubation period, equilibrate one glutathione spin column for each sample in the extraction assay.

3.4. Perform pull down on chaperones to isolate extracted material.

3.4.1. Once the 30-minute incubation is finished, add 200 µl of extraction buffer to the tube to bring total volume to 400 µL. Add to equilibrated glutathione resin and allow to bind on wheel at 4° C for 30 minutes.

3.4.2. Spin the columns at 700 x g for 2 minutes at room temperature to collect the flow through. Take 10 µL for the FLOW THROUGH tube. This sample contains substrates which are still integrated in the lipid bilayer.

3.4.3. Wash resin twice with 400 µL of extraction buffer, discarding the flow through. On the third wash, keep the flow through and take 50 µL for the WASH tube.

3.4.4 Prepare 5 mL of Elution Buffer by adding reduced glutathione to a final concentration of 5 mM in Extraction Buffer. Prepare this buffer fresh each time.

3.4.5. Add 200 µL of Elution Buffer to the spin column. Incubate at room temperature for 5 minutes. Spin at 700 x g for 2 minutes at room temperature to elute. Keep the flow through. Repeat the process a second time so that the total elution volume is 400 µL.

3.4.6. Take 50 µL of sample from the Elution sample and add it to the ELUTE tube.

3.5. Analyze extraction activity using SDS-PAGE and Western blot.

NOTE: As a western blot is a fairly standard procedure, a basic protocol is provided that highlights a few of the details unique to this assay.

3.5.1. Load samples into a stain free polyacrylamide gel (4% stacking, 15% separating) and run at 200 V for 50 minutes in Tris-glycine buffer. If space permits, use both an unstained and stained ladder to permit visualization on stain-free gel imager and transfer to PVDF membrane respectively. The stain free gel allows quantitative visualization of tryptophan containing proteins upon activation with ultraviolet light, while still allowing the gel to be used for a western blot.

3.5.2. Image the stain free gel to confirm that there is equal loading across all samples. This is an essential control that ensures any changes in signal detected by western blot are not a result of variable protein loading. There should only be visible bands for the chaperones (GST-calmodulin and GST-SGTA) in the INPUT and ELUTION samples. Recall that the ELUTE sample will be more concentrated than the INPUT sample.

3.5.3. Assemble a Western blot cassette using a 45 um PVDF membrane. Transfer at a constant current of 300 mA for 60 minutes.

3.5.4. After blocking the membrane, bind to the primary antibody for 16 h at 4 °C with gentle shaking ~15 rpm. Blot for substrate with rabbit Anti-FLAG at a 1:1,000 dilution.

NOTE: The primary and secondary antibodies used will be substrate specific and the concentration for use may need to be optimized.

3.5.5. Wash membrane and incubate with secondary antibody, goat anti-rabbit at a 1:10,000 dilution, with gentle shaking for 1 hour at room temperature.

3.5.6. Wash membrane and image for analysis using western blotting detection agent.

REPRESENTATIVE RESULTS:

To properly interpret the results, the stain free gel and the western blot must be viewed together. The stain free gel ensures equal loading across all samples. When viewing the stain free gel, the chaperones (GST-calmodulin and GST-SGTA) will be visible in the INPUT (I) and ELUTE (E) lanes. Double check that the intensity of these bands is uniform across all of the INPUT samples. Likewise, ensure that the intensity is uniform across the ELUTE samples. The ELUTE is 5x more concentrated than the INPUT and this difference in intensity will be visible in the gels.

After using the stain free gel to confirm proper loading, examine the western blot to determine extraction activity. Measure extraction activity by comparing the amount of substrate in the ELUTE (E) fraction relative to the INPUT (I) fraction. The signal in the Flow Through (FT) shows some variability, but is generally similar to the INPUT fraction. There should be no signal in the WASH (W) fraction. Typically, there is ~10% extraction efficiency for the positive control and 1-2% extraction efficiency in the negative control (**Figure 2**). Recall that the ELUTE fraction is 5x as intense as the INPUT fraction, so this needs to be taken into account when judging extraction efficiency. If reconstitution conditions are not optimized, there is typically comparable extraction levels in both the + ATP and - ATP samples (**Figure 3**)**.** This result is attributed to a failure of Msp1 to efficiently reconstitute, resulting in numerous proteoliposomes without a functional Msp1 hexamer.

FIGURE AND TABLE LEGENDS:

Table 1: Sample calculations for liposome preparation. The key goals of this table are to calculate the concentration corrected average molecular weight of the lipid mixture (cell E8) and the volume of each lipid stock required to make the liposomes (column H). Columns A, B, C, and G are either taken from product labels (MW or stock concentration) or determined by user (lipids and mole %).

Figure 1: Cartoon of extraction assay and list of key steps.

Figure 2: Representative data showing a properly functioning assay. Extraction efficiency is determined by comparing the amount of substrate in the ELUTE fraction with the INPUT fraction. Recall that the gel has 5x higher loading of the ELUTE fraction relative to the INPUT fraction.

Figure 3: Representative data of a failed reconstitution and extraction assay. Here, the activity in the + ATP sample is comparable to the activity in the – ATP sample.

DISCUSSION:

Proper mitochondrial function depends upon a robust protein quality control system. Due to inherent limits in the fidelity of the TA protein targeting pathways, mislocalized TA proteins are

a constant source of stress for mitochondria. A key component of the mitochondrial proteostasis network is Msp1, which is a membrane anchored AAA+ ATPase that removes mislocalized TA proteins from the OMM. Here, we have described how to prepare proteoliposomes, co-reconstitute Msp1 and a model TA protein, and perform an extraction assay. We previously used this assay to demonstrate that Msp1 directly recognizes mislocalized TA proteins and is capable of extracting these proteins from a lipid bilayer without any accessory proteins or cofactors[57].

A drawback of the assay is that there is some variability in the reconstitution process. To control for prep-to-prep variability, we always include a positive and negative control on the same gel/western blot to ensure that our assay is working as intended. We avoid making comparisons between reconstitutions that were done on different days or drawing comparisons between different western blots. The only comparisons we make are for samples reconstituted in parallel and run on the same gel/western blot. We are also quite conservative in our data interpretation. Although it is possible to quantify extraction efficiency using ImageJ, we typically describe our experiments as having full activity, intermediate activity, or no activity.

One source of variability is the total amount of protein reconstituted. While the majority of unincorporated proteins are removed by the pre-clearing process, the less than perfect reconstitution efficiency of Msp1 can have an outsized effect on the observed efficiency of substrate extraction. This effect arises from the fact that Msp1 functions as a homohexamer, but purifies as a monomer[57]. Liposomes containing anything other than 6x copies of Msp1 will be inactive. For example, a liposome with only 5 copies of Msp1 will be inactive. The only way to form stable full-length Msp1 hexamers is to inactivate ATPase activity with non-hydrolyzable ATP analogs (ATPγS) or the inactivating E193Q Walker B mutant, neither of which are compatible with an activity assay. Overcoming this technical hurdle is an area of active research in our lab.

Another area of active research focuses on making the extraction assay more quantitative. The current method relies on pull downs and western blotting for signal detection, both of which are only semi-quantitative and show assay to assay variability. Covalent modification of extracted substrates would eliminate the variability that arises from the pull downs. Likewise, use of radioactive or fluorescent labels on substrates would eliminate the need for western blots and the associated variability.

A major strength of the assay is that the system is completely defined. The membrane proteins are recombinantly expressed and purified and it is possible to make defined mutants in both Msp1 and the substrate to study specific aspects of the reaction. The role of the lipid environment in proteostasis has been largely ignored due to the technical challenges of studying this in a detailed manner. Because our assay uses liposomes with a defined lipid composition, this allows for full experimental control of the lipid environment. We can easily modulate factors such as: lipid fluidity, bilayer thickness, headgroup identity, and liposome size. We are actively working to use our assay to examine the role of the lipid environment on Msp1 activity. It is our hope that the *in vitro* reconstitution and extraction assay described here can serve as a simplified model system to study the common cellular process of AAA+ ATPase mediated extraction of membrane proteins from a lipid bilayer.

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DISCLOSURES:

None

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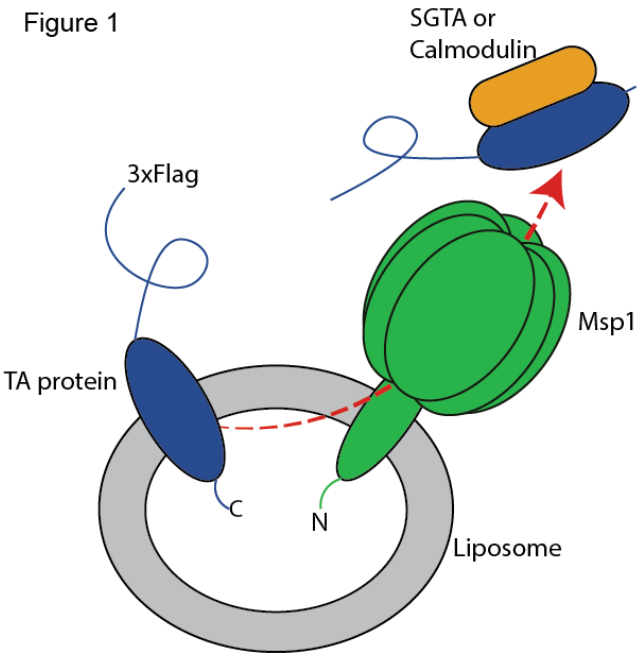
Figure 1



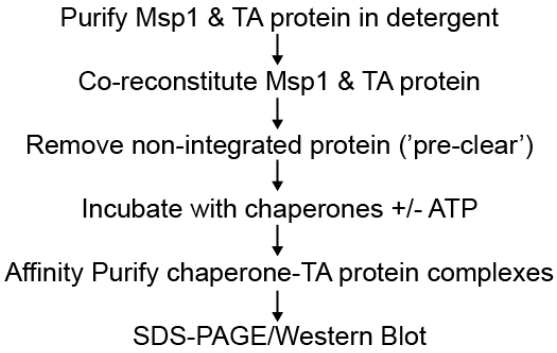


Figure 2

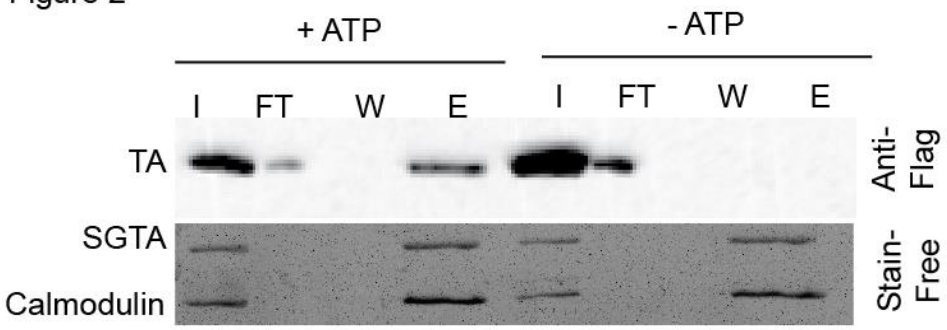


Figure 3

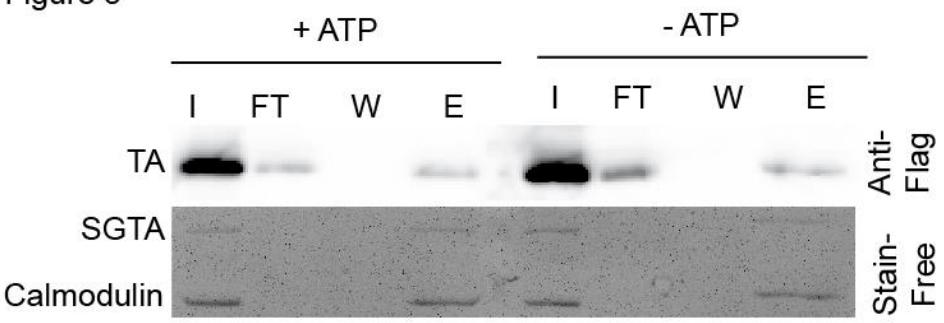


Figure 1 Adobe Illustrator File

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Figure 3 Adobe Illustrator File

Table 1

Lipid	Mole %	MW	Avg. MW	µmol in 25 mg	mg in 25 mg	Chlor stock (mg/mL)	µL for 25 mg
PC	48%	770	369.6	14.82	11.41	25	456.4
PE	28%	746	208.88	8.64	6.45	25	258.0
PI	10%	902	90.2	3.09	2.78	10	278.5
DOPS	10%	810	81	3.09	2.50	10	250.1
TOCL	4%	1502	60.08	1.23	1.85	25	74.2
			Conc. Corr Avg MW	809.76			
			µmol in 25 mg	30.87			

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Table of Materials

JOVE_Materials_Revised_Final.xls

Dear Nilanjana,

Thank you for the helpful comments on our manuscript. We have incorporated the comments and suggestions in our revised manuscript. A point-by-point response to all editorial and reviewer comments is below, with our responses in green text. If you have any questions, please do not hesitate to contact me.

Sincerely,
Matthew L. Wohlever

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. **Done**
2. Please revise the following lines to avoid previously published work: 24-26, 36-39, 176-177. **Done**
3. Please ensure that abbreviations are defined at first usage. For example, please define GET/EMC in Line 56. **Done**
4. Please avoid the use of personal pronouns (you, your, etc.). **Done**
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. Please sort the Materials Table alphabetically by the name of the material. **Done**
6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step**.** **Done**
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. **Done**
8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. **Done**
9. Please add more details to your protocol steps.

Step 1.2: Please specify the spinning speed. **Done**

Step 1.6.1: Please provide details regarding the filter support used, such as filter membrane and pore size. Please remove the commercial term from the Protocol step and add it to the table of Materials. **Done**
Step 1.6.3: Please specify the syringe used. **Done**
Step 1.3.4: How to ensure that the DBC is not below the critical micelle concentration? **We have added information to step 2.1 to address this point.**
Step 2.2.1: Please use "centrifuge tube" instead of "Falcon tube." **Done**
Step 2.4/2.8.2: Please provide more details regarding the protein purification steps if this needs to be filmed. **These steps do not need to be filmed. Full details on purification of these proteins would put the articles over the page limit and significantly shift the focus of the article. We have added some details regarding buffer composition and stock concentrations for these proteins to steps 2.4.2. and 2.8.3.**
Step 2.5.2: How to ensure that air is not trapped? **Done**
Step 2.6/2.8.5: Rotation/spinning speed? Please mention. **Done**
Step 2.8.6/3.4.2: Please mention the centrifugation temperature. **Done**
Step 3.4: Please describe how the step is performed. **This is detailed in steps 3.4.1-3.4.6.**
Step 3.5.4: Please specify the shaking speed. **Done**
10. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.). **Done**

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This is a nice methods review for Msp1 activity in removing tail-anchored proteins from defined liposome systems in vitro. Overall, the detail provided is sufficient to reproduce these experiments. In fact, I appreciate some of the finer points that are included that highlight little intricacies that are often missing in these methods papers.

Major Concerns:
Nothing too major, but I think addressing these concerns will provide clarity.

1. What is the TA protein? This is not described in the paper. I know that is can be any protein of your choosing, but it would be nice to provide an example. Later, you can note the applicability of this method for other TA proteins. This will help later when you are doing analysis and probing for FLAG tag. **We have added step 2.4.1 to address this point.**

2. Similarly, I know that there must be detergent in the isolated Msp1 and TA protein, but how much is there? Is there any consideration for the type of detergent used for this method? **We have added step 2.4.2 to address this point.**

3. Can the chaperones be purchased? Or are these only available from the authors of the previous papers? And are these specific chaperones needed for this assay? **We added a note to step 2.8.3.**

Minor Concerns:
Mostly clarification or typos.
Line 56 - Please define the GET or EMC pathways. Not familiar with them and additional information would help. **Done**
Line 102 - rather than saying that your are preparing 25 mg of liposomes, I would be more specific and stat that you are prepare a total of 25 mg of the lipid mixture. **Done**
Line 165 - You say to use a 200 nM polycarbonate membrane. I think you mean 200 nm. But I would also be less specific here and suggest using a polycarbonate membrane with a pore size of your choosing. This leads nicely into your next comment on the impact of liposome size. **Done**
In step 2.2, what are the recommended volumes for methanol wetting and washes with ddH2O? **Done**

Reviewer #2:
Manuscript Summary:
This manuscript describes a protocol to investigate quality control of tail-anchored proteins by the mitochondrial outer membrane ATPase Msp1. Reconstitution of Msp1-dependent tail-anchored protein extraction from synthetic liposomes was an important step forwrd in the field of membrane protein quality control and this assay is a very useful tool. Thus, it is of great interest to the field to have a detailed and accessible protocol and I fully support its publication. Below I list a few minor concenrs that may help improving the manuscript.

Major Concerns:
I have no major concerns

Minor Concerns:
1- Define Proteostasis. the term is used in the abstract and introduction but it is jargon and unlikely to be familiar to readers less familiar with the field. **Defined in line 22.**
2- Line 40: transmembrane domains, the S is missing **Done**
3- line 97. Which criteria was used for the lipid molar ratio to mimic mitochondria outer membrane? is this the standard in the field? are there other possibilities? **Step 1.1.1. has been updated to reflect that this is a previously established mixture. In the discussion we mention the possibility of altering liposome composition as an area of active research.**
4- line 120. It should be noted that chloroform is neurotoxic. The reader should be made aware that these experiments should be performed in a fume hood. **Done**

5- lines 249-253. it should be mention that these number are an estimate and that on average there will be 2 Msp1 hexamers and 10 TA molecules. Some background information on Msp1 oligomer formation would be useful. **Done. Background on Msp1 oligomerization is included in the discussion.**
6- line 255. Mention the buffer composition in which Msp1 and TAs are present **We have added step 2.4.2 to address this point.**
7- line 368. Explain what stain-free gel is. **Description added in step 3.5.1.**
8- Resolution of FLAG blot (in Figure 2) is poor. **We have uploaded the vector image file for each figure.**