PROTOCOL

Isolation of plasma membrane–associated membranes from rat liver

Jan M Suski[1,2], Magdalena Lebiedzinska[1,2], Aleksandra Wojtala[1], Jerzy Duszynski[1], Carlotta Giorgi[2], Paolo Pinton[2] & Mariusz R Wieckowski[1]

[1]Nencki Institute of Experimental Biology, Department of Biochemistry, Warsaw, Poland. [2]Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Interdisciplinary Center for the Study of Inflammation (ICSI) and Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, Ferrara, Italy. Correspondence should be addressed to M.R.W. (m.wieckowski@nencki.gov.pl).

Published online 16 January 2014; doi:10.1038/nprot.2014.016

Dynamic interplay between intracellular organelles requires a particular functional apposition of membrane structures. The organelles involved come into close contact, but do not fuse, thereby giving rise to notable microdomains; these microdomains allow rapid communication between the organelles. Plasma membrane–associated membranes (PAMs), which are microdomains of the plasma membrane (PM) interacting with the endoplasmic reticulum (ER) and mitochondria, are dynamic structures that mediate transport of proteins, lipids, ions and metabolites. These structures have gained much interest lately owing to their roles in many crucial cellular processes. Here we provide an optimized protocol for the isolation of PAM, PM and ER fractions from rat liver that is based on a series of differential centrifugations, followed by the fractionation of crude PM on a discontinuous sucrose gradient. The procedure requires ~8–10 h, and it can be easily modified and adapted to other tissues and cell types.

© 2014 Nature America, Inc. All rights reserved.

INTRODUCTION

Structural interaction between the ER and the PM was originally described in yeast by Pichler *et al.*[1]. It has been demonstrated that specialized ER microdomains are located in the proximity of the PM, resulting in PAMs upon subfractionation[1]. On the basis of the analysis of a series of ultrathin electron microscopy sections, it seems that associations between the ER and the PM occur approximately ten times more often frequently than associations between the mitochondria and the ER (in an average series of 1,100 and 80–110 sections, respectively)[1,2]. These findings have been confirmed in more recent studies[3]. Compared with the mitochondria-associated membrane (MAM) fraction, the molecular composition of the PAM fraction is much less well characterized.

The PAM fraction: its role in cellular physiology

PAMs are dynamic membrane domains that mediate interactions between, as well as transport of, proteins, lipids, ions and metabolites. On the basis of an analysis of the molecular components of the ER-PM junctions, it has been suggested that the PAM fraction may be involved in signal transduction pathways (e.g., cellular response to oxidative stress)[4], synthesis and trafficking of lipids[5], nonvesicular cholesterol transport (oxysterol-binding protein)[6,7] and capacitative calcium influx[8]. However, the presence of mitochondrial markers in the PAM fraction may indicate that the PM and mitochondria can also form stable structures distinct from PAMs, which are formed predominantly by the PM and ER[8].

To date, a variety of ER-PM interactions have been identified. It has been shown that soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), together with lipids exposed at the surface of the ER and PM, can be involved in linking these membranes through ionic interactions[9]. These contacts can also be formed through the ER membrane–resident vesicle-associated membrane protein–associated protein (VAP)[10,11], which forms complexes with peripheral PM proteins. Moreover, specialized types of ER-PM associations facilitated by junctophilins have been observed in muscle cells[12]. ER-PM contact sites can also be enriched in proteins involved in signal transduction pathways. For example, interactions occur between the ER membrane–resident protein tyrosine phosphatase 1B (PTP1B) and the insulin receptor localized in the PM[13,14], as well as other cell surface receptors involved in endocytosis[15]. In light of the above information, it becomes clear that the ER is a complex structure that participates in an array of processes within the cell, such as lipid synthesis, protein synthesis, modification and quality control of proteins, vesicular transport and nuclear pore formation (for reviews, see Helle *et al.*[16] and Leitman *et al.*[17]). It seems that some of these functions are allocated to different parts of the ER network. Indeed, a proteomic analysis reveals heterogeneity of the ER (for a comprehensive review, see Helle *et al.*[16]). Moreover, ER is the main intracellular calcium store in resting eukaryotic cells, and it has an important role in calcium ion homeostasis. This intracellular calcium pool is mobilized in activated cells and that event triggers the activation of store-operated calcium entry (SOCE)[18]. In relation to these phenomena, functional ER-PM contacts are formed between complexes of stromal interaction molecule 1 (STIM1) and calcium release–activated calcium channel protein 1 (ORAI1), which are involved in capacitative calcium influx[8]. This process requires translocation of mitochondria to the proximity of the PM, enabling proper calcium signaling[19]. In addition, mitochondria interact with the PM Na^+/Ca^{2+}-exchanger (NCX), a protein involved in calcium extrusion that is able to operate in reverse mode to mediate calcium entry[20]. Part of the mitochondrial network[21] supplies ATP for the activity of calcium pumps located on the PM (PMCA). This can explain the presence of voltage-dependent anion channel (VDAC)—the outer mitochondrial membrane protein in the PAM fraction.

Although the ER-PM interactions are quite strong, it is possible to disrupt them by using low pH (pH 6.0). This feature allows PM- and PAM-enriched fractions to be obtained via crude PM subfractionation, as described previously for yeast by Pichler *et al.*[1], and as described by us for Jurkat cells[8] and mouse liver[4].

© 2014 Nature America, Inc. All rights reserved.

Overview of the procedure

A protocol for PAM isolation was originally described by Pichler *et al.*[1], but that protocol referred only to its isolation from yeast and not from other biological material. The protocol described here can be used to obtain highly purified PAM and PM fractions from rat liver and, if appropriately modified, it can also be used to isolate PAM fractions from other organs, tissues and cell lines. By using this protocol, we have isolated the PAM fraction from Jurkat cells and detected the presence of two proteins involved in the SOCE (ORAI1 and STIM1)[8]. Moreover, we used this protocol to investigate the role of the p66Shc protein in the cellular response to oxidative stress related to aging. We have investigated age-related changes in the amount of p66Shc and its Ser36-phosphorylated form in PAM and MAM fractions[4]. In our opinion, this protocol can be successfully used in studies of intracellular organelle interaction with the PM and involvement of such interactions in many physiological and pathological cellular processes, such as calcium homeostasis. This protocol will be particularly useful for studies on spatial heterogeneity of the PM and relevant intracellular membranes, allowing their lipid and protein contents to be investigated by both lipidomics and proteomics. In addition, the PAM fraction contains protein complexes in their native state, enabling studies of interactions between them and other cellular components.

The protocol is based on a series of differential centrifugations, followed by the fractionation of crude PM on a discontinuous sucrose gradient. The procedure can be divided into two main sections. In the first section, a crude PM fraction is isolated from the tissue (**Fig. 1**). In the second section, crude PM is subfractionated into PM- and PAM-enriched fractions (**Fig. 2**). The protocol allows simultaneous isolation of microsomes, the cytosolic fraction (C) and crude mitochondrial fraction (CMF). Crude mitochondria can subsequently be subfractionated into mitochondria and MAM-enriched fractions, as described in our previous protocol[22]. Note that we do not recommend this protocol for the isolation of highly purified PM. A much better method based on biotinylation of cell-surface proteins and separation of the PM with the use of immobilized NeutrAvidin beads is described in our previous work[8].

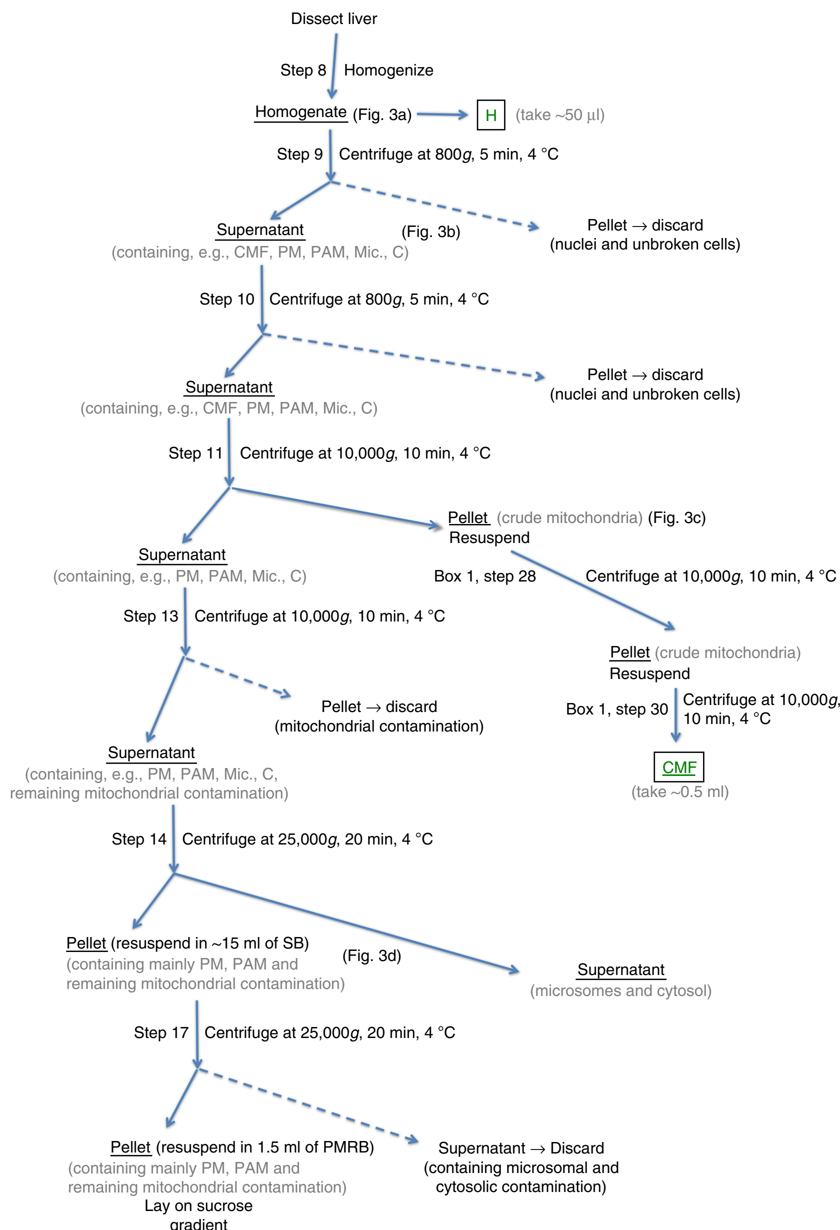

Figure 1 | Timeline and schematic steps for crude PM isolation from rat liver (Steps 1–18). Mic., microsomes.

Experimental design

Starting material. Our protocol shows how to isolate the PAM fraction from the rat liver as an example. The protocol is calculated for one rat liver (9–10 g of wet tissue), and this is enough to isolate a sufficient amount of PAM for proteomic studies (~5–10 mg ml^{-1} in a total volume of ~0.05 ml). Our experience shows that 9 g of liver is an optimal amount of starting material. When, e.g., liver from mouse is used, we recommend using at least 2–3 mouse livers for one isolation.

Scaling (limitation of the protocol). It is very difficult to scale down the procedure even if smaller centrifuge tubes are used. This will result in an insufficient amount of protein content in isolated fractions (especially PAM) for further studies. Moreover, handling much smaller pellets (mainly PAM) could be problematic. Of course, hypothetically it is possible to scale the procedure down, but we took into consideration that the material obtained

(mainly PAM) will be sufficient to run at least 10 electrophoreses to identify the proteins that reside in this fraction.

Using other tissues or organs. When adapting this procedure for other organs and tissues and for cell lines, special attention should be paid to crude PM purification from the mitochondrial fraction. The crucial step of PAM isolation includes crude PM subfractionation on a discontinuous sucrose gradient. As described in the protocol, this step (Step 23) will result in three dense bands containing PAM-, mitochondrial- and PM-enriched fractions from the top to the bottom of the ultracentrifuge tube, respectively. If a tissue or organ or other material characterized by high mitochondrial content is used, there is a risk that the middle band containing mitochondria can be particularly large in comparison with the PAM and PM. This may lead to contamination of the surrounding bands or even make it impossible to separate these fractions. Thus, to optimize PAM and PM isolation, we recommend removing the majority of the mitochondrial fraction in Steps 11–13 by centrifugations at 10,000*g*. If the biological material is characterized by a small amount of mitochondria (e.g., Jurkat cells), the risk of contamination is diminished and a crude PM fraction can be obtained by proceeding directly from the low-speed centrifugation of the homogenate (H) at 800*g* (Steps 9 and 10) to a centrifugation at 25,000*g* (Step 14) (omitting steps to isolate the mitochondrial fraction). The mitochondrial fraction will be obtained together with the PAM and PM.

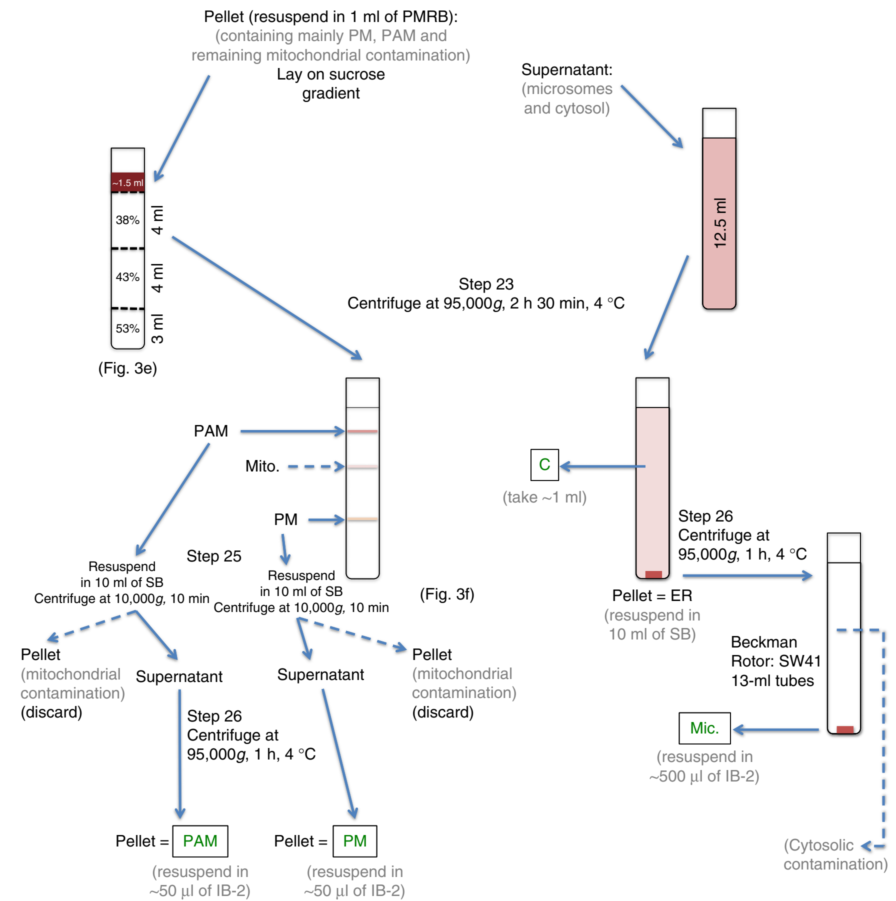

Figure 2 | Timeline and schematic steps for PAM, PM, crude mitochondria, microsome and cytosolic fraction isolation from rat liver (Steps 19–27).

© 2014 Nature America, Inc. All rights reserved.

npg

MATERIALS

REAGENTS

- Rats **! CAUTION** All experiments that use animals should be performed in accordance with institutional guidelines and local, national and, if applicable, international regulations.
- BSA (Sigma-Aldrich, cat. no. A6003) **▲ CRITICAL** BSA is used to remove (bind) free fatty acids. For this reason, it is important to use BSA that is essentially fatty acid free.
- D-Mannitol (Sigma-Aldrich, cat. no. M4125) **▲ CRITICAL** Originally, when low-calcium sucrose was not available, mannitol was used as a substitute. This limitation no longer exists (low-calcium sucrose is available), but we recommend using mannitol owing to its antioxidant properties, which may minimize the presence of free radicals (e.g., those produced by mitochondria) that are responsible for lipid peroxidation of biomembranes[23].
- Sucrose (Merck, cat. no. 100892.9050) **▲ CRITICAL** To isolate highly purified PAM that is not contaminated by fragments of swollen mitochondria, it is necessary to use sucrose with low calcium contamination, which will prevent calcium-dependent mitochondrial swelling.
- Trizma base (Sigma-Aldrich, cat. no. T1503)
- Bis-Tris (Sigma-Aldrich, cat. no. 14879)
- Disodium EDTA (EDTA; Sigma-Aldrich, cat. no. ED2SS)
- Disodium hydrogen phosphate (Na_2HPO_4; Sigma-Aldrich, cat. no. S5136)
- Dulbecco's PBS, liquid, without Ca^{2+} or Mg^{2+} (D-PBS; Invitrogen, cat. no. 70011036)
- EGTA (Sigma-Aldrich, cat. no. E4378)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Magnesium chloride ($MgCl_2$; Sigma-Aldrich, cat. no. M2670)
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P5405)
- Phosphatase inhibitor cocktail 3 (Sigma-Aldrich, cat. no. P0044)
- Protease inhibitor cocktail (Sigma-Aldrich, cat. no. P8340)
- KOH
- Bidistilled water

EQUIPMENT

- Motorized stirrer with an electronic speed controller (Cole-Parmer, cat. no. EW-04369-10)
- Motor-driven tightly fitting glass/Teflon Potter-Elvehjem homogenizer
- Loose-fitting glass/Teflon Potter-Elvehjem homogenizer
- Nalgene 30-ml Oak Ridge tubes for the Sigma angled rotor, 6 × 30 ml (model 12139)
- Ultraclear 14-ml polypropylene tubes for SW41 rotor (Beckman, cat. no. 344060)
- Polycarbonate tubes with cap assembly for 70Ti rotor (Beckman, cat. no. 355618)
- Eppendorf microcentrifuge test tubes, 1.5 ml (cat. no. 0030 120.086)
- Refrigerated low-speed centrifuge, tabletop, for 30- or 1.5-ml tubes depending on the rotor type (or equivalent; Sigma (Braun), model 2K15)
- Sigma angled rotor, 6 × 30 ml (model 12139)

- Optima L-100 XP ultracentrifuge (Beckman Coulter)
- SW 41 Ti Rotor, swinging bucket, 6 × 14 ml, 40,000 r.p.m., 285,000*g* (Beckman, cat. no. 331302)
- Type 70 Ti Rotor, fixed angle, 8 × 39 ml, 70,000 r.p.m., 504,000*g* (Beckman, cat. no. 337922)
- pH meter

REAGENT SETUP

General reagent preparation guidelines Wash all glassware with bidistilled water to avoid Ca^{2+} contamination. Calcium contamination can cause swelling of mitochondria and rupture of the outer mitochondrial membrane. Extreme care should be taken to avoid contamination from ice and tap water in all preparations because they contain calcium.

Tris-HCl, 1 M, pH 7.4 Dissolve 121.14 g of Trizma base in 500 ml of bidistilled water at 22–24 °C, adjust the pH to 7.4 by using HCl and bring the solution volume to 1 liter with bidistilled water. Cool down the solution and check the pH at 4 °C. Adjust the solution, if necessary, to pH 7.4. Store the solution at 4 °C indefinitely.

HEPES, 0.5 M, pH 7.4 Dissolve 59.57 g of HEPES in 400 ml of bidistilled water at room temperature (22–24 °C), adjust the pH to 7.4 with KOH and bring the solution volume to 500 ml with bidistilled water. Cool down the solution and check the pH at 4 °C. Adjust the solution, if necessary, to pH 7.4 and store it at 4 °C indefinitely.

EGTA, 100 mM, pH 7.4 Dissolve 3.8 g of EGTA in 70 ml of bidistilled water, adjust the pH to 7.4 with KOH and bring the solution volume to 100 ml with bidistilled water. Store the solution at 4 °C indefinitely.

EDTA, 100 mM, pH 7.4 Dissolve 2.92 g of EDTA in 70 ml of bidistilled water, adjust the pH to 7.4 with KOH and bring the solution volume to 100 ml with bidistilled water. Store the solution at 4 °C indefinitely.

Starting buffer (SB) Starting buffer contains 225 mM mannitol, 75 mM sucrose and 30 mM Tris-HCl (pH 7.4). It is used to make isolation buffer 1 (IB-1) and isolation buffer 2 (IB-2). Dissolve 20.5 g of mannitol and 13 g of sucrose in 400 ml of bidistilled water and add 15 ml of Tris-HCl (1 M, pH 7.4). Place the buffer at 4 °C for ~30 min to allow it to cool. Check the pH of the buffer and adjust (if necessary) it with KOH (if too low) or HCl (if too high). Bring the solution to a final volume of 500 ml with bidistilled water and store it at 4 °C for up to 2 weeks. A volume of 300–400 ml of SB is enough for one isolation of the PAM and other cellular fractions from the liver. ▲ CRITICAL SB must be made fresh, i.e., not more than 1 d before the experiment.

Isolation buffer 1 IB-1 contains 225 mM mannitol, 75 mM sucrose, 0.5% (wt/vol) BSA, 0.5 mM EGTA and 30 mM Tris-HCl, pH 7.4. Dissolve 0.75 g of albumin in 150 ml of SB, add 750 µl of 100 mM EGTA (pH 7.4) and store it at 4 °C for up to 2 weeks. A suitable volume of IB-1 used for homogenization should be supplemented with protease inhibitor cocktail (20 µl/4 ml) and 1% (vol/vol) phosphatase inhibitor cocktail (final concentration) to avoid sample degradation. ▲ CRITICAL EGTA is recommended for removing traces of Ca^{2+}. It can be replaced by EDTA; however, a lower concentration (not greater than 0.1 mM) should be used. ▲ CRITICAL IB-1 must be freshly made, i.e., not more than 1 d before the experiment.

Isolation buffer 2 IB-2 contains 225 mM mannitol, 75 mM sucrose and 30 mM Tris-HCl (pH 7.4) supplemented with protease inhibitor cocktail (20 µl/4 ml) and 1% (vol/vol) phosphatase inhibitor cocktail (final concentration) to avoid sample degradation. ▲ CRITICAL IB-2 must be freshly made, i.e., not more than 1 d before the experiment.

Mitochondria resuspension buffer (MRB) MRB contains 250 mM mannitol, 5 mM HEPES (pH 7.4) and 0.5 mM EGTA. To prepare 100 ml of MRB, dissolve 4.56 g of mannitol in 80 ml of bidistilled water. Add 1 ml of 0.5 M HEPES (pH 7.4). Place the buffer at 4 °C for ~30 min to cool. Check the pH of the buffer and adjust it (if necessary) with KOH (if too low) or HCl (if too high). Bring the solution to a final volume of 100 ml with bidistilled water and store it at 4 °C for up to 2 weeks.

PM resuspension buffer (PMRB) PMRB contains 5 mM Bis-Tris and 0.2 mM EDTA, pH 6.0. To prepare 500 ml of PMRB, dissolve 0.523 g of Bis-Tris in 400 ml of bidistilled water and add 1 ml of 100 mM EDTA (pH 7.4). Place the buffer at 4 °C for ~30 min to cool. Check the pH of the buffer and adjust it (if necessary) with KOH (if too low) or HCl (if too high). Bring the solution to a final volume of 500 ml with bidistilled water and store it at 4 °C for up to 2 weeks.

Sucrose gradient solution, 38% (wt/wt) Dissolve 19 g of sucrose in 31 g of PMRB. Store it at 4 °C for up to 1 week.

Sucrose gradient solution, 43% (wt/wt) Dissolve 21.5 g of sucrose in 28.5 g of PMRB. Store it at 4 °C for up to 1 week.

Sucrose gradient solution, 53% (wt/wt) Dissolve 26.5 g of sucrose in 23.5 g of PMRB. Store it at 4 °C for up to 1 week.

© 2014 Nature America, Inc. All rights reserved.

PROCEDURE

Isolation of the crude PM fraction from rat liver ● TIMING ~2.5 h

▲ CRITICAL Before starting the PROCEDURE, precool the glassware and homogenizer with a pestle (required at Step 7) in an ice bath for 5 min.

1| Starve the rat (~180–250 g) overnight before isolation of subcellular fractions (**Fig. 1**). Typically, a 180–250-g rat has a 9–10-g liver, and such an amount of tissue is required for one isolation. Note that the crude PM fraction can be isolated from different tissues (here, rat liver is used).

▲ CRITICAL STEP If isolation is to start in the morning, we recommend starving the animals overnight; high levels of glycogen in the liver of nonstarved animals can affect the purity of PAMs by contaminating the mitochondrial fraction[24]. Glycogen does not directly influence the quality (activity) of mitochondria, but most glycogen grains are of a similar size to mitochondria and they will therefore sediment together during centrifugation. Glycogen-contaminated mitochondrial pellets will be whitish in color. Moreover, the presence of glycogen can make protein measurement with the biuret method problematic; glycogen will make the samples muddy.

2| Kill the rat by decapitation via the most ethical and approved method. Dissect the liver and immediately wash it in ice-cold SB.

! CAUTION Different methods of killing animals have little effect on the outcome of the isolation procedure. Only methods approved by the local ethics committee are acceptable (e.g., decapitation, slow replacement of O_2 by CO_2 and breaking the spinal cord) depending on the animal species and animal size, but we strongly suggest using the method that is least stressful for the animal.

▲ CRITICAL STEP Rats do not have a gallbladder. If animals other than rats are used, be sure to carefully remove the gallbladder.

3| Transfer the liver (typically, a 180–250-g rat has a 9–10-g liver) to a 50-ml beaker.

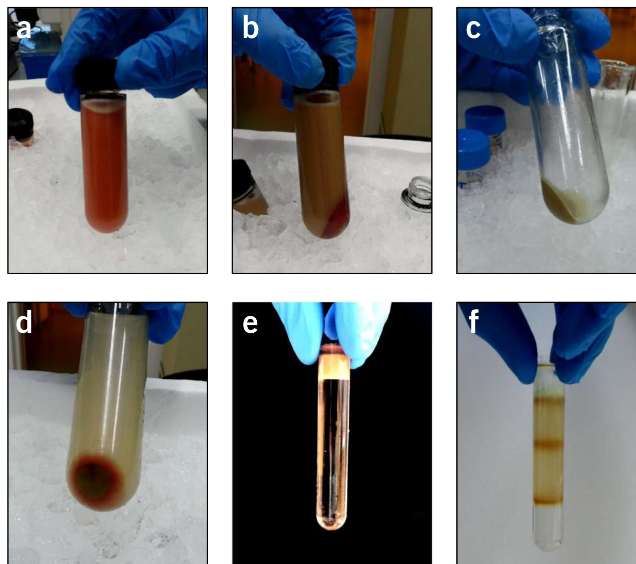

Figure 3 | Images of different types of pellets and fractions obtained during the isolation process. The figure shows a typical result of rat liver fractionation. The final steps of the PROCEDURE result in the isolation of PM- and PAM-enriched fractions. In addition, the isolation protocol enables purification of the CMF, as well as microsomal and cytosolic fractions. The size of the obtained pellets depends on the amount of material used for fractionation. (**a**,**b**) Homogenate (**a**); result of 800*g* for 5 min at 4 °C (Step 9) (**b**); the pellet contains nuclei and unbroken cells. The supernatant contains all the other fractions. (**c**) Pellet of crude mitochondria obtained in Step 12. (**d**) Pellet of crude PM obtained in Step 15. (**e**) Crude PM fraction layered on sucrose gradient (Step 22). (**f**) Result of 2.5 h at 95,000*g* centrifugation (Step 23). The top band contains PAMs, the bottom band contains PM and the middle band represents mitochondrial contamination.

4| Wash the liver three or four times with ice-cold SB to remove blood.

5| Use scissors to cut the liver into small pieces.

6| Discard the bloody SB and wash it once again with 10 ml of fresh ice-cold SB. Discard the bloody SB.

7| Transfer the liver pieces to the 50-ml glass/Teflon Potter-Elvehjem homogenizer. Add IB-1 by using a ratio of 4 ml of IB-1 per 1 g of liver.
▲ **CRITICAL STEP** Homogenization and the subsequent steps must be performed at 4 °C to minimize the activation of proteases and phospholipases.
? TROUBLESHOOTING

8| Homogenize the liver with a Teflon pestle by using 8–10 strokes at 1,500 r.p.m.
! CAUTION Wear protective gloves while using the homogenizer to avoid possible injuries in the unlikely event that the instrument breaks.
▲ **CRITICAL STEP** Our protocol is calculated for one rat liver (9–10 g of wet tissue), and this should be enough to isolate enough PAMs for proteomic studies. If more tissue is to be homogenized, it is necessary to keep the following ratio: 4 ml of IB-1 buffer per 1 g of liver. This increases the volume of homogenate, and thus more centrifuge tubes may be needed to perform subsequent centrifugations.
▲ **CRITICAL STEP** The following homogenization parameters can result in increased contamination of PAMs by broken mitochondria: longer durations; higher forces; higher speed; and a too-tight glass/Teflon homogenizer. Contamination can also be minimized by moving the pestle slowly and smoothly.
? TROUBLESHOOTING

9| Transfer the homogenate to a 30-ml polypropylene centrifugation tube (**Fig. 3**) and centrifuge it at 800*g* for 5 min at 4 °C (this can be performed in a Sigma refrigerated low-speed centrifuge).

10| Discard the pellet (unbroken cells and nuclei, **Fig. 3b**) and centrifuge the supernatant again for 5 min at 800*g* and 4 °C (Sigma refrigerated low-speed centrifuge).

11| Discard the pellet (if present) and centrifuge the supernatant for 10 min at 10,000*g* and 4 °C (Sigma refrigerated low-speed centrifuge).

12| Collect both the supernatant and the pellet: the supernatant contains PM, microsomes and cytosolic proteins; the pellet contains the crude mitochondrial fraction (**Fig. 3c**). To process the supernatant, continue with Step 13. To process the mitochondrial pellet, gently resuspend it in 20 ml of ice-cold SB—it can then be stored on ice or further processed to isolate mitochondria-associated membranes and purified mitochondria as described in **Box 1**.
▲ **CRITICAL STEP** Resuspension of mitochondria at this step with a pipetter or tight Potter can result in the rupture of the outer mitochondrial membrane. It is necessary to transfer the mitochondrial pellet from the centrifuge tube to the homogenizer with a 1-ml automatic pipette with the end of the tip cut off. For the resuspension process, a loose-fitting glass/Teflon Potter-Elvehjem homogenizer (two or three strokes by hand are enough) or a soft paintbrush should be used.
? TROUBLESHOOTING
■ **PAUSE POINT** If it is not being processed straight away, the resuspended crude mitochondrial fraction should be stored on ice (4 °C) until centrifugation at Step 28 (**Box 1**), but for no longer than 3–3.5 h. Do not freeze this fraction if you want to isolate the MAM fraction. Freezing will cause damage to the integrity of mitochondrial membranes.

© 2014 Nature America, Inc. All rights reserved.
npg

Box 1 | Purification of the crude mitochondrial fraction ● TIMING ~1 h

To save time, the following procedure can be performed simultaneously with Steps 13–16 of the PROCEDURE for crude PM isolation or during the centrifugation step described in Step 23 of the PROCEDURE.
1. Centrifuge the mitochondrial suspension obtained in Step 12 of the PROCEDURE for 10 min at 10,000*g* and 4 °C (Sigma refrigerated low-speed centrifuge).
2. Discard the supernatant and gently resuspend the crude mitochondrial pellet in 20 ml of ice-cold SB.
▲ CRITICAL STEP To avoid breaking the outer mitochondrial membrane or detaching the MAM fraction from mitochondria, transfer the mitochondrial pellet from the centrifuge tube to the homogenizer by using a 1-ml automatic pipette with the end of the tip cut off. For the resuspension process, a loose-fitting glass/Teflon Potter-Elvehjem homogenizer is used (two or three delicate strokes by hand are enough).
3. Centrifuge the mitochondrial suspension again for 10 min at 10,000*g* (Sigma refrigerated low-speed centrifuge).
▲ CRITICAL STEP It is important to perform all three centrifugation steps (at 10,000*g*) to remove any PM or microsomal contamination.
4. Resuspend the crude mitochondrial pellet in 2 ml of ice-cold MRB supplemented with protease inhibitor cocktail (20 μl/4 ml) and 1% (vol/vol) phosphatase inhibitor cocktail (final concentration) to avoid sample alteration. The obtained crude mitochondrial fraction can be used for further subfractionation to isolate MAMs and mitochondria of increased purity as described by us before[22].
■ PAUSE POINT A small amount (0.5 ml) of the crude mitochondrial fraction should be stored for further investigations (e.g., western blotting) in a 1.5-ml Eppendorf microcentrifuge tube. This material should be frozen at −80 °C for up to 6 months or at −20 °C for up to 1 month if it is not used immediately.

© 2014 Nature America, Inc. All rights reserved.

npg

13| Centrifuge the supernatant for 10 min at 10,000*g* and 4 °C to remove any mitochondrial contamination (Sigma refrigerated low-speed centrifuge).

14| Centrifuge the supernatant for 20 min at 25,000*g* and 4 °C to obtain the crude PM fraction (this step can be performed in the Beckman ultracentrifuge with the 70Ti rotor or equivalent).

15| Collect both the supernatant (this is the fraction containing microsomes and cytosolic proteins) and the pellet (**Fig. 4**; containing the crude PM fraction). The supernatant can be stored on ice or it can be processed to further purify microsomal and cytosolic fractions; proceed directly to Step 23. Proceed with Step 16 to prepare the crude PM pellet for further fractionation.
▲ CRITICAL STEP Transfer the supernatant to a 14-ml thin-walled polyallomer ultracentrifuge tube. Note that complete filling of the tubes is important to prevent damage during centrifugation.
? TROUBLESHOOTING
■ PAUSE POINT The supernatant containing microsomes and cytosolic proteins should be stored on ice (4 °C) until centrifugation at Step 23, but for no longer than 3–3.5 h. This supernatant cannot be frozen before further isolation of microsomal and cytosolic fractions because freezing will cause damage to microsomal membrane integrity and contaminate the cytosolic fraction with microsomal proteins.

16| Gently resuspend the crude PM pellet in 15 ml of ice-cold SB.

17| Centrifuge the crude PM suspension for 20 min at 25,000*g* and 4 °C to remove microsomal and cytosolic contamination (Beckman ultracentrifuge and 70Ti Rotor or equivalent).

18| Gently resuspend the crude PM pellet in 1.5 ml of ice-cold PMRB.

Fractionation of crude PM ● TIMING ~5–6 h
19| Create a discontinuous sucrose gradient as described in Reagent Setup (**Fig. 2**). Add 3 ml of 53% (wt/wt) sucrose gradient solution to a 14-ml thin-walled polyallomer ultracentrifuge tube.

20| Layer 4 ml of the 43% (wt/wt) sucrose solution on top of 3 ml of 53% (wt/wt) sucrose gradient solution in the ultracentrifuge tube.

21| Layer 4 ml of the 38% (wt/wt) sucrose gradient solution on top of 4 ml of 43% (wt/wt) sucrose medium in the ultracentrifuge tube.

22| Layer the suspension of crude PM collected in Step 18 on top of the 11-ml discontinuous sucrose gradient in the ultracentrifuge tube (**Fig. 3e**). Subsequently, gently layer PMRB solution (~2 ml) on top of the crude PM suspension to fill up the centrifuge tube (the top of the suspension should remain 4–5 mm below the edge of the 14-ml tube).
▲ CRITICAL STEP Complete filling of the tubes is important to prevent damage of the centrifuge tubes during centrifugation.
? TROUBLESHOOTING

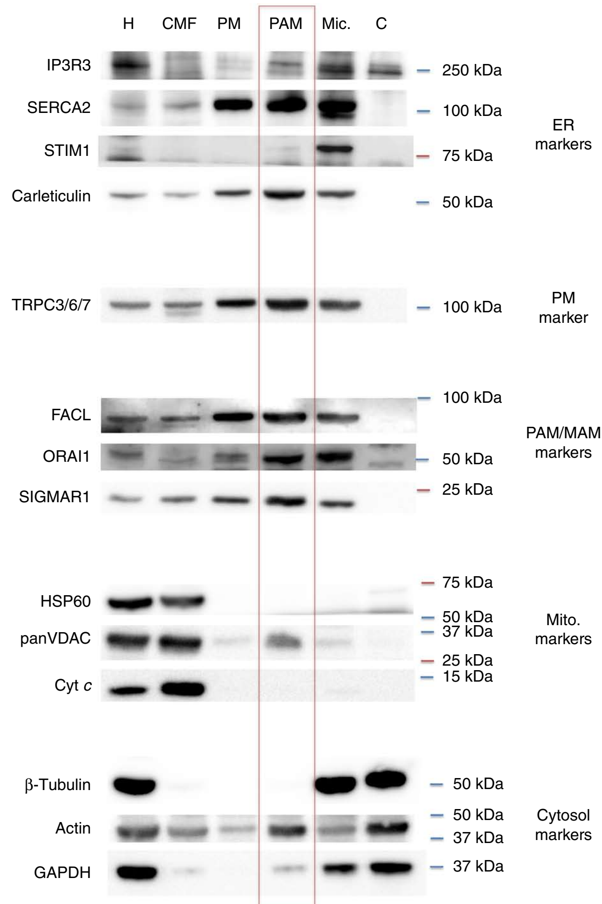

Figure 4 | Intracellular distribution of SIGMAR1 and other markers. Proteins were quantified by using the Bradford assay (Bio-Rad Laboratories), and 15 μg of protein was separated on a Novex NuPage Bis-Tris 4–12% (wt/vol) precast gel (Invitrogen) and transferred to polyvinylidene fluoride membranes for standard western blotting. The membranes were blocked by using 2% (wt/vol) nonfat milk in PBS buffer with 0.01% (vol/vol) Tween-20 (Sigma-Aldrich) for 1 h. Proteins were detected with anti-IP3R3 (1:500; Santa Cruz Biotechnology), anti-SERCA2 (1:1,000; Santa Cruz Biotechnology), anti-STIM1 (1:500; Santa Cruz Biotechnology), anti-carleticulin (1:1,000; Upstate Biotechnology), anti-TRPC3/6/7 (1:1,000; Santa Cruz Biotechnology), anti-FACL (1:500; Santa Cruz Biotechnology), anti ORAI1 (1:1,000; Santa Cruz Biotechnology), anti-SIGMAR1 (1:500; Sigma), anti-HSP60 (1:1,000; Santa Cruz Biotechnology), anti-panVDAC (1:1,000; Abcam), anti-cyt *c* (1:5,000; Santa Cruz Biotechnology), anti-β-tubulin (1:5,000; Santa Cruz Biotechnology), anti-actin (1:5,000; Sigma) and anti-GAPDH (1:1,000; Abcam), followed by secondary horseradish peroxidase (HRP)-conjugated antibodies (1:2,500) from Santa Cruz Biotechnology. H, homogenate; Mic., microsomes; C, cytosol.

© 2014 Nature America, Inc. All rights reserved.

23| Centrifuge the mixture for 2.5 h at 95,000*g* in a Beckman Coulter Optima L-100 XP ultracentrifuge (Beckman SW41 rotor) (accel = 5, decel = 5). If you wish to isolate microsomal and cytosolic fractions, include the supernatant from Step 15 at this centrifugation step.
▲ **CRITICAL STEP** For the crude PM sample, this step should result in three dense bands containing the following: purified PM located approximately at the interface between the 53% and 43% (wt/wt) sucrose gradient solutions (**Figs. 2** and **3f**); PAM, visible as a band located approximately on top of the 38% (wt/wt) sucrose gradient solution (**Figs. 2** and **3f**); and a third band located approximately at the interface between the 43% and 38% (wt/wt) sucrose gradient solutions (**Figs. 2** and **3f**), which is composed of mitochondria tightly interacting with the PM.

24| From the crude PM sample, collect the PAM and PM fractions from the sucrose gradient with a Pasteur pipette and dilute them 10 times with SB solution (~10 ml). The band containing mitochondria can be discarded because crude mitochondria have been obtained in Step 12, and afterward they can be purified to a high-quality crude mitochondrial fraction (**Box 1**). From the second centrifuge tube, collect the supernatant (cytosolic proteins) and pellet containing microsomes. Resuspend the pellet containing the microsomal fraction in 10 ml of SB. To eliminate cytosolic contamination from the microsomal fraction, process it simultaneously with the PAM and PM fractions as described in Step 26 below.
■ **PAUSE POINT** Store the supernatant containing the cytosolic fraction for further investigations (e.g., western blotting) in a 1.5-ml Eppendorf microcentrifuge tube. Freeze the tube at –80 °C for up to 6 months or at –20 °C up to 1 month if it is not used immediately.

25| Centrifuge the PAM and PM suspensions at 10,000*g* for 10 min (Sigma refrigerated low-speed centrifuge) to eliminate any mitochondrial contamination (present as a pellet that can be discarded).

26| Gently transfer the PAM and PM supernatants to polycarbonate tubes and centrifuge them at 95,000*g* for 1 h (Beckman rotor SW41) together with the microsomal fraction obtained at Step 24.

27| Discard the supernatants and resuspend the PAM, PM and microsomal pellets in 50 μl, 50 μl and 500 μl of IB-2, respectively.
? TROUBLESHOOTING
■ **PAUSE POINT** Store PM-, PAM- and microsomal (ER)-enriched fractions for further investigations (e.g., western blotting) in 1.5-ml Eppendorf microcentrifuge tubes. Freeze the tubes at –80 °C up to 6 months or at –20 °C up to 1 month if it is not used immediately.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible cause	Solution
7, 8, 27	PAM band is not visible	Not enough material was used for isolation	Use more material or tissue for homogenization
		Homogenization was insufficient to rupture cells sufficiently	Check the effectiveness of homogenization and homogenize longer or with a tighter pestle Avoid homogenization of the material when it is too dense. Dilute it with homogenization buffer
		Pellets were lost after one of the centrifugations	Try to discard supernatants more gently to avoid detaching pellets
		Not all of the material was collected from the sucrose gradient	Collect the PAM and PM fractions from the sucrose gradient more carefully. When the supernatant is poured off, the PAMs or PM pellets (thin bright 'membranes') may be detached. Remove PAMs and PM membrane directly without discarding the supernatant
8, 12, 15	PAMs are highly contaminated by the markers characteristic for the other organelles (e.g., mitochondria)	Crude PM was not purified well	Dilute tissue homogenate before first centrifugation (Step 9) Add one more centrifugation step (10 min at 10,000*g*) after Step 13 or Step 25 Dilute crude PM suspension (Step 16)
		Too much material was loaded on the gradient	Load less crude PM on the top of the gradient (in this case, we recommend loading less than half of the original amount)
		Mitochondria were broken or became swollen during isolation and contaminated the crude PM fraction	If PAMs and PMs are highly contaminated with mitochondrial markers (especially from the inner mitochondrial membrane), we recommend resuspending the PAM and PM fractions obtained in Step 24 in 10 mM Tris-HCl (pH 7.4) instead of SB solution If PAM and PM is highly contaminated with mitochondrial markers (especially from the inner mitochondrial membrane) Check whether the animals were starved before the experiment
		Homogenization performed for too long with too high a force, or with too tight glass/Teflon homogenizer, resulting in higher contamination of PAMs by destroyed mitochondria	Decrease homogenization time or use a less tightly fitting glass/Teflon homogenizer
22	After sucrose gradient centrifugation, it is difficult to distinguish three dense bands containing purified PMs, PAMs and mitochondria	Incorrect density of individual sucrose gradients solutions	Check calculations and/or prepare new sucrose gradient solutions
		Gradient break	Be more careful when creating the discontinuous sucrose gradient Be more careful when layering the crude PM suspension on the discontinuous sucrose gradient (Step 22)
		Too much material was loaded on the top of the gradient	Load less crude PM on the top of the gradient (in this case, we recommend loading less than half of the original amount)
	PAM band appears fragmented	Gradient break	Be more careful when creating the discontinuous sucrose gradient Be more careful when layering the crude PM suspension on the discontinuous sucrose gradient (Step 22)

© 2014 Nature America, Inc. All rights reserved.

● TIMING

Steps 1–18, isolation of crude PM fraction from rat liver: ~2.5 h
Steps 19–27, fractionation of crude PM: ~5–6 h (including purification of microsomal and cytosolic fractions)
Box 1, purification of the crude mitochondrial fraction: ~1 h

ANTICIPATED RESULTS

The typical yield of PAM preparation is ~5–10 mg ml^{-1} in a total volume of ~0.05 ml. The quality of the PAM preparation can be checked by western blot analysis. By using different markers for the obtained fractions, it is possible to check the purity of the PAM fraction and identify contaminants from other cellular compartments, as well as to identify new proteins that reside in the PAM.

As an example, **Figure 4** shows the presence of different marker proteins in the fractions isolated from rat liver. We demonstrated that three proteins, ORAI1, long-chain fatty-acid CoA synthase (FACL) and SIGMAR1, are considered to be members of the PAM fraction.

A summary of the expected distribution of characteristic protein markers can be found in **Table 2**. Additional information about specific markers can be found below.

SERCA2

This is an ER marker, but it should also be present in the PAM, representing the PM-ER contact sites. Its presence in the PM fraction can be an indication that some intracellular components have been trapped in the PM, forming vesicles after cell

TABLE 2 | Expected distribution of characteristic protein markers in the isolated fractions.

Cellular compartment	Marker	Fraction						Comment
		PAM	MAM	ER	PM	Mitochondria	Cytosol	
PAM/MAM	ORAI1	+						Known as a protein directly interacting with stromal-interacting molecule 1 (STIM1)
	FACL	+++	+++	+		+		Long-chain fatty-acid CoA synthase is a single-pass membrane protein
	SIGMAR1	+++	+++	+	+			Sigma-1 receptor. Regulator of inter-organellar membrane microdomains
ER	IP_3R	+	+	+++				Inositol 1,4,5-triphosphate receptor. The ER channel responsible for agonist-dependent ER-Ca^{2+} release
	SERCA2	++		+++	++			Sarco(endo)plasmic reticulum Ca^{2+} ATPase 2. The ATP-dependent calcium pump responsible for calcium storage in the ER
	STIM1	+/–		+++				Stromal-interacting molecule 1 is the ER protein mediating store-operated Ca^{2+} entry (SOCE). It acts as a Ca^{2+} sensor in the ER
	Carleticulin	++		+++	+	+/–		KDEL-containing protein
PM	TRPC3/6/7	+++		+	++			Transient receptor potential cation channels 3/6/7 are the PM proteins involved in the store-operated Ca^{2+} entry
Mito	VDAC	+	+			+++		The voltage-dependent anion channel
	Cytochrome *c*					+++		Component of electron transfer chain
	HSP60					+++		Mitochondrial matrix protein
Cytosol	Tubulin						+++	
	Actin						+++	
	GAPDH						+++	Glyceraldehyde 3-phosphate dehydrogenase

The relative amount of protein markers is indicated as follows: +++, highly enriched; ++, enriched; +, present; +/– present in low amount or absent. If not otherwise indicated, the protein was not found in the fraction.

© 2014 Nature America, Inc. All rights reserved.

npg

damage caused by homogenization. This interpretation can be supported by the presence of a low amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a cytosol marker.

Carleticulin
This is an ER protein, but its presence in the other subcellular compartments has been confirmed. Carleticulin has been found in nuclear and cytosolic fractions, Golgi compartment and in the PM vesicles[25].

TRPC3/6/7 (transient receptor potential cation channels)
Their enrichment in the PAM fraction is in agreement with the previous studies of Lillemeier *et al.*[26]. The group postulated that the PM proteins in Jurkat cells are clustered into separated 'islands' and that the PAM fraction isolated from these cells preferentially contains these subregions of the PM. Such regions contain proteins responsible for interaction with intracellular organelles and, e.g., store-operated Ca^{2+} entry. This can explain quite a high level of these proteins in the PAM fraction. Moreover, the presence of these proteins in the ER fraction can be explained by the contamination of the ER with very small PM vesicles. A method for purification of the PM from the microsomal fraction has been described in detail by Hinton[27].

Voltage-dependent anion channel (VDAC)
This is also known as mitochondrial porin, and it is localized on the outer mitochondrial membrane and is responsible for the permeability of that membrane. It can also be present in the PAM fraction, representing the PM-mitochondria contact sites.

Cytochrome *c* (cyt *c*)
Its presence in the other fraction indicates mitochondrial contamination. Cyt *c* detected in the cytosolic fraction is a signal that the outer mitochondrial membrane was broken (caused, e.g., by mitochondrial swelling or by extensive homogenization) and mitochondrial proteins present in the mitochondrial intermembrane space were released.

HSP60 (heat-shock protein 60)
This is a mitochondrial matrix protein. Similarly to cyt *c*, its presence in other fractions indicates mitochondrial contamination.

Tubulin, GAPDH
As cytosolic markers, these proteins should be absent from CMF, PM and PAMs. Their presence in the PM fraction can indicate that some of the cytosolic compartment has been trapped in the PM, forming vesicles after cell damage caused by homogenization. These vesicles can contaminate the ER, resulting in the presence of GAPDH in the ER fraction.

Actin
As a cytosol marker, it should be enriched in the cytosolic (C) fraction. It can also be detected in other fractionsor organelles that interact with the cytoskeleton.

© 2014 Nature America, Inc. All rights reserved.

ACKNOWLEDGMENTS This research was supported by grants from the Polish National Science Centre (UMO-2011/01/M/NZ3/02128), Iuventus Plus (UMO-0531/IP1/2011/71) and Polish Ministry of Science and Higher Education grant W100/HFSC/2011 to J.M.S., M.L., A.W., J.D. and M.R.W.; by the Italian Association for Cancer Research to C.G. and P.P.; and by Telethon (GGP11139B), the Italian Ministry of Education, University and Research, and the Italian Ministry of Health to P.P. J.M.S. was also supported by a Ph.D. fellowship from The Foundation for Polish Science (FNP), and by the EU, the European Regional Development Fund and the 'Innovative economy' Operational Programme. M.L. was supported by the 'Mobility Plus' fellowship from the Polish Ministry of Science and Higher Education.

AUTHOR CONTRIBUTIONS J.M.S., M.L., A.W. and C.G. carried out all experimental steps and performed characterization of the isolated subcellular fractions. J.M.S., J.D., P.P. and M.R.W. developed the concept, designed experiments and wrote the paper. All authors discussed the results and commented on the manuscript at all stages.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Pichler, H. *et al.* A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids. *Eur. J. Biochem.* **268**, 2351–2361 (2001).
2. Achleitner, G. *et al.* Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. *Eur. J. Biochem.* **264**, 545–553 (1999).
3. Wu, M.M., Buchanan, J., Luik, R.M. & Lewis, R.S. Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. *J. Cell Biol.* **174**, 803–813 (2006).
4. Lebiedzinska, M., Duszynski, J., Rizzuto, R., Pinton, P. & Wieckowski, M.R. Age-related changes in levels of p66Shc and serine 36-phosphorylated p66Shc in organs and mouse tissues. *Arch. Biochem. Biophys.* **486**, 73–80 (2009).
5. Vance, J.E. & Tasseva, G. Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. *Biochim. Biophys. Acta.* **1831**, 543–554 (2013).
6. Perry, R.J. & Ridgway, N.D. Oxysterol-binding protein and vesicle-associated membrane protein-associated protein are required for sterol-dependent activation of the ceramide transport protein. *Mol. Biol. Cell* **17**, 2604–2616 (2006).
7. Yan, D. & Olkkonen, V.M. Characteristics of oxysterol binding proteins. *Int. Rev. Cytol.* **265**, 253–285 (2008).

8. Kozieł, K. *et al.* Plasma membrane associated membranes (PAM) from Jurkat cells contain STIM1 protein: is PAM involved in the capacitative calcium entry? *Int. J. Biochem. Cell Biol.* **41**, 2440–2449 (2009).
9. Camici, O. & Corazzi, L. Phosphatidylserine translocation into brain mitochondria: involvement of a fusogenic protein associated with mitochondrial membranes. *Mol. Cell Biochem.* **175**, 71–80 (1997).
10. Lapierre, L.A., Tuma, P.L., Navarre, J., Goldenring, J.R. & Anderson, J.M. VAP-33 localizes to both an intracellular vesicle population and with occludin at the tight junction. *J. Cell Sci.* **112**, 3723–3732 (1999).
11. Lebiedzinska, M., Szabadkai, G., Jones, A.W., Duszynski, J. & Wieckowski, M.R. Interactions between the endoplasmic reticulum, mitochondria, plasma membrane and other subcellular organelles. *Int. J. Biochem. Cell Biol.* **41**, 1805–1816 (2009).
12. Kakizawa, S., Moriguchi, S., Ikeda, A., Iino, M. & Takeshima, H. Functional crosstalk between cell-surface and intracellular channels mediated by junctophilins essential for neuronal functions. *Cerebellum* **7**, 385–391 (2008).
13. Anderie, I., Schulz, I. & Schmid, A. Direct interaction between ER membrane-bound PTP1B and its plasma membrane-anchored targets. *Cell Signal* **19**, 582–592 (2007).
14. Goldstein, B.J., Bittner-Kowalczyk, A., White, M.F. & Harbeck, M. Tyrosine dephosphorylation and deactivation of insulin receptor substrate-1 by protein-tyrosine phosphatase 1B. Possible facilitation by the formation of a ternary complex with the Grb2 adaptor protein. *J. Biol. Chem.* **275**, 4283–4289 (2000).
15. Hernández, M.V., Sala, M.G., Balsamo, J., Lilien, J. & Arregui, C.O. ER-bound PTP1B is targeted to newly forming cell-matrix adhesions. *J. Cell Sci.* **119**, 1233–1243 (2006).
16. Helle, S.C. *et al.* Organization and function of membrane contact sites. *Biochim. Biophys. Acta* **1833**, 2526–2541 (2013).
17. Leitman, J., Ron, E., Ogen-Shtern, N. & Lederkremer, G.Z. Compartmentalization of endoplasmic reticulum quality control and ER-associated degradation factors. *DNA Cell Biol.* **32**, 2–7 (2013).
18. Lam, A.K. & Galione, A. The endoplasmic reticulum and junctional membrane communication during calcium signaling. *Biochim. Biophys. Acta* **1833**, 2542–2559 (2013).
19. Quintana, A. *et al.* Sustained activity of calcium release-activated calcium channels requires translocation of mitochondria to the plasma membrane. *J. Biol. Chem.* **281**, 40302–40309 (2006).
20. Poburko, D. *et al.* Mitochondria buffer NCX-mediated Ca^{2+}-entry and limit its diffusion into vascular smooth muscle cells. *Cell Calcium* **40**, 359–371 (2006).
21. Giorgi, C., De Stefani, D., Bononi, A., Rizzuto, R. & Pinton, P. Structural and functional link between the mitochondrial network and the endoplasmic reticulum. *Int. J. Biochem. Cell Biol.* **41**, 1817–1827 (2009).
22. Wieckowski, M.R., Giorgi, C., Lebiedzinska, M., Duszynski, J. & Pinton, P. Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells. *Nat. Protoc.* **4**, 1582–1590 (2009).
23. England, M.D. *et al.* Influence of antioxidants (mannitol and allopurinol) on oxygen free radical generation during and after cardiopulmonary bypass. *Circulation* **74**, 134–137 (1986).
24. Nur, T., Sela, I., Webster, N.J. & Madar, Z. Starvation and refeeding regulate glycogen synthase gene expression in rat liver at the posttranscriptional level. *J. Nutr.* **125**, 2457–2462 (1995).
25. Gold, L.I. *et al.* Calreticulin: non-endoplasmic reticulum functions in physiology and disease. *FASEB J.* **24**, 665–683 (2010).
26. Lillemeier, B.F., Pfeiffer, J.R., Surviladze, Z., Wilson, B.S. & Davis, M.M. Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. *Proc. Natl. Acad. Sci. USA* **103**, 18992–18997 (2006).
27. Hinton, R.H. Purification of plasma membrane fragments. In *Subcellular components. Preparation and fractionation* 2nd edn, (ed. Birnie, G.D.) 119–156 (Butterworths, 1972).

© 2014 Nature America, Inc. All rights reserved.

npg