Subcellular fractionation

Cox B, Emili A. Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics. *Nat. Protoc.* 1:1872–1878 (2006).

Materials Needed

* Homogenization
  + Cell lifters (Corning 3008), 1 per treatment
  + Protease and phosphatase inhibitors
  + Dounce homogenizers, 7 mL or 15 mL glass, 1 per group ([Wheaton 357544](http://wheaton.com/15-ml-tissue-grinder-dounce.html))
  + Other standard cell culture supplies
* Pipettes (air and positive displacement, pipet aid)
* Centrifuge
* Centrifuge tubes
* Ultracentrifuge
* Ultracentrifuge tubes (Beckman Coulter 344059)
* Reagents listed below

Prep

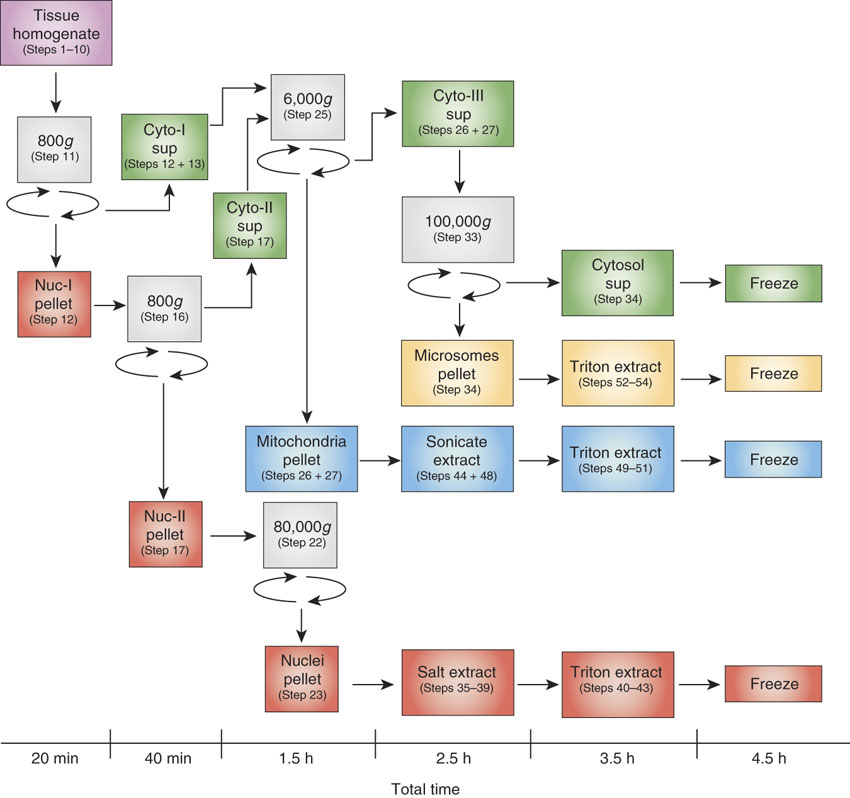
Advance prep

1. If harvesting cells, split into the desired number of dishes far enough in advance for 70% confluency on the day of the experiment. For HEK-293 cells, this would be 5 days in advance if seeded at a 1:30 dilution of a cell suspension with 105 cells, or 4 days at 1:20, with medium replenished once. ≥20 150 mm dishes may be required to get a measurable protein yield from HEK cells, especially for the microsomal fractions. The 500 cm2 square culture dishes can also be used (1 can replace 3 dishes).
2. **Reserve ultracentrifuge.** You will need it late in the day.
3. **Set out tubes.**
   1. Microcentrifuge tubes per sample/treatment
      1. 5-10 1.5 mL tubes for aliquotting cyto-I supernatant before centrifugation (number of tubes depends on volume used for homogenization)
      2. 1 1.5 mL tube for extraction of microsome proteins
      3. 2 1.5 mL tubes for microsomal affinity purification and input
      4. 1 tube for extraction of nuclear proteins
      5. 2 1.5 mL tubes for nuclear affinity purification and input
   2. 2 15 or 50 mL tubes per sample/treatment
   3. Tubes do not need to be thoroughly labeled beforehand. Most of them will not be saved.
4. **Prep reagents on next page.** Protease and phosphatase inhibitors, spermine and spermidine can be added to buffers the day before if needed (see reagent prep below). If treating cells, add after beginning treatments.
5. If treating cells, **set out medium the night before** at room temp or in 37°C bead bath.
6. **Refrigerate PBS for harvesting cells.**
7. **On the day of fractionation, set out ice, buffers, Dounce homogenizers, and tubes.**

Reagent prep

* **250-STM Buffer:** Stock buffer for initial homogenization.Filter-sterilize and store at 4°C. Consists of 250 mM sucrose (85.575 g/L), 50 mM Tris-HCl (pH 7.4) (50 mL 1 M Tris-HCl/L), 5 mM MgCl2 (5 mL 1 M MgCl2/L).
  + **Add before use:**
    - **Protease and phosphatase inhibitors**: ThermoFisher Halt 78441: dilute 1:100 (10 μL/mL). Add 450 μL to 50 mL so you have 50 μL left in 1 mL tube for 5 mL lysis buffer. Sigma P8340: dilute 1:50-1:100, add phosphatase inhibitors. Sigma P8340 not recommended because AEBSF can shift mass spec peaks. See datasheets for ThermoFisher Halt and Sigma MS SAFE. PMSF is also not recommended because it is highly unstable and only inhibits serine proteases. See Sigma P7626 datasheet.
* **~~250-STMDPS Buffer:~~** ~~Working buffer for initial homogenization.~~ **~~10 mL total per sample: 5 mL/tube of cells (10 dishes, each dish scraped in 5 mL PBS) for homogenization, 5 mL to balance ultracentrifuge tube. Make 10% extra (35 mL for 60 dishes).~~**
  + **~~Add before use:~~**
    - **~~Protease and phosphatase inhibitors~~**~~: ThermoFisher Halt 78441: dilute 1:100 (10 μL/mL). Add 450 μL to 50 mL so you have 50 μL left in 1 mL tube for 5 mL lysis buffer. Sigma P8340: dilute 1:50-1:100, add phosphatase inhibitors. Sigma P8340 not recommended because AEBSF can shift mass spec peaks. See datasheets for ThermoFisher Halt and Sigma MS SAFE. PMSF is also not recommended because it is highly unstable and only inhibits serine proteases. See Sigma P7626 datasheet.~~
    - **~~Spermine and spermidine~~** ~~to 25 μg/mL (1.25 mg/50 mL, or 50 μL 1:1000 stock solution)~~
      * ~~These are cationic polyamines used to precipitate DNA.~~
      * ~~124 μM spermine (MW: 202.34), 172 μM spermidine (MW 145.25)~~
      * ~~Spermine and spermidine stock solutions (make by weight rather than molarity):~~ 
        + ~~Dissolve 25 mg in 1 mL np H~~~~2~~~~O: tare an empty microtube, add 25 mg, then add H~~~~2~~~~O, mix with Eppendorf ThermoMixer. Store at -20°C. Dilute 1:1000 to 25 μg/mL.~~
        + ~~MilliporeSigma: Soluble at 50 mg/mL in H~~~~2~~~~O. 0.22 μM filter. Will oxidize over time. Stable at -20°C for 1 month (only validated for spermidine).~~
        + ~~Also see Cold Spring Harbor Protocols 2006 (doi:10.1101/pdb.rec8627).~~
    - ~~DTT: Dilute NuPAGE reducing agent (500 mM DTT) 1:500 (100 μL/50 mL).~~ **~~DTT may leach antibody from ThermoFisher 88837 magnetic beads. Omit unless necessary.~~**
* **2 M STM Buffer:** Stock buffer for second homogenization and cushion during preparation of nuclear lysate. Filter-sterilize and store at 4°C. Consists of 2 M sucrose (684.60 g/L), 50 mM Tris-HCl (pH 7.4) (50 mL 1 M Tris-HCl/L), 5 mM MgCl2 (5 mL 1 M MgCl2/L).
  + Dissolve the sucrose by mixing at an elevated temperature. Set hot plate to 95°C, and add sucrose gradually. Will take 30-60 minutes to dissolve. The sucrose can add 5-600 mL to the buffer volume. Begin with 300 mL and gradually bring up to 1 L volume. This solution will rapidly clog a 0.22 μm filter, but 0.45 μm filters can be used.
  + **10 mL/sample, 35 mL/60 dishes.**
  + **Add protease and phosphatase inhibitors, spermine and spermidine to 2 M STM**.
* **~~2 M STMDPS Buffer:~~** ~~Working buffer for second homogenization and cushion.~~
* **Lysis buffer for protein extraction**
  + **BWS: ~2 mL lysis buffer required/sample: 1 mL for nuclear pellet, 0.5 mL for microsomal pellet, additional buffer for adjusting [protein] after BCA. 5 mL adequate for 3 samples.**
  + **Add before use:**
    - **Protease and phosphatase inhibitors**: ThermoFisher Halt 78441: dilute 1:100 (10 μL/mL).
  + **BWS:** RIPA buffer should not be used because of potential disruption of protein complexes. RIPA contains 25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS. Denaturing urea or SDS-based buffers can disrupt protein complexes. This is why there is a separate IP lysis buffer recommended for IP instead of RIPA. See [Broad proteomics lectures](https://www.broadinstitute.org/partnerships/education/broade/proteomics-everything-you-always-wanted-know-were-afraid-ask).
  + **IP lysis buffer:** 25 mM Tris•HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol
  + **ME Buffer:** Membrane extraction buffer for mitochondria and microsomes. Filter-sterilize and store at 4°C. Consists of 20 mM Tris-HCl (pH 7.8), 0.4 M NaCl, 15% glycerol, 1 mM DTT, 1 mM PMSF and 1.5% Triton-X-100.
    - ME buffer is similar to IP lysis buffer, with Triton X-100 instead of NP-40. NP-40 solubilizes ER well (Babu Nature 2012 Figure S1), but Triton X-100 is most effective for membranes.
    - 2 mL 1 M Tris-HCl/100 mL ME
    - 2.34 g NaCl/100 mL ME
    - 15 mL glycerol/100 mL ME
    - 15 mL 10% Triton X-100/100 mL ME
  + **NE Buffer:** For extraction of nuclear proteins. Filter-sterilize and store at 4°C. Consists of 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.5 M NaCl, 0.2 mM EDTA and 20% glycerol.
  + **NET Buffer:** Make up to 1% Triton-X-100, 1 mM DTT, 1 mM PMSF in NE Buffer.
    - 2 mL 1 M HEPES/100 mL NET.1 M HEPES: 11.92 g/50 mL
    - 150 μL 1M MgCl2
    - 2.92 g NaCl/100 mL NET
    - 1 mL 0.5 M EDTA
    - 10 mL 10% Triton X-100/100 mL NET
  + **~~HDP:~~** ~~Hypotonic lysis buffer for extraction of soluble mitochondrial proteins. Filter-sterilize and store at 4°C. Consists of 10 mM HEPES (pH 7.9), 1 mM DTT and 1 mM PMSF.~~

Procedure



***Figure 1***

**Flow chart of the different centrifugation steps and the supernatant and pellets recovered from them.**

Along the bottom is shown an approximate time scale for the protocol. The indicated steps should be preformed within the appropriate time range to minimize the time required to complete the entire procedure. Sup, supernatant.

Initial homogenization

**Treat cells**

I am currently treating cells for 6 hours with 100 μM cholesterol or 50 nM epoxomicin. Procedures are in separate cell treatment protocols.

**Space treatments 1 hour apart to allow time for harvesting and lysing cells.**

**To cut down the wait time during treatment, treat the cholesterol and epoxomicin cells first, then add medium to control cells. Harvest controls 1 hour before cholesterol.**

Cells

**Harvest cells: 1 hour/treatment**

* 1. Prep
     1. Set centrifuge to 4°C
     2. Do 5-10 dishes at a time, and one cell treatment group at a time.
  2. Remove dish lids and set above dishes.
  3. Aspirate and discard medium.
  4. Wash once with 10 mL 4°C PBS.
  5. Add 4 mL 4°C PBS per dish.
  6. Scrape cells with a cell lifter. Re-use the cell lifter for dishes with the same treatment, and keep the package so you can set down the cell lifter if necessary.
  7. Add to 50 mL tube with pipet-aid.
     1. If using 20 dishes per treatment, add to 2 50 mL tubes
  8. Centrifuge 1500 rpm 5 min 4°C to pellet cells.
  9. Aspirate and discard PBS supernatant.
  10. Bring tube out to bench and place on ice.
  11. Resuspend cells in 5 mL STMDPS buffer
      1. If using 20 dishes per treatment in 2 50 mL tubes, resuspend each tube with 5 mL STMDPS, and then combine for homogenization.
  12. Transfer to Dounce homogenizer tube by decanting or with pipet-aid.
  13. **Homogenize by 30-50 strokes of a manual Wheaton glass Dounce homogenizer with tight fitting pestle. Press pestle to bottom of tube, turn halfway, slowly raise, and repeat.** 
      1. To check homogenization, pipette 1 μL Hoechst 33342 stain (ThermoFisher H3570) onto a glass microscope slide, and add 20 μL homogenate. Add cover slip. Visualize by fluorescence microscopy in blue wavelength (510-540 nm), and flip back and forth between fluorescence and white light. Nuclei should appear distinct from other unstained cytoplasmic debris.
  14. Transfer lysate to 15 mL centrifuge tube.
  15. **Repeat for next treatment group either now or after starting centrifuge at step 10.**

Animals

**30 min**

1. Anesthetize and euthanize animal
   1. **Critical step:** Time and temperature are critical: there are a few pause points, but typically **all fractions are collected and extracted before freezing.**
   2. Work quickly to remove and rinse tissues/organ of interest, as cell death will begin to occur only a few minutes after cardiopulmonary arrest, resulting in gene turnover and proteome changes.
   3. Keep all tissue and buffers ice-cold to prevent protein degradation.
   4. Pre-chill the glass homogenizer on ice and pre-cool the centrifuge and rotors. If multiple centrifuges are not available, the samples will keep on ice for the short centrifuge times.
   5. Kinase and phosphatase inhibitors could be added to buffers if desired, but have not been tested.
   6. **Critical step:**Consider the method of sacrifice carefully. Killing by CO2 can affect the activity of many enzymes and kinases, thereby altering the phosphorylation state of proteins. Cervical dislocation as a means of killing may damage tissues of interest. In all cases, you should adhere to your institute's or facility’s animal care procedures.
   7. **Caution:**If using primary human tissues, these must be handled in a biohazard containment hood or as per your institute's guidelines. Extra care should be taken as the tissues or cells may contain human pathogens. Immunizations may be required to work with tissues or cell lines.
2. If possible, **clear blood from the organ by perfusing the organs of interest with cold PBS**, as detailed in Box 1. If a suitable vein or artery is not available for the perfusion, or the tissue is not amenable to perfusion, proceed directly to Step 3.
   1. **Critical step:**If perfusion is not carried out, the sample may contain large amounts of blood proteins that may need to be accounted for during data analysis.
   2. Box 1: perfusion of organs or tissues to clear blood
      1. Insert the cannula needle into a vein or artery in the direction of blood flow, and clamp with a pair of forceps or a bulldog clamp to prevent backflow. For example, the hepatic portal vein is a good choice for perfusing the liver.
      2. Pump the PBS using either a large volume syringe (60 ml) or peristaltic pump. Critical: maintain only enough pressure to clear the blood; high pressure will rupture the smaller vessels and prevent clearing.
      3. Occasionally, gently massage the organ to facilitate the flow of buffer and blood.
3. Remove the organ and trim away any membranes or other adherent tissue that is not desired. For small samples, this can be performed under a dissecting microscope with the tissue in cold PBS.
4. Place the tissue in a Petri dish and mince the tissue with sharp scissors.
5. **Rinse the minced tissue in cold PBS** according to either option A or option B. If the tissue was perfused in Step 2, follow option A. If the tissue was not perfused, follow option B:
   1. **If the tissue was perfused, rinse once in 5–10 volumes of cold PBS** as follows:
      1. **BWS: put each liver in 50 mL tuzbe with 20 mL cold PBS, keep on ice.**
      2. Place the tissue in a tube, preferably with a screw-cap that will fit into the rotor of the low-speed centrifuge. **Caution:** Always balance the centrifuge sample to prevent rotor imbalance.
      3. Add 5 volumes or more of cold PBS.
      4. Gently spin (100g for 1 min or less) to pellet the minced tissue.
      5. Decant or remove liquid with a pipette.
   2. If the tissue was not perfused, and needs to be cleared of excess blood, rinse four times in cold PBS as follows:
      1. Place the tissue in a tube, preferably with a screw-cap that will fit into the rotor of the low-speed centrifuge. **Caution:** Always balance the centrifuge sample to prevent rotor imbalance.
      2. Add 5 volumes or more of cold PBS.
      3. Gently spin (100g for 1 min or less) to pellet the minced tissue.
      4. Decant or remove liquid with a pipette.
      5. Repeat from Step (ii) three more times.
6. **Rinse the tissue once in 5–10 volumes ice-cold 250-STMDPS Buffer.**
   1. **BWS: without protease inhibitors. Add buffer, spin 100 g 1 min, remove supernatant.**
7. Place the tissue into a pre-chilled dounce glass tube and add 4–8 volumes of ice-cold 250-STMDPS Buffer. Place into an ice bucket and secure the glass tube in place with a retort stand.
   1. **Caution:** Wear safety glasses and a face shield, as the glass tube may shatter if too much pressure is applied or if the drill is moved at an angle other than vertical.
   2. **Critical step:** The volume of buffer may need to be optimized for a particular tissue or organ; 4–8 ml of buffer per gram of tissue is a good start.
8. Homogenize for a minimum of 2 min using a tight-fitting Teflon pestle attached to a power drill (set to >1,000 rpm) by slowly stroking the pestle up and down, taking 15 s per stroke and 30 s to complete a down and up cycle.
9. Inspect the homogenate; if intact tissue is still apparent, re-homogenize for an additional 1 min.
   1. **If desired, the homogenization can be checked by placing 10 μl of homogenate on a microscope slide with a glass cover slip. Visualize by phase-contrast microscopy for cell lysis. Efficient lysis should have small nuclei floating freely in the homogenate, with few intact cells visible.**
10. **Centrifuge 1000g 15 min 4°C** (Cox and Emili step 10) toseparate nuclei from other organelles.
11. **Transfer cyto-I supernatant into 1.5 or 2 mL tubes for isolation of mitochondria, cytosol and microsomes.** Proceed to isolation of mitochondria.
    1. BWS: Transfer supernatant with Eppendorf Repeater M4 positive displacement pipette. The nuclear pellet is not lodged securely enough in the bottom of the tube for decanting.
    2. BWS: Use filter tips to prevent lysate from being aspirated into pipette barrel.
    3. BWS: If you have a centrifuge that can spin 15 mL tubes at 6000xg, transfer to one 15 mL tube.
    4. **BWS: keep the cyto-I supernatant on ice until nuclei-I is in the ultracentrifuge.**
12. **Label and save the pellet on ice as nuclei-I.** Proceed to isolation of nuclei**.**
13. If a cleaner preparation is required, an optional additional centrifuge step can be included. Re-spin the supernatant at 800–1,000g for 15 min to pellet any nuclei that have come free from the pellet during decanting. Decant and save the supernatant as cyto-I for isolation of mitochondria, cytosol and microsomes. The pellet may be discarded.

Isolation of nuclei

**Timing: 1 h**

1. ~~Add 5–10 volumes of 250-STMDPS Buffer to nuclei-I (from Step 12) and re-homogenize for 1 min in the dounce homogenizer.~~
2. ~~Decant the homogenate into an appropriately-sized centrifuge tube. For large samples, conical-bottomed 50 or 14 ml polypropylene tubes are handy, as they seal, are clear and have volume markings.~~
3. ~~Centrifuge at 800~~*~~g~~*~~for 15 min.~~
4. ~~Decant and save the supernatant as cyto-II (for use in Step 25) and save the pellet as nuclei-II.~~
   1. **BWS: skip the re-homogenization (steps 14-17). It is not necessary and would require double the buffer volume. Homogenize well the first time.**
5. Re-solubilize the nuclei-II pellet in 4 volumes of 2 M STMDPS Buffer by repeated pipetting~~, and homogenize with a single stroke of the dounce homogenizer.~~
   1. **BWS: Resuspend in 6-7 mL 2 M STMDPS with a pipet aid. Skip re-homogenization.**
6. ~~Filter the re-suspension through several layers of cheesecloth or gauze to remove debris.~~
   1. **BWS: This step may help for liver tissue, but is not necessary for cells. The nuclear pellet could become trapped in the gauze.**
7. Prepare a 4-ml cushion of **2 M** STMDPS Buffer in a 14-ml ultracentrifuge tube.
   1. **Beckman XL-90 ultracentrifuge** 
      1. Turn on Beckman XL-90 ultracentrifuge and press Vacuum to begin cooling.
      2. Remove tube holders with screw caps from fridge.
      3. Zero the balance with a Styrofoam tube rack.
      4. Add tube holder and screw cap to tube rack.
      5. Add 4 mL 2 M STMDPS to tube. (Cox and Emili step 20).
8. Layer the nuclear suspension onto the cushion by filling a pipette with the suspension and dispensing slowly with the tip touching the side wall of the tube.
   1. **Critical step:**If there is less than 10 ml of sample, add more 2 M STMDPS Buffer to the sample and mix before pipetting onto the cushion.
   2. **BWS:** Slowly dispense nuclear suspension with positive displacement pipette or pipet-aid.
   3. **BWS:** Nuclear suspension should appear cloudy above the 4 mL cushion.
9. **Centrifuge at 21,600 RPM (80,000g max) 35 minutes 4°C (Cox and Emili step 22).**
   1. **Beckman XL-90 ultracentrifuge** 
      1. [Beckman rotor calculations](https://www.beckmancoulter.com/wsrportal/wsrportal.portal?_nfpb=true&_windowLabel=UCM_RENDERER&_urlType=render&wlpUCM_RENDERER_path=%2Fwsr%2Fresearch-and-discovery%2Fproducts-and-services%2Fcentrifugation%2Frotors%2Findex.htm&wlpUCM_RENDERER_t=3)
      2. We have an extra rotor and buckets in the 4°C walk-in fridge. There is another ultracentrifuge with the GCD shared equipment on the first floor.
      3. Insert ultracentrifuge tube into bucket.
      4. **Opposing tubes should be balanced to <0.1 g (~120 g). Extra spaces on rotor should be filled with empty buckets. Do not get any buffer on the outside of the tube.**
      5. Screw on tube cap. Threads can be greased with spinkote lube for best seal. There is a rubber o-ring below the cap that must be present for proper vacuum.
      6. Repeat for next sample.
      7. Press vacuum to depressurize. Load tube holders into rotor. **Ensure hooks are inserted.**
      8. Load SW-41 TI rotor into ultracentrifuge. Spin lightly to ensure it is properly loaded.
      9. Set speed, time, and temperature, pressing Enter to set each time. Select max acceleration/deceleration. Select the correct rotor from the on-screen menu and press Enter. Press Enter again, then press Vacuum. This reduces the air pressure for less friction. Wait until vacuum is below 300 μm before beginning run (5 min).
      10. Press Enter, and then Start to begin the run.
      11. **BWS: isolate mitochondria during the run (step 25).**
      12. After run, press Vacuum to depressurize. Open chamber and remove rotor. Remove tube holders and set in rack. Record run conditions and RPM in log book.
      13. Unscrew caps and lift tubes out of tube holders with forceps.
10. Slowly aspirate the supernatant with a pipette, and then remove the last 1 ml with a p1000 pipette to prevent disturbing the pellet, which contains pure nuclei.
    1. **BWS note: there will be multiple layers in the tube: a gelatinous film on top, a clear layer, a cloudy layer, and the pure nuclear pellet at the bottom. Use positive displacement pipette to remove the upper layers to avoid contaminating pellet. The pellet is lodged securely in the bottom of the tube, so decanting also works if necessary.**
    2. **Wash pellet gently with 1 mL 2 M STM to prevent contamination with layers above.**
11. To extract nuclear proteins from the pellet, proceed to Step 35. To continue with tissue fractionation of the supernatant (cyto-I), proceed to Step 25. These steps can be carried out in parallel, as outlined in Figure 1.

Isolation of mitochondria

**Timing: 45 min to 1 h**

1. Centrifuge cyto-I supernatant 6000*g* 15 min 4°C to pellet mitochondria (Cox and Emili step 25).
   1. **BWS: you will not keep these tubes, just label simply.**
   2. If using cyto-II (from Step 17), spin in separate tubes.
2. Decant the cyto-I supernatant and save as cyto-III for use in Step 32; the pellet is the mitochondria.
3. ~~Decant the cyto-II supernatant and optionally combine with cyto-III for use in Step 32; the pellet is mitochondria.~~
4. ~~Gently resuspend both mitochondria pellets and combine in 10 or more volumes of 250-STMDPS Buffer with a pipette.~~
5. ~~Spin again at 6,000~~*~~g~~*~~for 15 min.~~
6. ~~Decant the supernatant and save the pellet as the mitochondria.~~
   1. **BWS: not currently saving mitochondria.**
7. To extract mitochondrial proteins from the pellet, proceed to Step 44. To continue with tissue fractionation of the supernatant, cyto-III (from Steps 26 and 27), proceed to Step 32. These steps can be carried out in parallel, as outlined in Figure 1.
   1. **Pause Point:**The pellet may be frozen at −70 °C for protein extraction at a later date.

Isolation of cytosol and microsomes

**Timing: 1 h 15 min**

1. Place the cyto-III supernatant (from Steps 26 and 27) into ~~two~~ ultracentrifuge tubes of appropriate volume and balance.
   1. **Beckman XL-90 ultracentrifuge** 
      1. Turn on Beckman XL-90 ultracentrifuge and press Vacuum to begin cooling.
      2. Remove tube holders with screw caps from fridge.
      3. Zero the balance with a Styrofoam tube rack.
      4. Add tube holder and screw cap to tube rack. Unscrew cap and insert ultracentrifuge tube.
      5. Transfer cyto-III into 14 mL ultracentrifuge tube (Cox and Emili step 32).
      6. **Opposing tubes should be balanced to <0.1 g (~120 g). Extra spaces on rotor can be filled with empty buckets. Do not get any buffer on the outside of the tube. If blank tube is light, add weight with NaCl.**
      7. Screw on tube cap.
2. **Centrifuge at 24,200 RPM (100,000g max) 1 hour 4°C (Cox and Emili step 33).**
   1. **Beckman XL-90 ultracentrifuge** 
      1. [Beckman rotor calculations](https://www.beckmancoulter.com/wsrportal/wsrportal.portal?_nfpb=true&_windowLabel=UCM_RENDERER&_urlType=render&wlpUCM_RENDERER_path=%2Fwsr%2Fresearch-and-discovery%2Fproducts-and-services%2Fcentrifugation%2Frotors%2Findex.htm&wlpUCM_RENDERER_t=3)
      2. Press vacuum to depressurize. Load tube holders into rotor. **Ensure hooks are inserted.**
      3. Load SW-41 TI rotor into ultracentrifuge. Spin lightly to ensure it is properly loaded.
      4. Set speed, time, and temperature, pressing Enter to set each time. Select max acceleration/deceleration. Select the correct rotor from the on-screen menu and press Enter. Press Enter again, then press Vacuum. This reduces the air pressure for less friction. Wait until vacuum is below 300 μm before beginning run.
      5. Press Enter, and then Start to begin the run.
      6. **BWS: begin nuclear protein extraction during the run (step 35), then take a break.**
      7. After run, press Vacuum to depressurize. Open chamber and remove rotor. Remove tube holders and set in rack. Record run conditions and RPM in log book.
      8. Unscrew caps and lift tubes out of tube holders with forceps.
3. Decant the supernatant and save as pure cytosol. Save the pellet as the microsomal fraction. To extract microsomal proteins from the pellet, proceed to Step 52.
   1. **Pause Point:**The supernatant can be frozen at −70 °C until required. The pellet may be frozen at −70 °C and processed at a later date.
   2. **Aspirate supernatant with positive displacement pipette to prevent contamination.**

Extraction of nuclear proteins

**Timing: 2 h**

1. ~~Resuspend the pure nuclei (from Step 23) in five volumes of NE buffer with a pipette. This can typically be performed in a 1.5-ml snap-cap microcentrifuge tube.~~
2. ~~Incubate the nuclei for 30 min with gentle rocking at 4 °C.~~
3. ~~Lyse the nuclei with 10 passages through an 18-gauge needle.~~
4. ~~Centrifuge the lysate at 9,000~~*~~g~~*~~for 30 min in a microcentrifuge.~~
5. ~~Save the supernatant as Nuc-S. This will contain many of the soluble proteins and those not so tightly bound to DNA. Optionally, take a small aliquot to determine the concentration of protein; this will prevent having to thaw the whole sample at a later date.~~
   1. **~~Pause Point:~~**~~The sample and aliquot can be frozen at −70 °C until needed.~~
6. ~~Resuspend the pellet in 5 volumes of NET buffer with a pipette and gently rock for 30 min at 4 °C.~~
7. ~~Extract the nuclei debris by 10 passages through an 18-gauge needle.~~
8. ~~Centrifuge at 9,000~~*~~g~~*~~for 30 min in a microcentrifuge.~~
9. ~~Save the supernatant as Nuc-T. This will contain nuclear membrane proteins and other proteins that are tightly bound to DNA, such as histones. Optionally, take a small aliquot to determine the concentration of protein; this will prevent having to thaw the whole sample at a later date.~~
   1. **~~Pause Point:~~**~~The sample and aliquot can be frozen at −70 °C until needed.~~
   2. **BWS modifications:**
      1. Add 1000 μL lysis buffer to ultracentrifuge tube (2 mL/60 dishes).
      2. Pipette repeatedly to suspend pellet and transfer to 1.5 mL tube. May take several tries. Repeatedly pipette until no pellet remains in the tube.
      3. Incubate 30-60 min 4°C on tube rotator, pipetting every 10 minutes to mix. The pellet will not be completely solubilized because some of it is DNA.
      4. Centrifuge 15-30 min 9,000g 4°C. Save supernatant. Proceed to BCA and IP.

Extraction of mitochondrial proteins

**Timing: 2.5 h**

**BWS: not currently extracting mitochondrial proteins.**

1. ~~Resuspend the mitochondrial pellet (from Step 31) in 0.5 ml HDP buffer in a 1.5-ml snap-cap microfuge tube; if 0.5 ml is less than 10 volumes, split the pellet into multiple tubes.~~
2. ~~Incubate on ice for 30 min.~~
3. ~~Sonicate the suspension at a high setting to lyse the mitochondria. Keep the mitochondria on ice during sonication and use four 5–10-s bursts with 30-s pauses to prevent sample heating.~~
4. ~~Microcentrifuge the lysate at 9,000~~*~~g~~*~~for 30 min.~~
5. ~~Save the supernatant as Mito-S fraction, the soluble mitochondrial matrix proteins.~~
6. ~~Resuspend the pellet in 0.5 ml of ME buffer and incubate for 30 min with gentle rocking.~~
7. ~~Microcentrifuge at 9,000~~*~~g~~*~~for 30 min.~~
8. ~~Save the supernatant as Mito-M, the mitochondrial membrane proteins.~~
   1. **~~Pause Point:~~**~~Freeze the extracts at −70 °C.~~

Extraction of microsome proteins

**Timing: 1.5 h**

1. ~~Resuspend the microsome pellet (from Step 34, erroneously listed in the paper as step 35) with 0.5 ml of ME buffer and incubate for 1 h with gentle rocking.~~
2. ~~Microcentrifuge at 9,000~~*~~g~~*~~for 30 min.~~
3. ~~Save the supernatant as Micro, the microsome proteins.~~
   1. **~~Pause Point:~~**~~Freeze the extract at −70°C.~~
   2. **BWS modifications:**
      1. Ensure protease inhibitors have been added to extraction buffer.
      2. Add 250-500 μL lysis buffer to ultracentrifuge tube (1 mL/60 dishes).
      3. Pipette repeatedly to suspend microsome pellet and transfer to 1.5 mL tube.
      4. Incubate 30-60 min 4°C on tube rotator, pipetting every 10 minutes to mix.
      5. Centrifuge 15-30 min 9,000g 4°C. Save supernatant. Proceed to BCA and IP.

Abbreviated subcellular fractionation

Adapted by Scott Widenmaier from Cox B, Emili A. Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics. *Nat. Protoc.* 1:1872–1878 (2006).

Also similar to the method used in Wang W, Chan JY. Nrf1 is targeted to the endoplasmic reticulum membrane by an N-terminal transmembrane domain: Inhibition of nuclear translocation and transacting function. *J. Biol. Chem.* 281:19676–19687 (2006).

Materials Needed

* Homogenization
  + Cell lifters (Corning 3008), 1 per treatment
  + Lysis buffer
  + Protease inhibitors
  + Dounce homogenizers, 15 mL glass, 1 per group ([Wheaton 357544](http://wheaton.com/15-ml-tissue-grinder-dounce.html))
* Centrifuge
* Ultracentrifuge
* Centrifuge tubes
* Ultracentrifuge tubes (Beckman Coulter 344059)
* Forceps
* Balance
* Reagents listed below

Prep

Advance prep

1. **Reserve ultracentrifuge.** You will need it late in the day.
2. **Label tubes.**
   1. 11 microcentrifuge tubes per sample/treatment
      1. 5 2 mL tubes for aliquotting cyto-I supernatant before centrifugation
      2. 1 1.5 mL tube for extraction of microsome proteins
      3. 2 1.5 mL tubes for microsomal affinity purification and input
      4. 1 tube for extraction of nuclear proteins
      5. 2 1.5 mL tubes for nuclear affinity purification and input
   2. 2 15 or 50 mL tubes per sample/treatment, 1 with 10 mL STMDPS for homogenization.
   3. 1 ultracentrifuge tube/sample

Reagent prep

1. **250-STM Buffer** 
   1. Stock buffer for initial homogenization.
   2. Filter-sterilize and store at 4°C.
   3. Consists of 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2.
2. **250-STMDPS Buffer** 
   1. Add protease inhibitors and 1 mM DTT to 250-STM buffer.
   2. Spermine and spermidine are not essential for this abbreviated fractionation.
3. **RIPA buffer**
   1. Add protease and phosphatase inhibitors
4. **Commercial hypotonic cell fractionation buffer**
   1. PARIS cell fractionation buffer (Life Technologies AM1921)

Procedure

Initial homogenization

**Timing: 60-90 min**

1. Harvest and lyse cells
   1. Do five dishes at a time, and one cell treatment group at a time.
   2. Remove dish lids and set above dishes.
   3. Aspirate and discard medium.
   4. Add 5 mL ice cold PBS per dish.
   5. Scrape cells with a cell lifter. Re-use the cell lifter for dishes with the same treatment.
   6. Add to 15 or 50 mL tube.
   7. Centrifuge 1500 rpm 5 min 4°C to pellet cells.
   8. Aspirate and discard PBS supernatant.
   9. Resuspend cells in 15 mL STMDPS buffer.
   10. **Homogenize by 30 strokes of a manual Wheaton glass Dounce homogenizer with tight fitting pestle. Press pestle to bottom of tube, turn halfway, slowly raise, and repeat.**
2. Harvest and lyse tissues
   1. Euthanize mice by cervical dislocation or ketamine/xylazine.
   2. Open along midline and remove blood from the heart.
   3. Perfuse liver using a syringe filled with cold PBS. Note that perfusing the liver ex vivo does not work well.
   4. Weigh 0.5 g and transfer to a weigh boat or 100 mL beaker with 5 mL STMDPS buffer.
   5. Mince liver with scissors into <1 mm pieces.
   6. Pour into glass dounce homogenizer tube.
   7. Homogenize with Wheaton overhead stirrer with Dounce attachment, gradually ramping up to speed 4. If glass tube is too small for 10 mL, do two homogenizations of 5 mL each.
   8. Transfer lysate to 15 mL centrifuge tube.
3. Centrifuge 800g 15 min 4°C (Cox and Emili step 10) toseparate nuclei from other organelles.
4. **Transfer cyto-I supernatant to 15 mL tube, then into 1.5 or 2 mL tubes for isolation of mitochondria, cytosol and microsomes.** Proceed to isolation of mitochondria.
   1. Supernatant can be decanted, or transferred with a 10 mL stripette followed by 1 mL pipette.
   2. Use filter tips to prevent lysate from being aspirated into pipette barrel.
   3. If you have a centrifuge that can spin 15 mL tubes at 6000xg, no need to transfer to 2 mL tubes.
5. **Label and save the pellet on ice as nuclei-I.** Proceed to extraction of nuclear proteins**.**
6. Turn on Beckman XL-90 ultracentrifuge and press Vacuum to begin cooling.

Isolation of nuclei

**Timing: 15 min**

1. Resuspend nuclei-I pellet in 1 mL PARIS cell fractionation buffer. Pipette 5x to mix.
   1. The original Cox and Emili protocol would continue here at step 14 using STMDPS buffer.
2. Leave at 4°C or on ice 10 min with gentle agitation.
3. Centrifuge 600g 4 min 4°C. Discard supernatant (would be equivalent to cyto-II).
4. Resuspend pellet again in 1 mL cell fractionation buffer.
5. Centrifuge 600g 2 min 4°C. Discard supernatant.

Proceed to extraction of nuclear proteins or nuclear fixation for ChIP.

Isolation of mitochondria

**Timing: 15 min**

1. Centrifuge cyto-I supernatant 6000g 15 min 4°C to pellet mitochondria (Cox and Emili step 25). The supernatant (cyto-III) will be used for isolation of cytosol and microsomes (Cox and Emili step 32).

Proceed to isolation of cytosol and microsomes.

Nuclear fixation for ChIP

1. Prepare a solution of PBS with 10% CCS and 1% formaldehyde (diluted from Thermo 28906 MeOH-free 16%). Add 8 mL to nuclear pellet and allow to fix for 30 min RT.
2. Add 950 μL 10x glycine for a [final] of 1x. Allow to sit 5 min RT.
3. Centrifuge 800g 5 min 4°C.
4. Wash 2x with 10 mL PBS each time.
5. Resuspend in 1 mL ChIP buffer with protease inhibitors.
6. Sonicate.

Isolation of cytosol and microsomes

**Timing: 90-120 min**

1. Turn on Beckman XL-90 ultracentrifuge and press Vacuum to begin cooling.
2. Carefully balance the ultracentrifuge tubes.
   1. Remove tube holders with screw caps from fridge.
   2. Zero the balance with a Styrofoam tube rack.
   3. Add tube holder and screw cap to tube rack. Unscrew cap and insert ultracentrifuge tube.
   4. Transfer cyto-III into 14 mL ultracentrifuge tube (Cox and Emili step 32).
   5. Add STMDPS buffer to fill tube (should be 118-119 g).
   6. Screw on tube cap.
   7. Repeat for next sample. Tubes should be balanced to within 0.1 g with STMDPS buffer If a blank tube is needed, fill with STMDPS.
3. Centrifuge at 24,200 RPM (100,000g) 1 hour 4°C (Cox and Emili step 33).
   1. Press vacuum to depressurize. Load tube holders into rotor. **Ensure both hooks are inserted.**
   2. Load SW-41 TI rotor into ultracentrifuge. Spin lightly to ensure it is properly loaded.
   3. Set speed, time, and temperature, pressing Enter to set each time. Select max acceleration/deceleration. Select the correct rotor from the on-screen menu and press Enter. Press Enter again, then press Vacuum. This reduces the air pressure for less friction. Wait until vacuum is below 300 μm before beginning run.
   4. Press Start to begin the run.

Proceed to extraction of microsome proteins.

Extraction of nuclear proteins

1. Resuspend nuclear pellet in 5-800 μL RIPA buffer with protease inhibitors. Vortex and pipette to mix.
2. Freeze at -80°C, then thaw to fully fracture membranes.
3. Centrifuge 15 min 18,000g (max speed) 4°C to pellet nucleic acid
4. Aspirate supernatant containing nuclear proteins and transfer to 1.5 mL tube. Discard pellet.

Proceed to protein quantification.

Extraction of mitochondrial proteins

Mitochondria were not used for this protocol.

Extraction of microsome proteins

1. After ultracentrifugation, press Vacuum to depressurize. Open the chamber and remove the rotor. Remove tube holders and set in rack. Record run conditions and RPM count in log book, and turn off.
2. Unscrew caps and lift tubes out of tube holders with forceps. Discard the cytosol supernatant.
   1. The pellet is lodged in bottom of tube, so supernatant can be poured off.
3. Add 500-1000 μL RIPA buffer with protease inhibitors to the ultracentrifuge tube.
4. Pipette repeatedly to suspend microsome pellet and transfer to 1.5 mL tube.
5. Incubate 30 min 4°C on tube rotator, pipetting every 10 minutes to mix.
6. Centrifuge 15 min 9,000g 4°C

Proceed to protein quantification.

Protein quantification

Quantify total protein present in the fractions with a BCA assay (see BWS gel and blot protocol). Adjust volumes to normalize protein concentrations across fractions.

Proceed to affinity purification (separate protocol).

Additional procedures

1. Quantify total protein present in cell fractions with a BCA assay before affinity purification.
   1. Adjust volumes to normalize protein concentrations across fractions.
   2. The BCA assay can also be used after affinity purification if 0.1 M glycine was used for elution.
   3. The Bio-Rad RC-DC assay or the Pierce 660 nm protein assay reagent (22660) with ionic detergent compatibility reagent (22663) can be used if total protein quantification is desired after elution in non-reducing SDS-PAGE loading buffer.
2. Cell fractionation can be verified by Western blot for fraction-specific proteins. See Figure 2.

Troubleshooting

| **Step** | **Problem** | **Possible reason** | **Solution** |
| --- | --- | --- | --- |
| 11 | Crude nuclear pellet contains large amount of red blood cells | Highly vascularized tissue was not perfused to remove blood | Perfuse next time; spin at 250*g* for 5 min to spin down the red blood cells before the nuclei |
| 11 | Small pellet or supernatant from 800*g* spin is murky | Nuclei are not pelleted; poor cell lysis | Increase speed of centrifuge to 1,000*g* and re-spin |
| 11 | Nuclear pellet becomes viscous during resuspension | Nuclei have lysed; homogenization is too harsh | Increase spermine and spermidine concentration; reduce time of dounce homogenization; reduce speed of homogenizer; decrease fit of Teflon/glass pestle to reduce shearing force  **Scott Widenmaier: A microccocal nuclease may help reduce nuclear viscosity.**  **Sonication can reduce nuclear viscosity, but could potentially disrupt protein-protein interactions.** |
| 37, 41 | Nuclei clog the needle | The nuclear pellet is very large | Shear with a larger gauge needle (16 gauge) and then shear with an 18-gauge needle |

Useful resources

* GE protein purification handbooks
* [Rotors and Tubes for Preparative Ultracentrifuges – Beckman Coulter](https://www.beckmancoulter.com/wsrportal/techdocs?docname=LR-IM-24)

Notes

* DTT may be included in the STMDPS buffer to prevent protein aggregation.
* Nuclei can also be separated from other organelles by passage through a 27 g needle 12 times (Sancak *Science* 2013).
* Cross-linkers can be used to keep protein complexes together. The Sabatini lab uses DSP (Di(N-succinimidyl) 3,3′-dithiodipropionate), which is a homobifunctional crosslinker (has identical reactive groups). DSP is reversible after affinity purification. Note that the tandem mass tags used for quantification react with primary amine groups. Most cross-linkers also react with amine groups. Even if reversible, this could affect the ability of the peptide to bind a TMT for quantification. We could try a carboxy-reactive cross linker.

Notes from Cox and Emili *Nat. Protoc.* 2006

Introduction

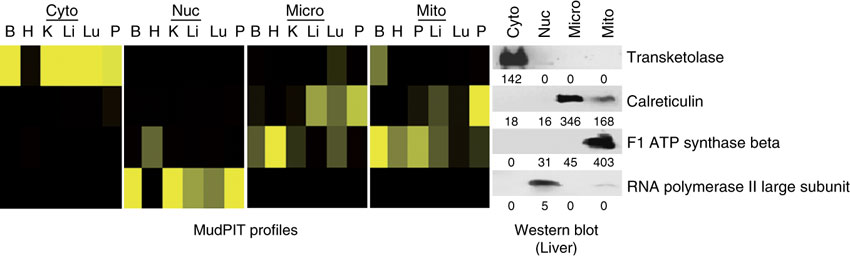
* We have found this protocol to be readily applicable to cell culture; however, frozen tissue shows poor results as the freeze–thaw step generally lyses the cells and its subcellular components, resulting in higher cross-contamination of the fractions (B.C. and A.E., unpublished observations).
* The homogenization is based on shearing with a Teflon-glass dounce homogenizer and an electric drill. We have found that glass–glass homogenizers and homogenization by hand also work (B.C. and A.E., unpublished observations). Rotary-blade homogenizers, such as a Polytron, will lyse tissues but will also tend to lyse nuclei and mitochondria and cause higher cross-contamination of the subcellular fractions (B.C. and A.E., unpublished observations).
* This protocol may not reveal the proteome of a specific subcellular fraction to the same depth as methods that are specifically optimized to generate highly purified subcellular components. However, our methodology does afford the advantage of comparison of multiple fractions so that different subcellular compartments from the same sample can be compared to judge the relative enrichment of a protein. The generation of a general microsomal fraction does combine many membranous fractions together, such as Golgi, ER and intracellular vesicles. In addition, we have noted that the ribosome is also highly enriched with this fraction, probably in association with the rough ER, as the lysis method is gentle and detergent-free5.
* We have utilized Triton-X-100 for the extraction of proteins from the insoluble portion of many of the fractions. Detergents can interfere with the collection of high-quality mass spectra; however, Triton-X-100 is easily removed during sample clean-up.
* The protocol presented here is optimized to provide a survey of the cell or tissue proteome and may not be optimized for individual organelle or cellular subcomponents. Nevertheless, this provides an excellent starting point for global cellular protein analysis. We typically run the protocol from dissection to protein extraction in 5–6 h and freeze the samples for processing at a later date for analysis using Multidimensional Protein Identification Technology (MudPIT)5.

Timing

* Although the entire protocol timing totals over 9 h, many of the steps can be carried out in parallel such that the entire procedure can be completed in less than 6 h. See Figure 1 for a flow chart and timing coordination.

Anticipated results

* After fractionation and extraction, typical protein yields are all in the several mg ml−1 range, as determined by the Bradford assay10. As we have previously published, each fraction is highly enriched for proteins that are known to be localized to these fractions. Figure 2 shows a western blot of a preparation made from liver blotted for markers of cytosol, nuclei, microsomes (ER) and mitochondria. Also shown are the MudPIT profiles for the same proteins in six different organ preparations. As can be seen, the nuclei and cytosol show the highest degree of purity, with the mitochondria and microsomes being more variable depending on the tissue source. The depth of coverage is sufficient that numerous proteins specific to tissue types are readily identified in their correct subcellular location.



***Figure 2***

**MudPIT profiles and western blot of markers for the four subcellular fractions: nuclei (Nuc), cytosol (Cyto), microsomes (Micro) and mitochondria (Mito).**

Markers for cytosol (Transketolase), nuclei (RNA polymerase II large subunit), microsomes (Calreticulin) and mitochondria (F1 ATP synthase beta subunit) were blotted against extracts from liver subcellular fractions. Note the high degree of specificity. Quantification values obtained by densitometry are displayed below each lane. MudPIT profiles from brain, heart, kidney, liver, lung and placenta for these same proteins are shown alongside the western blots. Intensity of yellow corresponds to the relative quantity of protein detected. Note that the cytosol and nuclei show a high degree of purity for most tissues, whereas the microsomes and mitochondria specificity vary depending on the tissue source. B, brain; H, heart; K, kidney; Li, liver; Lu, lung; P, placenta. Reprinted and modified from ref. 5, with permission from Elsevier.

**BWS: other fraction-specific proteins**

Nuclear: TFIIB, Lamin A/C (common nuclear loading controls)

Microsomal: Calreticulin is best. Could also use chaperones.

Mitochondrial: COX IV

Other published fractionation protocols

Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS. Crucial step in cholesterol homeostasis: Sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell 110:489–500 (2002).

**Identification of SCAP-Interacting Proteins**

* Stably transfected human 293S cells (Reeves et al., 1996) expressing SCAP(TM1-6) and SCAP(TM1-5) were generated as described in Supplemental Experimental Procedures available at above URL.
* 293S/SCAP(TM1-6) and 293S/SCAP(TM1-5) cells were grown in sus- pension for 5 days in medium B at 37C (without CO2). On day 6, each cell line received 1 ug/ml 25-HC, was incubated for 24 hr, and then harvested. The 1000 *g* pellet of cells was washed once with phosphate-buffered saline (PBS) and frozen at -80C until use.
* All subsequent operations were carried out on ice or at 4C. Each thawed cell pellet (representing 10 liters of 293S/SCAP(TM1-5) cells or 6 liters of 293S/SCAP(TM1-6) cells) was incubated at 4C for 30 min with buffer containing 50 mM HEPES-KOH [pH 7.4], 1.5 mM MgCl2, 10 mM KCl, 250 mM sucrose, 5 mM sodium EDTA, 5 mM sodium EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 􏰃g/ml leupeptin.
* Cells were lysed with 30 strokes of a Dounce homogenizer.
* Each cell lysate was centrifuged at 1000 *g* for 5 min to remove nuclei and unlysed cells.
* Each supernatant was centrifuged at 10,000 *g* for 15 min to isolate a membrane fraction, which was solubilized with buffer A (50 mM HEPES-KOH [pH 7.4], 150 mM NaCl, 0.5% (w/v) Chapso, 1 mM PMSF, and 10 􏰃g/ml of leupeptin).
* After incubation for 1 hr, the insoluble material was removed by centrifugation at 105 *g* for 30 min.
* The supernatant was incubated with an irrelevant control mouse monoclonal antibody IgG-2001 (Tolleshaug et al., 1982) and Protein G Sepharose 4 beads for 1 hr.
* The supernatant was bound to anti-FLAG-M2 Agarose Affinity Gel in batch, and the suspension of beads was rotated for 3 hr.
* The beads were washed 5 times (10 min each) with buffer containing 50 mM HEPES-KOH [pH 7.4], 150 mM NaCl, and 0.2% Chapso, after which the proteins captured by the affinity resin were eluted for 12 hr with buffer A containing 0.25 mg/ml of FLAG peptide.
* Each eluate was adjusted to a final concentration of 2 mM CaCl2 and applied to calmodulin affinity resin in batch for 2 hr, after which each resin was washed 3 times (10 min each) with buffer containing 50 mM HEPES-KOH [pH 7.4], 0.5 M NaCl, 0.2% Chapso, and 2 mM CaCl2. Proteins were eluted for 1 hr in buffer containing 50 mM HEPES- KOH [pH 7.4], 0.3 M NaCl, 2 mM sodium EGTA, and 0.5% Chapso.
* Each eluate was precipitated overnight at -80C with 5 volumes of cold acetone and centrifuged at 20,000 *g* for 10 min. Each pellet was dried and resuspended at room temperature in 80 ul of buffer containing 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 1% (w/v) SDS.
* The proteins were subjected to SDS-PAGE on 12% gels, visualized with Coomassie blue stain, and processed for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described in Supplemental Experimental Procedures available at <http://www.cell.com/cgi/content/full/110/4/489/DC1>.

Radhakrishnan Cell Metab 2008

RESULTS

Isolation of Pure ER Membranes from CHO Cells

We developed a fractionation scheme to isolate pure ER mem- branes from CHO-K1 cells, as outlined in Figure 1 (left panel). Efficient and reproducible cell homogenization was achieved through the use of a ball-bearing homogenizer (Balch and Roth- man, 1985). The cell homogenate (designated Fraction A in Fig- ure 1) was subjected to three centrifugation steps, which pro- gressively separated ER membranes from other organelles. The results of the first two centrifugation steps are summarized in Figure 1 (right panel). Centrifugation of the homogenate at 3000 *g* eliminates unbroken cells and the heaviest membranes. As assessed by immunoblot analysis of the fractions, a soluble nuclear protein marker (CREB) and a mitochondrial membrane protein marker (Prohibitin-1) are mostly retained in the 3000 *g* pellet (Fraction B), whereas significant amounts of all other mem- brane markers escape into the 3000 *g* supernatant (Fraction C). Fraction C was then subjected to centrifugation through a dis- continuous sucrose gradient and yielded two distinct bands of membranes (Fractions D and E). The small amount of CREB in Fraction C is absent in Fractions D or E and likely represents a portion that was released into the cytosol, owing to rupture of the nuclear membranes during the fractionation procedure. The Golgi membrane proteins (GM130 and GRASP65), plasma membrane proteins (transferrin receptor, caveolin-1, and Na+/ K+ ATPase), and the early endosomal membrane protein (EEA1) are found in the light membrane Fraction D and are almost completely ab- sent from the heavy membrane Fraction E. The peroxisomal membrane protein (PMP70) is localized predominantly in Fraction E, and a lysosomal membrane protein (LAMP1) is present in both Frac-

tions D and E. ER-resident membrane proteins (Sec61a and ACAT-1) are localized almost completely to Fraction E. Scap, a membrane protein that cycles between ER and Golgi mem- branes (Goldstein et al., 2006), is found in both Fractions D and E. The two forms of site-1 protease, the inactive precursor form and the processed active form (Espenshade et al., 1999), are separated into Fraction E and Fraction D, respectively.

The aforementioned data suggest that Fraction E contains partially purified ER membranes devoid of membranes from the nucleus, mitochondria, plasma membrane, Golgi, and early endosomes, but still contaminated with lysosomal and peroxi- somal membranes. In order to further purify ER membranes, we subjected Fraction E to an additional centrifugation step through a continuous iodixanol gradient (van Veldhoven et al., 1996). As shown in Figure 2A, the lysosomal and peroxisomal membrane proteins, LAMP1 and PMP70, are exclusively local- ized to a floating membrane layer (tube 13, which is designated as Fraction F). On the other hand, ER membrane proteins (Sec61a, ACAT-1, and Scap) divide into two portions. A portion of all three of these proteins float (tube 13, Fraction F), but the majority of all three are found in denser regions of the gradient (tubes 2–7), which were pooled and designated Fraction G. Frac- tion G is devoid of the lysosomal protein LAMP1 and the perox- isomal protein PMP70. Moreover, Fraction G did not show any detectable signal for plasma membrane markers (Na+/K+ ATPase and transferrin receptor), even when we analyzed ali- quots that were 10-fold higher than those shown in Figure 2.

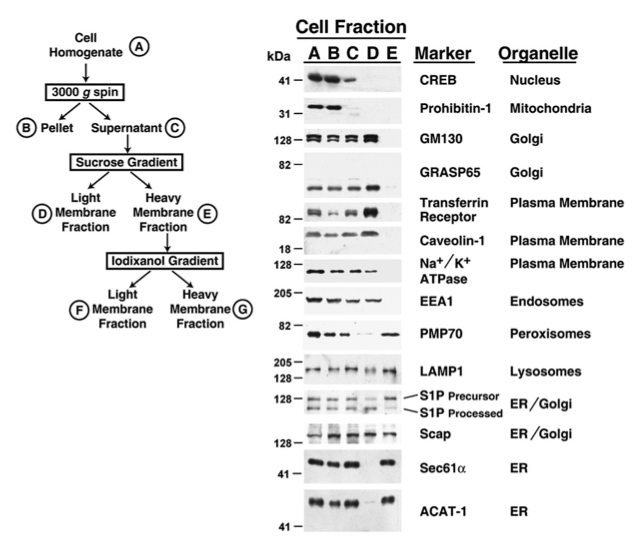


Figure 1. Step 1 in Purification of ER Mem- branes from CHO-K1 Cells: Elimination of Nuclear, Mitochondrial, Plasma, Golgi, and Early Endosomal Membranes

(Left panel) Diagram of ER membrane fraction- ation scheme. (A)–(G) denote major fractions recovered and analyzed by immunoblot analysis. (Right panel) CHO-K1 cells (􏱰2 3 108) were treated according to the fractionation scheme as described in Experimental Procedures and shown at the left. Cells were disrupted using a ball-bearing homogenizer and centrifuged at 3000 *g*. The supernatant was then loaded at the top of a dis- continuous sucrose gradient and centrifuged at 100,000 *g* for 1 hr, yielding two distinct membrane layers. After this step, aliquots representing equal volumes of each fraction (A–E) were subjected to immunoblot analysis for the indicated organelle markers.

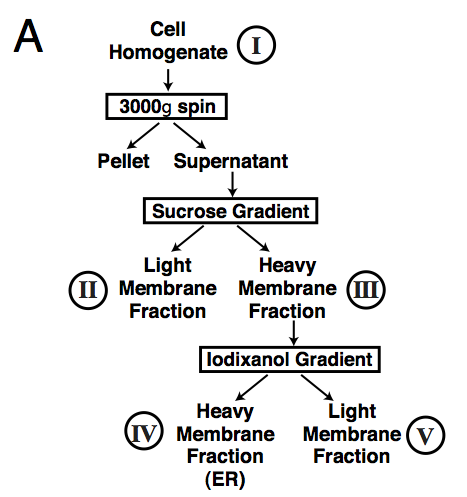
Abi-Mosleh 2009:

SI Methods

Purified endoplasmic reticulum (ER) membranes were isolated from human fibroblasts by a fractionation scheme described for CHO cells by Radhakrishnan et al. (7).

* Briefly, cells were disrupted by using a ball-bearing homogenizer and centrifuged at 3,000 x *g*.
* The super- natant was loaded at the top of a discontinuous sucrose gradient and centrifuged at 100,000 x *g* at 4 °C for 1 h, yielding two distinct membrane layers.
* The heavy membrane fraction was loaded below a continuous 19–25% iodixanol gradient and centrifuged for 2 h at 110,000 x *g* at 4 °C, after which fractions were collected from the bottom.

Fig. 5

Cholesterol content of purified endoplasmic reticulum (ER) membranes from control and NPC1 mutant human fibroblasts. (*A*) Endoplasmic reticulum membrane fractionation scheme. (*B* and *C*) Immunoblot and enzymatic analysis of membrane fractions. On day 0, 13 dishes of hTERT-control and hTERT-NPC1 fibroblasts were set up in medium A with 10% FCS and 4 x 105 cells per 100-mm dish. On day 3, the cells were harvested, and the ER membranes were prepared as described in *SI Materials and Methods* and shown in *A*.

Steffen Mol Cell 2010

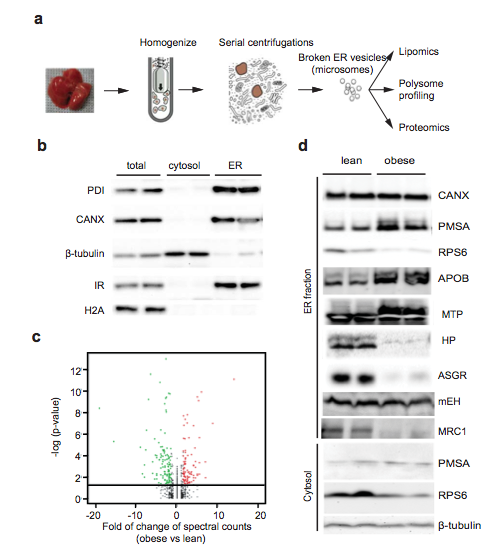
* For isolation of nuclear and non-nuclear proteins cells were resuspended in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, ETDA free Complete (Roche), phosSTOP (Roche), 10 μM MG132) and incubated on ice for 20 min.
* Afterwards NP-40 was added to a final concentration of 0.5 % and the mixture was incubated for additional 5 min.
* The nuclei were pelleted by centrifugation at 1000 xg, 10 min, 4 ° C; the supernatant corresponds to the non-nuclear fraction.
* To isolate nuclear proteins the nuclei were lysed in buffer B (20 mM HEPES pH 7.9, 500 mM KCl, 1 mM EDTA, 1 mM DTT, 10 % Glycerol, EDTA free Complete, phosSTOP, 10 μM MG132) on ice for 30 min.
* The debris was removed by centrifugation at 12000 xg for 10 min at 4 °C.
* To separate cytosolic, membrane bound and nuclear proteins, cells were resuspended in 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl2, Complete, phosSTOP, 10 μM MG132 and lysed with three rounds of freeze/thawing in liquid nitrogen.
* Membranes and nuclei were pelleted at 1000 x g for 10 min, 4 °C and the supernatant was kept as cytosolic fraction.
* Membrane bound proteins were extracted in the same buffer containing 0.5 % NP40 and the nuclei were pelleted by centrifugation at 6000 x g for 10 min at 4 °C.
* Nuclear proteins were obtained by incubating the nuclei in RIPA buffer for 30 min on ice and centrifugation at 6800 x g 10 min 4 °C.

Variations for membrane proteins: babu nature 2012 1% Triton X-100

In contrast to the success of tandem affinity purification (TAP) procedures for characterizing soluble-protein complexes in yeast6,7, native MP complexes are more difficult to purify owing to their hydrophobic nature1. We therefore developed alternative TAP extraction and affinity isolation procedures, using buffers containing one of three different mild, non-denaturing detergents optimized for MP solubiliza- tion. Based on pilot studies, we selected Triton X-100, DDM (n-dodecyl- b-D-maltopyranoside), and C12E8 (octaethylene glycol monododecyl ether), as these were most effective and extracted complementary sets of yeast MPs (Supplementary Fig. 1a–c and Supplementary Text).

Fu Nature 2011:

**ER fractionation**

ER fractionation protocols were adapted from Cox and Emili (2006)22. Briefly, male mice at three months of age (unless otherwise noted) with or without overnight fasting were anesthetized by tribromoethanol and perfused with 20ml 0.25M sucrose solution before tissue harvesting. Fresh liver tissue (1.0g for lean and 1.2g for obese mice produced an equal amount of ER) was immediately transferred to 10ml ice cold STM buffer (0.25M sucrose, 50mM Tris pH7.4, 5mM MgCl2), chopped into small pieces and homogenized by 6 strokes in a motor- driven, loose-fit, teflon-glass homogenizer at speed setting of 3.5 (Wheaton, NJ). The whole lysates were first cleared by centrifugation at 3000g for 10 minutes followed by a series of centrifugations to obtain the final ER pellet. The pellet was washed with 11ml of ice-cold 0.25M sucrose solution and was subjected to centrifugation to obtain the final ER preparation which was either snap frozen in liquid nitrogen or used directly for biochemical and other analysis.

**Supplementary Figure 1: ER fractionation and validation.**

Supplementary Figure 1: ER fractionation and validation.

a, Illustration of ER fractionation procedure for proteomic and lipidomic analyses and polysome profiling. b, Validation of ER fractionation methodology by immunoblot analyses of subcellular markers. PDI: protein disulfide isomerase, CANX: Calnexin, IR: Insulin receptor, H2A: Histone 2A. c, Volcano plot of the fold changes of median spectral counts of proteins from obese and lean samples against the significance of differential expression (log-normalized p-Values). Proteins of interest are highlighted (red: p<0.05, fold of change (obese/lean) ≥1.5, average spectral counts ≥ 5; green: p<0.05, fold of change (lean/obese) ≥ 1.5, average spectral counts ≥ 5). d, Immunoblot of differentially regulated proteins identified from the proteomic study for protein lysates prepared from cytosolic and ER fractions of unfasted lean and obese liver. PMSA: Proteasome and polysome small subunit a, RPS6: Ribosomal small subunit 6, APOB: Apolipoprotein B, MTP: Microsmal triglyceride transfer protein, HP: Hepatoglobin, ASGR: Asialoglycoprotein receptor, mEH: Microsomal epoxide hydrolase, MRC1: Mannose receptor, C type 1.

Arruda Nature Medicine 2014:

This was a mitochondria and mitochondria-associated membrane purification.

**Subcellular fractionation.** The subcellular fractionation of the liver was per- formed based on published protocols54 with minor modifications. 8- to 10-week- old WT mice, *ob/ob* mice and 16 weeks HFD-fed and lean controls were fasted overnight in order to deplete glycogen stocks. The following morning, the ani- mals were killed, and 1 g of liver was immediately weighed and washed 3 times in cold Buffer 1 (content described below). The tissue was minced and immersed in Buffer 2. The tissue was filtered to eliminate the blood and transferred to a glass potter in 30 mL of Buffer 1 and further disrupted by Dounce homogenization. The homogenate was centrifuged at 740*g* for 5 min twice in a Sorvall instrument; the supernatant was recovered and further centrifuged for 10 min at 8000*g*, 3 times. The resulting pellet (crude mitochondrial fraction) (**Supplementary Fig. 3**) was collected and the supernatant was saved for gathering the ER frac- tion. The crude mitochondrial fraction was layered in a 30% Percoll gradient in Buffer 3 and centrifuged at 95,000*g* for 30 min using a Beckman ultracentrifuge in a 41.1 SW rotor. The obtained pure mitochondrial fraction was recovered at the bottom of the tube, whereas the mitochondrial-associated membranes (MAMs) were identified as an intermediate layer between the light membrane and the pure mitochondrial fractions. The pure mitochondria fraction was col- lected, diluted in MRB buffer and further centrifuged at 10,000*g* (11,300 r.p.m.). The pellet was resuspended in 2 mL of MRB buffer. The MAM fraction was collected and diluted 10× in MRB buffer. The suspension was centrifuged at 100,000*g* in a Beckman instrument. The pellet was resuspended in small volume of MRB buffer. The ER fraction was obtained by centrifuging the supernatant collected from the 8,000*g* centrifugation step at 100,000*g* for 60 min. Protein con- centration was determined by BCA, and 25 g of protein was separated by SDS- PAGE and immunoblotted as indicated in the figure legends. Buffer 1: mannitol 225 mM, sucrose 75 mM, Tris-HCl 30 mM, BSA 0.5% and EGTA 0.5 mM, pH 7.4. Buffer 2: mannitol 225 mM, sucrose 75 mM, Tris-HCl 30 mM, BSA 0.5%, pH 7.4. Buffer 3: mannitol 225 mM, sucrose 75 mM, Tris-HCl 30 mM, pH 7.4. MRB Buffer: mannitol 225 mM, HEPES 5 mM, EGTA 0.5 mM, pH 7.4.