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Biochemical Approaches to Studying *Caenorhabditis elegans* ESCRT Functions In Vitro

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Abstract

Our fundamental understanding of the roles played by the endosomal sorting complex required for transport (ESCRT) machinery in cells comes from interdisciplinary approaches that combine numerous in vivo and in vitro techniques. Here, we focus on methods used to biochemically characterize *Caenorhabditis elegans* ESCRT components in vitro, including the production and characterization of recombinant ESCRT complexes and their use in membrane interaction studies. Key methodologies used include gel filtration chromatography, glycerol density gradient analysis, multi-angle light scattering, liposome co-flotation, and single-liposome fluorescence microscopy. Collectively, these studies have enabled us to define subunit stoichiometry of soluble *C. elegans* ESCRT complexes and to demonstrate that the late-acting ESCRT-III complex facilitates membrane bending and remodeling, at least in part by virtue of its ability to sense the curvature of lipid bilayers.

Keywords

ESCRT; *C. elegans*; Biochemistry; Multi-angle light scattering; Liposome co-flotation

1 Introduction

Although *Caenorhabditis elegans* is generally known for its utility in genetic analysis of pathway function, we and others have found it to be equally amenable to biochemical approaches, including in vitro reconstitution-based studies [1–5]. In particular, components of the *C. elegans* ESCRT machinery can be purified recombinantly from *Escherichia coli* with limited proteolysis and, in some cases, superior stability as compared to analogous proteins from other species [6]. The early-acting ESCRT machinery is composed of three multi-subunit, soluble complexes (ESCRT-0, ESCRT-I, and ESCRT-II), which only assemble properly in vitro when their sub-units are co-expressed. The advent of polycistronic expression systems facilitated the production of these complexes in large quantities [7]. In contrast, the late-acting ESCRT-III complex forms polymers, mostly on lipid bilayers in vivo

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[8, 9]. Individual subunits can be expressed and purified using *E. coli*, albeit at differing levels of solubility, and they can subsequently be added together in the presence of membranes to generate higher-order oligomers. Among the *C. elegans* ESCRT-III subunits, our findings indicate that Did2/CHMP1, Vps24/CHMP3, and Vps20/CHMP6 are soluble at millimolar levels, while Vps2/CHMP2, Vps32/CHMP4, and Vps60/CHMP5 precipitate at elevated concentrations (above 10 μM) when purified in the presence of physiological levels of salt. The final component of the core ESCRT machinery is Vps4, an ATPase that functions in concert with ESCRT-III to promote membrane scission reactions in cells [10]. Upon binding ATP, *C. elegans* Vps4 subunits assemble into hexameric ring complexes in vitro. However, these complexes exhibit minimal intrinsic ATPase activity, in contrast to their yeast counterpart, although *C. elegans* Vps4 can be stimulated by the presence of ESCRT-III subunits, similar to mammalian orthologues [3]. Here, we will describe approaches to purifying components of the *C. elegans* ESCRT machinery. In addition, we will highlight the impact of hydrodynamic studies and imaging approaches to our understanding of ESCRT assembly and function on membrane surfaces.

For early-acting ESCRT complexes, we co-express individual subunits from the polycistronic expression plasmid pST39. In all three cases, only a single subunit of each complex is polyhistidine tagged to enable isolation from bacterial extracts on nickel-nitrilotriacetic acid (Ni-NTA) resin. For ESCRT-III subunits and Vps4, the expression occurs using the plasmid pGEX6P-1, which encodes a glutathione S-transferase (GST) tag followed by a PreScission protease cleavage site upstream of each ESCRT component. Bacterially expressed proteins are initially recovered on glutathione agarose and then cleaved using the protease to enable release of the untagged, soluble protein. In most cases, proteins are further purified using size-exclusion chromatography.

Following purification, ESCRT proteins can be used in numerous experimental approaches, including hydrodynamic analyses and interaction studies with membranes. In particular, we have developed approaches to analyze subunit stoichiometry in the early-acting ESCRT machinery, both in solution and on lipid bilayers [2, 4]. Influence of specific membrane lipids and bilayer curvature on ESCRT protein association can also be tested rigorously, using chemically defined liposomes and parallel fluorescence-based measurements [1–5]. In this chapter, we will describe some of the key strategies used to understand how ESCRT complexes assemble in solution and on membranes.

2 Materials

2.1 Recombinant Protein Purification and Hydrodynamic Analysis

1. Terrific Broth-modified powder autoclaved in H_2O to manufacturer's specifications (500 mL in 2.8 L Erlenmeyer flasks).
2. Lysogeny broth (LB) autoclaved in H_2O (100 mL in 250 mL flasks).
3. 100 mg/mL ampicillin in H_2O (1000× stock).
4. Incubator shaker for bacterial growth (temperature range from 18 °C to 37 °C).
5. 400 mM isopropyl β-D-1thiogalactopyranoside (IPTG) in H_2O.

6. Large-capacity centrifuge (Avanti J-26 XPI with JLA-8.1000 6 L rotor and 1 L polypropylene bottles).
7. GST lysis buffer: 1× PBS, 10 mM EGTA, 10 mM EDTA, 0.1% Tween-20, and 250 mM NaCl.
8. 8. GST wash buffer:1× PBS, 250 mM NaCl, 0.1% Tween-20, and 1 mM dithiothreitol (DTT).
9. 6xHis lysis buffer: 50 mM $NaHPO_3$ pH 8.0, 300 mM NaCl, 10 mM imidazole, and 0.15% Tween-20.
10. 6xHis wash buffer: 50 mM $NaHPO_3$ pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.1% Tween-20, and 2-mercaptoethanol.
11. Vortexer (standard for falcon tubes).
12. 50 mL polypropylene centrifuge tubes.
13. Sonicator (Branson Sonifier with macrotip).
14. 1.0 M phenylmethane sulfonyl fluoride (PMSF) in ethanol.
15. 1.0 M benzamidine in ethanol.
16. 16. 20 mg/mL lysozyme; egg white in 1 × PBS.
17. 2-Mercaptoethanol.
18. Floor model centrifuge (Sorvall RC-5B centrifuge with SS-34 rotor).
19. Glutathione agarose.
20. Ni-NTA agarose.
21. Tube rotator.
22. Clinical centrifuge (Thermo IEC31R with swinging bucket rotor).
23. Aspirator.
24. 5 mL syringes (to assemble gravity flow columns).
25. 6xHis elution buffer: 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.6, 100 mM NaCl, 300 mM imidazole, and 1 mM DTT.
26. GST elution buffer: 50 mM Tris pH 8.2, 75 mM KCl, 1 mM DTT, and 10 mM glutathione.
27. Cleavage buffer: 50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.01% Tween-20.
28. PreScission Protease.
29. Tabletop ultracentrifuge (Beckman TL100 with TLA-100.3 rotor and polycarbonate centrifuge tubes).

30. Fast protein liquid chromatography (FPLC) buffers: 50 mM HEPES pH 7.6, 100–500 mM NaCl, and 1 mM DTT.
31. Disposable 3 mL syringes and needles.
32. FPLC with size-exclusion chromatography columns (AKTA purifier with Superose 6 and/or S200 columns from GE).
33. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) supplies and Coomassie Blue.
34. Glycerol solutions: 50 mM Hepes pH 7.6, 100 mM NaCl, 1 mM DTT, and 10% or 30% glycerol.
35. Gradient Master and accessories (to pour glycerol gradients).
36. Gradient centrifuge tubes (7010, Seton).
37. Wyatt miniDAWN TREOS multi-angle light-scattering device.
38. Liquid nitrogen.

2.2 Protein and Membrane Interaction Studies

1. Avanti mini extruder.
2. Polycarbonate filters.
3. Purified lipids: phosphatidylcholine (POPC), phosphatidyleth-anolamine (POPE), phosphatidylserine (POPS), phosphatidy-linositol 3-phosphate (PI3P), biotinyl-PE, and rhodamine-labeled PE.
4. Tweezers.
5. 200 proof ethanol.
6. Deionized H_2O.
7. N_2 gas.
8. Lyophilizer.
9. Co-flotation buffer: 50 mM HEPES pH 7.6, 100 mM NaCl, and 1 mM DTT.
10. Vortexer.
11. 100% accudenz (wt/vol) in deionized H_2O).
12. Accudenz solutions: 30%, 35%, and 80% accudenz in co-flotation buffer.
13. Tabletop ultracentrifuge (Beckman TL100 with TLA-100 rotor and polycarbonate tubes).
14. Boron-dipyrromethene (BODIPY)-FL-labeled maleimide.
15. Glutathione.
16. Spectrofluorometer (PTI QM40).

17. Swept-field confocal microscope (Nikon Ti-E equipped with a Roper CoolSNAP HQ2 CCD camera and a 60×, 1.4 numerical aperture Plan Apo oil objective lens).
18. Nikon Elements Software.
19. Glass coverslips.
20. Polyethylene glycol (PEG) and biotin-PEG (40:1 ratio) solutions.
21. 1.0 M Avidin in wash buffer.
22. Wash buffer: 50 mM HEPES pH 7.6, 100 mM NaCl.
23. MetaMorph software.
24. MicroCal ITC200 calorimeter.

3 Methods

3.1 Recombinant Protein Purification and Characterization

3.1.1 ESCRT-0, ESCRT-I, and ESCRT-II Complex Expression and Purification

1. Transfect pST39-based expression plasmid into BL21 (DE3) competent cells. For ESCRT-0, the Hrs subunit harbors an amino-terminal polyhistidine tag. For ESCRT-I, the Tsg101 subunit harbors a carboxyl-terminal polyhistidine tag. For ESCRT-II, the Vps25 subunit harbors a carboxyl-terminal polyhistidine tag.
2. Set up an overnight culture (100 mL LB, 1× ampicillin in a 250 mL flask) with a single BL21 (DE3) transfected colony. Grow cultures at 25 °C (shaking at 240 RPM [revolutions per minute]) for 12–16 h.
3. Inoculate an Erlenmeyer flask containing 500 mL of sterile Terrific Broth media (containing 1× ampicillin) using 10 mL of the overnight LB culture. Place the flask in an incubator shaker and grow at 25 °C (shaking at 240 RPM) to an OD_{600} of approximately 2.0. Turn down the incubator shaker to 18 °C and add 300 μL of 400 mM IPTG to the culture (final concentration: 0.24 mM). Let the culture shake for approximately 12–14 h.
4. Centrifuge the cells in a large-capacity centrifuge (4785 RCF [relative centrifugal force], 4 °C) for 15 min (*see* Note 1). Pour off the majority of the media and transfer the pelleted bacteria to a 50 mL polypropylene centrifuge tube (by resuspending the pellet in the remaining media). Centrifuge again, aspirate the media, and resuspend the pellet in 6xHis lysis buffer by vortexing. Freeze the pellet at 80 °C (*see* Note 2).
5. Thaw the pellet at room temperature. Once thawed, transfer the lysate to a 50-mL glass beaker on ice and add 1 mL of 1.0 M benzamidine, 1 mL of 1.0 M PMSF, and 2 mL of 1 mg/mL lysozyme (*see* Note 3). Swirl gently on ice.

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1. RCF can be converted to RPM using the following equation: RCF = $r(1.118 \times 10^{-5})$ RPM, where r is the rotational radius in cm.
2. Pellets can be stored at −80 °C for up to 3 months.

6. Sonicate the cells by using a 400-watt ultrasonic homogenizer (70% amplitude, 0.3 s pulse on, 0.7 s pulse off, macrotip) for a total of 60 s in an ice bath. Avoid creating bubbles or froth. Add 40 μL of 2-mercaptoethanol and swirl gently on ice (*see* Note 4).

7. Centrifuge the lysate in a floor model centrifuge at 27,000 RCF, 4 °C, for 1 h. Carefully collect the supernatant into a 50 mL polypropylene centrifuge tube containing 1 mL Ni-NTA resin. Place the tube on a rotator and rotate for 1 h at 4 °C (*see* Note 5).

8. Centrifuge the resin in a clinical centrifuge (1000 RCF, 4 °C) for 2 min. Aspirate to the top of the resin and then resuspend the resin with cold 6xHis lysis buffer through gentle inversion (*see* Note 6). Repeat two times.

9. Transfer the resin to a 5 mL syringe column (with a porous frit at the bottom to prevent resin from leaking out) and wash with 200 mL of cold 6xHis wash buffer by gravity flow at 4 °C (add PMSF to the wash buffer immediately prior to use). Wash with 50 mL of cold 6xHis wash buffer without Tween-20 by gravity flow at 4 °C (add PMSF to the wash buffer immediately prior to use). Avoid allowing the column to run dry.

10. Cap the bottom of the syringe and add 1 mL 6xHis elution buffer. Plug the top of the syringe and rotate for 5 min at 4 °C. Remove the top plug first, and collect the eluate containing the protein of interest by gravity flow. Repeat twice.

11. Concentrate the sample to 1 mL total volume using a Vivaspin centrifugal concentrator and gel filter using a Superose 6 or S200 (GE) size-exclusion column equilibrated in 6xHis elution buffer without imidazole. Elute in 1 mL fractions.

12. Analyze fractions using SDS-PAGE followed by Coomassie blue staining. Combine fractions containing intact ESCRT complexes and snap freeze aliquots in liquid nitrogen and store at −80 °C. In general, only one or two of the fractions recovered from the gel filtration column will contain intact ESCRT complexes.

3.1.2 Vps4 and ESCRT-III Subunit Expression and Purification

1. Transfect pGEX6P-1-based expression plasmid into BL21 (DE3) competent cells. For all ESCRT-III subunits and Vps4, an amino-terminal GST tag is present to enhance solubility and enable purification.

2. Set up an overnight culture (100 mL LB, 1× ampicillin in a 250 mL flask) with a single BL21 (DE3) transfected colony. Grow cultures at 25 °C (shaking at 240 RPM) for 12–16 h.

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3. Benzamidine and PMSF are protease inhibitors that are added to prevent unwanted proteolysis of overexpressed protein. Lysozyme facilitates cell rupture by degrading the bacterial cell wall.
4. To avoid creating bubbles or froth, the tip should be submerged approximately ½ in. 2-mercaptoethanol is added after sonication to reduce oxidized protein.
5. It is important to avoid collecting insoluble material in the supernatant. Contamination will compromise the subsequent wash steps.
6. After the first centrifugation step, the pelleted resin may be difficult to resuspend through inversion. If this occurs, do not shake the tube. Use a pipette tip to gently break up the resin.

3. Inoculate an Erlenmeyer flask containing 500 mL of sterile Terrific Broth media (containing 1× ampicillin) using 10 mL of the overnight LB culture. Place the flask in an incubator shaker and grow at 25 °C (shaking at 240 RPM) to an OD_{600} of approximately 2.0. Turn down the incubator shaker to 18 °C and add 300 μL of 400 mM IPTG to the culture (final concentration: 0.24 mM). Let the culture shake for approximately 12–14 h.

4. Centrifuge the cells in a large-capacity centrifuge (4785 RCF, 4 °C) for 15 min. Pour off the majority of the media and transfer the pelleted bacteria to a 50 mL polypropylene centrifuge tube (by resuspending in the remaining media). Centrifuge again, aspirate the media, and resuspend the pellet in GST lysis buffer by vortexing. Freeze the pellet at −80 °C.

5. Thaw the pellet at room temperature. Once thawed, transfer the lysate to a 50 mL glass beaker on ice and add 1 mL of 1.0 M benzamidine, 1 mL of 1.0 M PMSF, and 2 mL of 1 mg/mL lysozyme. Swirl gently on ice. For Vps4, additionally add 1 mM ADP to the extract (*see* Note 7).

6. Sonicate the cells by using a 400-watt ultrasonic homogenizer (70% amplitude, 0.3 s pulse on, 0.7 s pulse off, macrotip) for a total of 60 s in an ice bath. Add 40 μL of 2-mercaptoethanol and swirl gently on ice.

7. Centrifuge the lysate in a floor model centrifuge at 27,000 RCF, 4 °C, for 1 h. Carefully collect the supernatant into a 50 mL polypropylene centrifuge tube with 1 mL glutathione agarose that has been equilibrated in GST lysis buffer. Place the tube on a rotator and rotate slowly for 1 h at 4 °C.

8. Centrifuge the resin in a clinical centrifuge (1000 RCF, 4 °C) for 2 min. Aspirate to the top of the resin and then resuspend the resin with cold GST lysis buffer through gentle inversion. Repeat two times.

9. Transfer the resin to a 5 mL syringe column (with a porous frit at the bottom to prevent resin from leaking out) and wash with 200 mL of cold GST wash buffer by gravity flow at 4 °C (add PMSF to the wash buffer immediately prior to use). Avoid allowing the column to run dry.

10. Wash with 20 mL of cleavage buffer by gravity flow at 4 °C. Avoid allowing the column to run dry.

11. Cap the bottom of the column and transfer resin using an equal volume of cleavage buffer to a screw-top polypropylene tube. For Vps4, supplement the cleavage buffer with 10 μM ADP. Add PreScission protease and rotate tube overnight at 4 °C (Fig. 1).

12. Centrifuge at 27,000 RCF and transfer the supernatant, which contains the cleaved protein, to a fresh tube.

13. Resuspend resin with 200 μL cleavage buffer, centrifuge, and add the supernatant to the previously collected, eluted protein.

[7]·ADP is required to stabilize monomeric Vps4 and prevent aggregation.

14. Centrifuge the eluted protein in a tabletop ultracentrifuge (100,000 RCF, 4 °C) for 15 min. Collect the supernatant, being careful not to disturb any pellet that may have formed during centrifugation (Fig. 1).

15. Gel filter the sample using a Superose 6 or S200 (GE) size-exclusion column equilibrated in GST elution buffer without glutathione. Elute in 1 mL fractions. For Vps4, supplement the buffer with 10 μM ADP.

16. Analyze fractions using SDS-PAGE followed by Coomassie blue staining. Combine fractions containing intact ESCRT subunits and snap freeze aliquots in liquid nitrogen and store at −80 °C. In general, only one or two of the fractions will contain the intact ESCRT components. In the case of Vps4, oligomers that form during initial purification from bacterial extracts are aggregates and will not respond to the addition of ATP. Only retain the final fraction of Vps4 that elutes from the gel filtration column, as it contains mostly monomers, which are capable of assembling into hexameric ring complexes (Fig. 1).

3.2 Hydrodynamic Analysis of ESCRT Components

1. Use a Gradient Master system (Biocomp Instruments) to pour 10–30% glycerol gradients.

2. Mark the center of the gradient centrifuge tubes and fill to approximately 2 mm above this point with 10% glycerol.

3. Using a 10 mL syringe, layer the 30% glycerol solution underneath the 10% solution, until the interface between solutions reaches the center of the centrifuge tube. Be careful not to introduce any air bubbles into the tube.

4. Cap and rotate the tubes in the Gradient Master to generate linear gradients, following the manufacturers' instructions.

5. Cool the gradients for 1 h at 4 °C.

6. Carefully remove the caps and load 100 μL of purified ESCRT protein on top of one gradient. In parallel, load standards (with known sedimentation values such as thyroglobulin, apoferritin, BSA, etc.) onto other gradients that you will centrifuge at the same time.

7. Balance tubes by weight and centrifuge for 8 h at 50,000 RPM (260,000 RCF) in an SW60Ti rotor (swinging bucket).

8. Collect fractions (200 μL each) by hand from the top of the gradient, being careful not to disturb the gradient during this process.

9. Analyze fractions using SDS-PAGE followed by Coomassie blue staining.

10. Create a standard curve by plotting the peak elution of known standards against their sedimentation values. At least three standards must be run in parallel with your ESCRT protein, although additional standards will generate a more precise standard curve.

11. Plot the peak elution of the ESCRT component on the standard curve to identify its sedimentation value.

12. In combination with its Stokes radius, derived from size-exclusion chromatography studies, the native molecular weight of the ESCRT component can be estimated (within approximately 10%).

13. To calculate the native molecular weight, the following equation can be used: $M = 6\pi\eta Nas/(1 - \upsilon\rho)$, where M is the native molecular weight, η is the viscosity of the medium, N is Avogadro's number, a is the Stokes radius, s is the sedimentation value, υ is the partial specific volume, and ρ is the density of the medium [11].

14. In cases where ESCRT proteins can be purified to high concentrations (greater than 50 μM), molecular mass can also be determined with higher accuracy using a combination of size-exclusion chromatography and multi-angle light scattering. Use a Wyatt miniDAWN TREOS three-angle light-scattering device, coupled to a high-resolution size-exclusion column, for this purpose.

15. Apply 500 μL of purified ESCRT protein onto a Wyatt WTC-030S5 gel filtration column.

16. Use a flow rate of no more than 0.5 mL/min to maintain resolution.

17. Use Astra software to determine molecular mass, based on UV detection and in-line light scattering [12].

3.3 Interaction Studies with Membranes

3.3.1 Production of Liposomes

1. Using a glass micropipette, transfer stock lipid solutions (in 100% chloroform) into a glass test tube. Dry lipids using a steady stream of N_2 gas and then lyophilize overnight. The composition of liposomes can vary. In general, use a combination of POPC, POPS, and POPE to mimic biological membranes. However, the addition of cholesterol, phosphoinositides, or other lipids can be performed. Incorporate 0.5% of rhodamine-labeled PE into liposomes if they are used in co-flotation assays or other membrane interaction studies that leverage fluorescence microscopy.

2. Add 250 μL of co-flotation buffer and resuspend by gently vortexing at 5 min intervals for 30 min.

3. Set up the mini extruder as described by the manufacturer, making sure to soak the polycarbonate filter in deionized H_2O. Draw up 500 μL of deionized H_2O into one of the syringes and pass through the filter ten times. Discard the water and repeat with co-float buffer. The size of the filter used will determine liposome size. Hydrated lipid solutions will initially form large, multilamellar vesicles. After the initial pass through a membrane, the particle size distribution will tend toward bimodal. After sufficient passes through the membrane, a unimodal, normal distribution can be obtained. However, liposomes will not

likely be homogenous in size using this method, and the use of dynamic light scattering to measure the size distribution of liposomes generated is highly recommended.

4. Draw up the resuspended lipids into one of the syringes and extrude them by passing them through the filter 19 times, making sure to collect the liposomes in the opposite syringe. Store the liposomes in a 1.5 mL polypropylene tube at 4 °C (*see* Note 8).

3.3.2 Co-flotation Assay

1. Thaw-purified ESCRT component on ice. While waiting, make up solutions of accudenz density media (30%, 35%, and 80%) in co-flotation buffer. Once the proteins are thawed, mix them with liposomes to a final volume of 60 μL and place the tube on ice for 30 min (*see* Note 9).
2. Add 60 μL of the 80% accudenz solution to the protein/liposome mixture, making sure to pipette up and down until the solution is homogenous. Add 100 μL of the solution to the bottom of a polycarbonate centrifuge tube (TLA 100).
3. Carefully layer 50 μL of the 35% accudenz solution on top. A clear phase boundary should develop. Repeat with the 30% accudenz solution. Then add 20 μL of co-float buffer to the top of the centrifuge tube (*see* Note 10).
4. Centrifuge in a tabletop ultracentrifuge for 1 h at 541,000 RCF. Collect the liposomes with associated proteins at the top of the tube (*see* Note 11).
5. Normalize the recovery of liposomes based on fluorescence intensity using a spectrofluorometer and analyze the samples using SDS-PAGE followed by Coomassie blue staining (*see* Note 12).
6. By varying the size of liposomes used, curvature sensing of ESCRT proteins can be measured (Fig. 2). By varying lipid composition, the dependence of ESCRT association with membranes on specific lipid headgroups can be determined.

3.3.3 Fluorescent Labeling and Imaging of ESCRT Proteins

1. Several *C. elegans* ESCRT subunits harbor endogenous cysteine residues that are amenable to direct labeling with fluorescent dyes. Use dye maleimides, including BODIPY-FL-maleimide, to label recombinant ESCRT proteins.
2. Incubate a 20-fold molar excess of BODIPY-FL-maleimide with the ESCRT protein of interest. Rotate the mixture overnight.

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8. After extrusion, it is important to clean the extruder materials to avoid buildup of salts and lipids. Soak the extruder apparatus in 100% ethanol, and wash out the syringes with 100% ethanol, followed by deionized H_2O.
9. Accudenz is a nontoxic, inert, nonionic, tri-iodinated derivative of benzoic acid with three aliphatic hydrophilic side chains. It has low osmolality and low viscosity, making it useful for the fractionation of many proteins.
10. To avoid mixing the layers, touch the pipette tip to the side of the tube just below the meniscus of the previous layer and pipette slowly.
11. The liposomes/proteins should form a distinct layer at the top of the tube, but they may occasionally form an aggregate just below the surface. If this occurs, try and collect as much of the aggregate as possible.
12. If the liposome-associated proteins are not visible after Coomassie blue staining, more sensitive dyes can be used. SYPRO Ruby is recommended for staining less than 50 ng of protein.

3. Quench the reaction by adding excess glutathione (fivefold molar excess), and remove the excess unbound dye using size-exclusion chromatography.
4. Measure dye-labeling stoichiometry based on the final concentration of recovered protein and its absorbance.
5. To visualize ESCRT protein association with membranes, fluorescently labeled liposomes can be used in combination with fluorescently labeled ESCRT subunits.
6. To generate immobilized-, fluorescently labeled liposomes, incorporate both rhodamine-PE and biotin-PE (0.5%) into lipid mixtures prior to extrusion. These liposomes can then be immobilized on avidin-coated glass coverslips.
7. Clean glass coverslips with H_2O and coat them with a solution of PEG (5000 Da) and biotin-PEG (40:1 ratio).
8. Incubate the PEG-coated coverslips with 1.0 μM avidin for 10 min.
9. Wash the coverslips 2–3 times with wash buffer to remove excess avidin.
10. Mix 100 μL of the biotinylated, rhodamine-labeled liposomes with the BODIPY-FL-labeled ESCRT protein (250 nM) and incubate the mixture on the avidin-coated coverslips for 30 min.
11. Aspirate unbound liposomes and protein. Add 3 μL of wash buffer and invert onto a depression slide for imaging.
12. Use a 60×, 1.4 numerical aperture Plan Apo oil objective lens for imaging.

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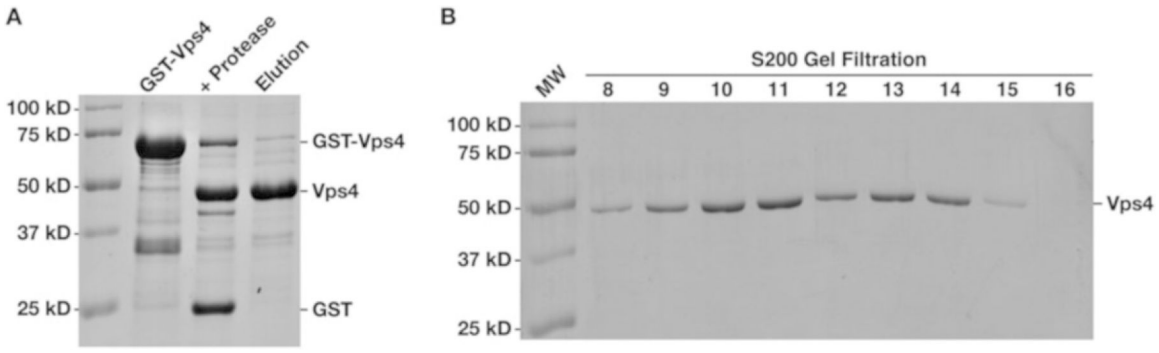

Fig. 1.

Purification of *C. elegans* Vps4. (**a**) Following the expression from pGEX6P-1, GST-Vps4 is purified initially on glutathione agarose beads (lane 2), cleaved using PreScission protease (lane 3, a sample of boiled resin), and the eluate is resolved by SDS-PAGE (lane 4). Molecular weight marker is also shown (lane 1). (**b**) Recombinant Vps4 cleaved from glutathione resin following purification as a GST fusion protein was applied onto an S200 gel filtration column and elutes as two distinct peaks. Only peak fractions 13–15 harbor monomeric protein, which is capable of homotypic assembly into hexameric protein complexes when ATP is supplemented into the buffer

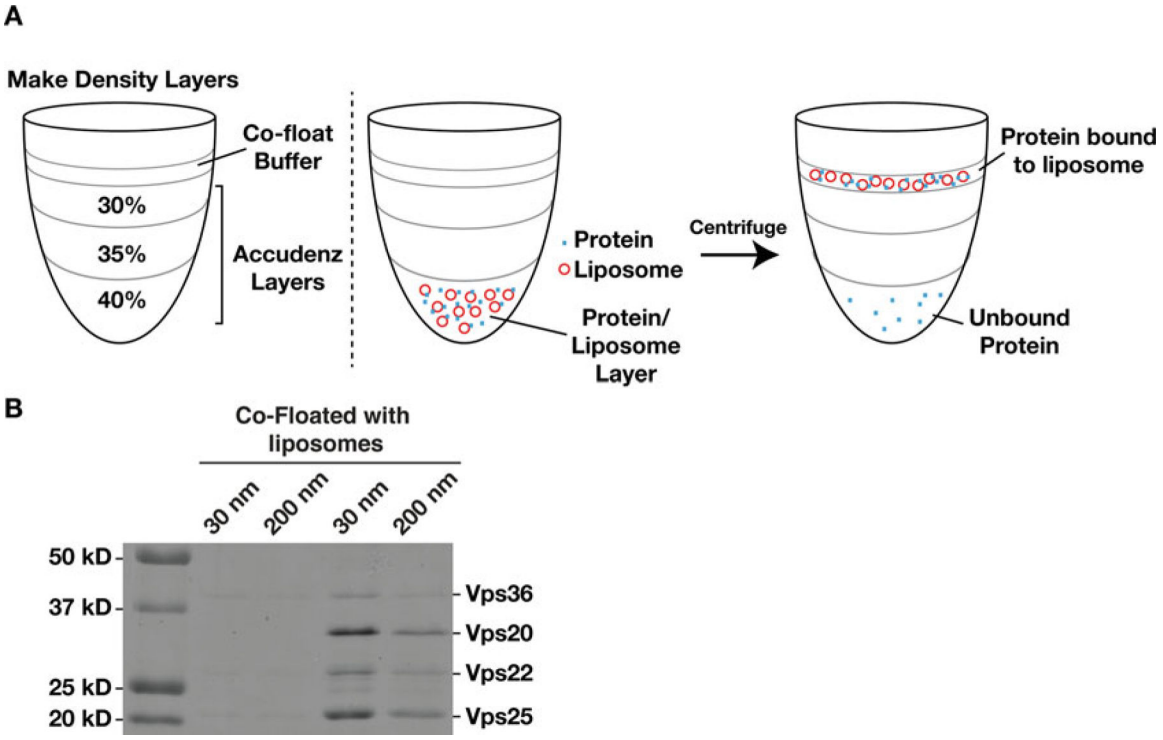

Fig. 2.

Liposome co-flotation analysis to measure curvature sensing in the ESCRT machinery. (**a**) A cartoon illustrating the steps involved in conducting a co-flotation study. First, varying concentrations of accudenz are layered in a centrifuge tube, with liposomes and ESCRT proteins mixed in the 40% layer at the bottom. During centrifugation, the liposomes and associated ESCRT proteins float to the top of the gradient, while unbound ESCRT proteins remain at the bottom of the centrifuge tube. (**b**) Image of SDS-PAGE analysis followed by Coomassie blue staining of co-flotation assay. The ESCRT-II complex composed of Vps36, Vps22, and Vps25 co-floats modestly with liposomes (55% PC, 30% PE, and 15% PS), irrespective of their size (lanes 2 and 3). However, when the ESCRT-III subunit Vps20 is added, the set of proteins co-float with liposomes more robustly, with a preference for more highly curved liposomes (lanes 4 and 5). A molecular weight marker is also included (lane 1)