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TITLE:

Expression, Purification, and Liposome Binding of Budding Yeast SNX-BAR Heterodimers

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SNX-BAR, purification, endosome, phospholipid, yeast, liposomes

SUMMARY:

Here, we present a workflow for the expression, purification and liposome binding of SNX-BAR heterodimers in yeast.

ABSTRACT:

SNX-BAR proteins are an evolutionarily conserved class of membrane remodeling proteins that play key roles in sorting and trafficking of protein and lipids during endocytosis, sorting within the endosomal system, and autophagy. Central to SNX-BAR protein function is the ability to form homodimers or heterodimers that bind membranes using highly conserved phox-homology (PX) and BAR (Bin/Amphiphysin/Rvs) domains. In addition, oligomerization of SNX-BAR dimers on membranes can elicit the formation of membrane tubules and vesicles and this activity is thought to reflect their functions as coat proteins for endosome-derived transport carriers. Researchers have long utilized in vitro binding studies using recombinant SNX-BAR proteins on synthetic liposomes or giant unilamellar vesicles (GUVs) to reveal the precise makeup of lipids needed to drive membrane remodeling, thus revealing their mechanism of action. However, due to technical challenges with dual expression systems, toxicity of SNX-BAR protein expression in bacteria, and poor solubility of individual SNX-BAR proteins, most studies to date have examined SNX-BAR homodimers, including non-physiological dimers that form during expression in bacteria. Recently, we have optimized a protocol to overcome the major shortcomings of a typical bacterial expression system. Using this workflow, we demonstrate how to successfully express and purify large amounts of SNX-BAR heterodimers and how to reconstitute them on synthetic liposomes for binding and tubulation assays.

INTRODUCTION:

Membrane-bound organelles such as the plasma membrane, the endoplasmic reticulum, the Golgi apparatus, lysosome (yeast vacuole), and endosome comprise the endomembrane system of the eukaryotic cell. Most organelles have the ability to communicate and exchange material with other organelles through vesicle transport carriers. How the cell coordinates the packaging and formation of vesicle transport carriers within the endomembrane system is not well understood. However, the proteins and lipids that constitute much of the endomembrane system are known to originate from internalizing endocytic vesicles from the plasma membrane (PM). The endosome is the primary acceptor organelle for these vesicles and is comprised of multiple interconnected sets of tubular organelles. The principal function of the endosome is to facilitate nutrient acquisition, regulate protein and lipid turnover, protect from pathogen infection, and to serve as the primary replenishing source of lipids for the plasma membrane. As the endosome receives the bulk of cargo proteins and lipids from the plasma membrane, it acts as a sorting compartment by isolating cargos into tubular endosomal transport carriers (ETCs). Any proteins not sequestered into ETCs are left to be degraded via the endo-lysosomal system. The dysregulation of cargo sorting into ETCs can lead to the loss of nutrient uptake, protein turnover or lipid homeostasis, resulting in numerous metabolic, developmental, and neurological disorders[1,2]. However, despite ETCs central role at the endosome, the underlying mechanism of how the endosome can selectively coordinate the packaging of a multitude of heterogeneous cargos into tubular carriers is not known.

The sorting nexin (SNX) family is an evolutionarily conserved class of proteins that have been found to be critical for many vesicle transport reactions in the cell[3-5]. Sorting nexins are recruited to the endosome membrane and aid in cargo capture via their characteristic phox homology (PX) domain, which binds phosphatidylinositol-3-monophosphate (PtdIns(3)P), a lipid enriched on the endosome membrane. Mammals encode thirty-three SNX proteins, which can be further divided into multiple subfamilies, according to the presence of other domains[1]. Most notably, the SNX-BAR subfamily is the largest subfamily consisting of twelve in human, while in budding yeast, *Saccharomyces cerevisiae*, the subfamily is reduced to just seven SNX-BARs. SNX-BAR proteins have both a PX domain and a Bin-Amphiphysin-Rvs (BAR) domain that triggers lipid reservoirs to bind positive curvature membranes. Consequently, the SNX-BAR family has a natural affinity for the endosome and can mediate ETC formation via their membrane remodeling abilities. In vitro, the remodeling properties of SNX-BARs can be induced by the addition of purified SNX-BARs to synthetic liposomes and the subsequent formation of narrow, coated tubules can be visualized by electron microscopy. Using these methods, researchers have determined that both oligomerization concentration and constriction strength appear to vary amongst the SNX-BAR family suggesting they could aid in both the formation and scission of ETCs.

The SNX-BARs can be further classified by their exclusive dimerization properties. In vitro binding assays and structural studies have demonstrated that SNX-BAR proteins can only form specific homodimers or heterodimers. Therefore, in principle, each potential SNX-BAR dimer-oligomer could provide a tubule coat for a cargo-specific trafficking pathway and likewise, the restricted oligomerization of the other SNX-BAR protomers, can also define distinct export pathways. However, due to the large number of SNX-BARs and diversity within the SNX family, a one sorting

nexin-one cargo hypothesis is highly unlikely. Instead a coordinated effort using a multitude of factors such as SNX-BARs, cargo, lipid specificity and other dependencies is more probable. Likewise, recent studies of the yeast SNX4 family revealed evidence for additional lipid specificity, beyond PtdIns(3)P, to potentiate endosome transport carriers[6]. In this study, SNX-BAR homodimer Mvp1-Mvp1 was purified from bacteria and native heterodimers Snx4-Atg20 and Vps5-Vps17 were expressed and purified in high yield from yeast, while only Snx4-Atg20 was found to preferentially bind phosphatidylserine (PS) and form narrow tube-like structures in liposome binding studies[6]. While others in the field have revealed important properties of the SNX-BARs using recombinantly purified SNX-BAR homodimers from bacteria, toxicity associated with expressing SNX-BAR heterodimers in similar systems have hindered their native characterization[7-10]. Therefore, without a reliable system to obtain pure recombinantly expressed native heterodimers, researchers must forgo these lines of investigation. In **Figure 1**, we present a four-part workflow to 1) construct a yeast strain overexpressing SNX-BAR heterodimers for tandem affinity purification, 2) express and purify native SNX-BAR heterodimers, 3) prepare unilamellar synthetic liposomes, and 4) set up a liposome tubulation or sedimentations assay, providing a vital tool for researchers to investigate the growing catalogue of sorting nexins found in nature.

PROTOCOL:

1. Yeast strain construction

1.1) Begin with TVY614 (*pep4Δ*::LEU2 *prb1Δ*::hisG *prc1Δ*::HIS3) [11] as the parent strain. This strain is deficient for vacuolar proteases, which contribute to the majority of protein degradation after cell lysis, and therefore allows for a cleaner and more efficient purification.

1.2) Design primers[12] and integrate tandem affinity purification (TAP) tag at the C-terminus of Atg20 (SNX-BAR ORF 1) using homologous recombination. Use polymerase chain reaction (PCR) to confirm integrations (**Figure 2**).

1.3) Perform a western blot of cell lysate against the TAP tag to confirm proper integration[13].

NOTE: We recommend harvesting 3 OD (1 OD ≈ 1 x 10^7 cells) of cells for SDS-PAGE and western blot verification. Note that integration of the TAP tag should occur prior to replacing the endogenous promoters with the GAL1 promoter to allow for easier TAP tag verification via western blot.

1.4) Replace endogenous Snx4 (SNX-BAR ORF1) and Atg20 (SNX-BAR ORF 2) promoters with untagged GAL1 promoter using sequential homologous recombination and transformation steps of each individual ORF[14].

1.5) Use flanking primers outside of the integration sites to PCR confirm successful integrations (**Figure 2**). This will result in a null phenotype of the targeted SNX-BARs in the absence of galactose in the growth medium.

2. Yeast induction and SNX-BAR dimer purification

NOTE: Yeast cells can be propagated on standard YPD (yeast extract, peptone, and 2% glucose) agar plates as the modifications are chromosomally integrated.

2.1) Inoculate a large swab of cells into 50 mL of standard YP (yeast extract and peptone) medium with 2% raffinose and 0.1% glucose as carbon source in a flask at least 4x the volume of the culture and grow overnight in 30 °C shaker to allow proper aeration. Expect that growth in this medium will be slower compared to standard YPD.

2.2) Next morning, use the 50 mL preculture to inoculate into 1 L of standard YP medium with 2% raffinose and 0.1% glucose and grow for 4–5 h in 30 °C shaker.

NOTE: The volume of preculture used to inoculate 1 L culture may be adjusted depending on the OD_{600} of the preculture. The OD_{600} of the 1 L culture after inoculation should be around 0.2 to allow for at least two doublings during the 4–5 h growth.

2.2.1) Use a baffled Fernbach flask for growth to allow proper aeration, a 2.8 L volume flask is sufficient. Less aeration may result in slower growth and smaller cell pellet upon harvesting.

2.3) Check OD_{600} to make sure culture is in the log phase (0.5–1) after 4–5 h of growth. Depending on the growth of the strain, make adjustments to the growth time to allow for at least two doublings. Add to 2% galactose and grow overnight in 30 °C shaker.

NOTE: The OD600 after the overnight growth may vary but culture should be saturated. Note that cells do not need to be removed from 0.1% glucose before addition of 2% galactose for this growth protocol. We recommend harvesting 3 OD of uninduced and induced cells at this step for SDS-PAGE and western blot for verification (**Figure 3A,B**, Lane 1–2).

2.4) Harvest cells by centrifugation at 4500 x *g* for 15 min. A swinging bucket rotor that accommodates the 1 L volume culture may be used here.

2.5) Transfer the yeast pellet into a 50 mL conical tube; a second centrifugation step may be performed as needed. The cell pellet will generally be around 10–15 mL in volume as measured by graduation markings and may be used immediately or stored at -80 °C.

2.5.1) Resuspend pellet in 15 mL purification buffer (50 mM Tris pH 7.4, 300 mM NaCl, 1.5 mM $MgCl_2$, 1 mM Dithiothreitol (DTT), protease inhibitor cocktail) to make final volume around 30 mL.

2.6) Chill a homogenizer 4 °C before use and equilibrate it with purification buffer. Lyse cells using a mechanical cell disruptor or homogenizer. Load sample into the homogenizer and lyse at 20,000–25,000 psi for 2–3 rounds; note that as cells become lysed, more input pressure is

required to maintain 20,000–25,000 psi. Collect the cell lysate in a 50 mL conical tube on ice.

NOTE: If additional samples need to be lysed, the homogenizer should be thoroughly cleaned and equilibrated before loading the next sample. Keep all cell lysates on ice.

2.7) Immediately clear the cell lysate at 35,000 x *g* for 1 h at 4 °C. Carefully transfer the supernatant into new tube. Note that the lipids from cell lysis will float to the top during centrifugation and will not affect purification.

NOTE: We recommend saving 0.5–1% of the lysate and pellet for SDS-PAGE samples. Typically, no major differences are observed and an additional western blot may be done to confirm TAP protein solubility (**Figure 4**, Lanes 1 and 2, respectively).

2.8) Equilibrate 300 µL of IgG sepharose beads with purification buffer. Add to the cleared cell lysate and incubate for 2 h, rotating at 4 °C.

2.9) Collect beads in a 10 mL chromatography column and allow unbound lysate to flow through.

2.10) Wash beads using 10 mL of wash buffer (50 mM Tris pH 7.4, 300 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT), adding 1 mL at a time and allowing it to flow through completely.

NOTE: We recommend saving 2% of the bound-IgG beads for SDS-PAGE sample. Typically, we observe four major bands; two SNX-BAR proteins and IgG Heavy and Light Chains (**Figure 4**, Lane 3). We recommend saving equivalent amounts of 'eluate' and 'IgG beads after TEV' to compare for TEV cleavage efficiency.

2.11) Collect beads and transfer them to a microcentrifuge tube. Add to 500 µL of total volume with fresh wash buffer and 2 µL of 10 mg/mL TEV protease and incubate overnight, rotating at 4 °C.

2.12) Next morning, remove the supernatant completely using a 27 G needle and assess protein purity by 10% polyacrylamide SDS-PAGE (**Figure 4**, Lane 4).

NOTE: We typically obtain 500 µL of 0.5–1 mg/mL of 95% pure heterodimer (**Figure 4**, Lane 4). Additional purification using calmodulin resin can also be done, however we typically see a significant reduction in yield and recommend stopping here if purity is >90%. TEV does not interfere with liposome binding assays, though TEV can be additionally removed using Ni-NTA agarose beads[15].

2.13) To concentrate, transfer the sample to a 0.5 mL centrifugal filter with 10 KDa cutoff and centrifuge according to manufacturer's instructions to 50 µL or less. Quantify the concentrated proteins using the Bradford protein assay. Store at 4 °C and use within one week.

3. Liposome preparation

3.1) Purchase commercially available lipids: phosphatidylserine (PS), PI3P, ergosterol, and phosphatidylcholine (PC). If needed, resuspend in recommended solvent to make stock lipids.

NOTE: Lipids are resuspended in a methanol/chloroform mixture per manufacturer's recommendations. Make sure lipid stocks are clear and warmed to room temperature before using. Resuspended lipids can be stored under argon gas and sealed using wax film (or equivalent) at -20 °C for 6–12 months or until loss of activity is observed.

3.2) Calculate the volume required of each lipid stock to create a mixture with the desired lipid composition (see **Table 1**). Assume a total of 1 mole of lipids in the lipid mixture.

3.3) Perform this step in a chemical fume hood. Clean glass syringes by drawing up a full syringe volume of chloroform and discarding it in a waste container. Repeat two more times. When transferring chloroform, use only glass syringes or pipettes. When drawing up chloroform, pull on the stopper slowly to prevent introduction of gas bubbles into the syringe.

3.4) Use glass syringes to transfer stock lipids as calculated into a clean glass culture tube to make a final lipid mixture of 1% PI3P, 10% ergosterol, 30% PS, PC (**Table 1**). Depending on the solvents each lipid is resuspended in, the mixture may turn cloudy upon addition of each lipid.

NOTE: To vary concentrations of PS (0–30%), adjust volumes accordingly and compensate with varying PC (**Table 1**).

3.5) Carefully dry down the lipid mixture using nitrogen gas directed at the lipid mixture in a circular motion to dry lipids uniformly. Use low gas flow to keep the lipids at the bottom of the glass tube during the drying process. Wrap the glass culture tube with foil, leaving the opening uncovered, and further dehydrate in vacuum for 1 h.

3.6) Add 400 µL of binding buffer (50 mM Tris pH 7.4, 300 mM NaCl, 1 mM $MgCl_2$) to completely dehydrate lipids to make a final liposome concentration of 2.5 mM.

NOTE: Final liposome concentration can be adjusted by adding more or less binding buffer. For instance, 200 µL binding buffer may be added to produce a liposome concentration of 5 mM if needed (see below).

3.6.1) Resuspend lipids by shaking on medium speed on a vortex at room temperature for 30 min. Buffer should appear cloudy as lipids are resuspended.

3.7) Transfer resuspended liposomes to a microcentrifuge tube. From this point on, plastic pipette tips may be used as lipids are no longer resuspended in chloroform. Note that the liposome solution should look cloudy.

3.8) Freeze-thaw liposomes seven to eight times by submerging the microcentrifuge tube first in

liquid nitrogen, then in a 37 °C water bath. The liposome mixture should appear completely frozen and solid by eye before thawing.

3.9) Perform steps involving chloroform in a chemical fume hood. Clean two 1 mL glass syringes by drawing up and discarding full syringe volumes of chloroform, three times each, to remove any residual lipids. Equilibrate each glass syringe with ultrapure water by drawing up two syringe volumes, then equilibrate with binding buffer by drawing up two syringe volumes.

3.10) Assemble the mini-extruder according to manufacturer's recommendations. Equilibrate one 200 nm membrane and two pieces of filter supports (see **Table of Materials**) by submerging each in binding buffer.

3.11) Sandwich the membrane between the filter supports and place in the mini-extruder. To reduce the dead volume in the assembled mini-extruder and to make sure the assembly is air-tight, pass a volume of binding buffer comparable to the volume of the liposome mixture through mini-extruder using the 1 mL glass syringes.

3.12) Use one of the 1 mL glass syringes and draw up the liposome mixture. Invert the microcentrifuge tube to collect the last of the liposome mixture in the tube cap for drawing up into the glass syringe.

3.13) Extrude liposomes by passing through the 200 nm membrane 19–21 times. Collect extruded liposomes in new a microcentrifuge tube.

NOTE: Extruded liposomes should appear less cloudy than liposomes before extrusion. Liposomes should be used the same day and stored on ice. The last extrusion should place liposomes in the syringe opposite of the one it began in.

4. SNX-BAR liposome binding and tubulation

4.1) Preclear purified protein at 100,000 x *g* in an ultracentrifuge for 20 min at 4 °C prior to conducting liposome binding and sedimentation experiments. Remove the supernatant and transfer it to a new microcentrifuge tube; do not disturb the pellet if there is one.

4.2) To perform liposome binding and tubulation assays, incubate 4 µM purified Snx4-Atg20 and 2.5 mM liposomes in a total reaction volume of 20 µL, varying the volume of liposomes added.

NOTE: In the same experiment, the same volume of liposomes should be used.

4.3) Incubate the reaction at 30 °C for 30 min.

NOTE: We suggest maximizing the amount of 2.5 mM liposomes added to each reaction by using no less than 10 µL liposomes to allow for visualization during sedimentation. If 10 µL of 2.5 mM liposomes is used in a 20 µL reaction, the final concentration of liposomes will be 1.25 mM. If the

purified proteins are dilute and more volume is required for 4 µM protein, the lipids may be resuspended in 200 µL during the rehydration step to double the concentration of liposomes (see Step 3.6); however, this will require knowing the protein concentration prior to making the liposomes.

4.4) Visualize and quantify liposome tubulation.

4.4.1) Process liposome binding reactions immediately for electron microscopy analysis. Spot samples onto a carbon-coated copper mesh grid and negative stain using 1% uranyl acetate (**Figure 5D**)[16].

4.4.2) Analyze samples on a transmission electron microscope (200 kV).

4.4.3) Use image analysis software to measure and quantify tubule diameter. To accurately quantify tubule diameter of a single tubule, take three diameter measurements along the length of a tubule and average.

NOTE: Two-way analysis of variance was used to determine statistical significance (**Figure 5E**). Uranyl acetate is both radioactive and toxic. Proper lab safety certification is required to perform this step.

4.5) Liposome binding and sedimentation.

4.5.1) Transfer the reaction (20 µL, from step 4.3) to a polycarbonate centrifuge tube and use a compatible rotor to spin at 100,000 x *g* in an ultracentrifuge for 20 min at 4 °C. Carefully remove the supernatant and transfer to new microcentrifuge tube. Note that the pellet should stay intact.

4.5.2) Resuspend the pellet in SDS-PAGE 40 µL of sample buffer and transfer to a new microcentrifuge tube. Add 20 µL of sample buffer to the supernatant. Load equivalent amounts of pellet and supernatant in a 10% polyacrylamide SDS-PAGE gel and perform Coomassie staining to visualize SNX-BARs bound to liposomes (**Figure 5A**).

4.5.3) To quantify the amount of SNX-BAR complex in the pellet fraction, quantify band intensities using densitometry and quantify the proportion of SNX-BAR proteins in the pellet fraction.

NOTE: Two-way analysis of variance was used to determine statistical significance (**Figure 5B–C**).

REPRESENTATIVE RESULTS:

This protocol describes a method for reproducible and robust production of endogenous yeast SNX-BAR complexes that can be used for downstream membrane remodeling assays (**Figure 1**). The construction of the yeast strain used for purification takes advantage of the efficiency of homologous recombination in budding yeast, allowing for modifications at the genomic loci of the targeted SNX-BARs (**Figure 2**). This design has two advantages, (i) as selection is not required to maintain the modifications, standard YP medium can be used, allowing for growth to a higher

cell density and thus higher production of protein and (ii) the expression levels of the targeted SNX-BARs will be even, optimizing production of the heterodimer complex. Prior to addition of galactose, the targeted SNX-BARs will exhibit a null phenotype and thus may result in a growth defect or other known defects specific to the targeted SNX-BARs. Furthermore, growth in 2% raffinose and 0.1% glucose is slower than growth in 2% glucose. Therefore, the growth period prior to galactose induction may require optimization for each particular strain. To check for proper induction of SNX-BAR expression, a western blot against the TAP tag is likely required since protein levels of the SNX-BARs may not be detected via Coomassie stain (**Figure 3**). However, because only one member of the SNX-BAR complex has a tag, expression of the untagged protein(s) cannot be confirmed unless all steps of the purification are completed. After purification of the SNX-BAR heterodimer, the bands of the two SNX-BARs should appear to be in 1:1 stochiometric ratio and there should be little to no contaminating bands (**Figure 4**, lane 4). If there are additional contaminating bands, and the starting yeast strain is already protease-deficient, more protease inhibitor may be added during cell lysis. Furthermore, a second purification step using calmodulin resin may be performed.

When conducting membrane remodeling assays (**Figure 5**), the same preparation of liposomes and purified proteins must be used in the same experiment. If multiple purification preparations of protein are required to reach the desired concentration, combine all of the protein purified prior to conducting experiment. Liposomes should be made and used within the same day (**Table 1**). When conducting liposome sedimentation assays, it is critical that the purified protein is precleared using the same sedimentation conditions of 100,000 x *g* for 20 minutes immediately prior to incubating with liposomes as precipitated protein may skew results. Furthermore, the liposome pellet should stay intact after sedimentation.

FIGURE AND TABLE LEGENDS:

Figure 1. SNX-BAR binding assay flow chart. Briefly, in steps 1–2, we engineer GAL promoters into two SNX-BAR genomic loci, replacing each of the endogenous promoters and engineer a C-terminal TAP tag into one of the two SNX-BAR loci. Next, in steps 3–7, we induce the cells with galactose and purify the SNX-BAR heterodimers to homogeneity. In steps 8–12, we calculate and prepare unilamellar liposomes. Lastly, we can combine the SNX-BAR heterodimers with unilamellar liposomes and perform two assays; Step 14a–16a involve membrane tubulation and 14b-16a involve the sedimentation assay. See text for more details.

Figure 2. SNX-BAR integration strategy. Two SNX-BAR loci were targeted for GAL expression using homologous recombination. SNX-BAR ORF1 (Atg20) was additionally targeted to express a C-terminal TAP tag.

Figure 3. Galactose induction verification. (**A–B**) In order to verify GAL induction of Atg20-TAP, we recommend an SDS-PAGE and western blot analysis of cell extracts induced with 2% galactose (A, B, lane 2) and uninduced (A, B, lane 1). (**C**) Western blot membrane is also stripped and probed with anti-pgk1 for a loading control.

Figure 4. Example purification of Atg20-Snx4 heterodimers. Yeast cells engineered to express Atg20-TAP and Snx4 driven by galactose promoters were induced with 2% galactose, lysed and bound using IgG sepharose, and eluted with TEV protease. Samples from each step of purification is shown in 10% SDS-PAGE. Lane 1: induced supernatant of lysate. Lane 2: induced pellet of lysate. Lane 3: sample of bound proteins to IgG sepharose. Lane 4: TEV eluate of pure Atg20-Snx4 heterodimers from IgG sepharose.

Figure 5. Representative liposome binding and tubulation assay. (**A–C**) SNX-BAR heterodimers, Atg20-Snx4 and Vps5-Vps17 from yeast, were expressed, purified, and bound to synthetic liposomes as described in text. Note that Mvp1 forms homodimers and was expressed in bacteria. (**B–C**) SNX-BAR binding to varying liposome compositions was quantified by densitometry. Graph indicates mean and standard error of the mean. *$p < 0.05$; ***$p < 0.001$. (**D**) EM micrographs of Snx4-Atg20 liposome tubulation assay. (**E**) Tubule diameters were quantified and graphed as described in the text. The scale bar represents 200 nm. Reproduced with permission from Ma et al.[6]. Error bars represent two-way analysis of variance.

Table 1. Liposome recipe. Synthetic liposomes were prepared using a combination of DOPC, DOPS, ergosterol, and PI3P. We calculate the final concentration to 1 mol of lipid. Our standard composition includes 20% ergosterol, 1% PI3P, DOPS (up to 30%), and varying amounts of DOPC. Table includes a typical formulation for 400 µL of 30% DOPS.

DISCUSSION:

Here, we demonstrate an optimized workflow to purify SNX-BAR dimers in yeast and two assays to evaluate their biophysical properties on synthetic liposomes. The main advantage over typical recombinant protein expression in *Escherichia coli* or other systems is the ability to evenly express SNX-BAR proteins in a native host, thus avoiding the toxicity and insolubility issues found in purifying SNX-BARs in other systems. It is also notable that our system does not require molecular cloning or the harboring of multiple expression vectors[17]. Our dual SNX-BAR yeast strains are driven by chromosomally engineered galactose promoters, thus ensuring even expression upon induction. An important consideration is that in the absence of galactose, there will be little to no expression of the targeted SNX-BARs, thus resulting in a null phenotype. Furthermore, we find that SNX-BARs tend to tolerate C-terminal tags well, allowing us to add the TAP tag at the C-terminus. However, depending on the protein being tagged, an N-terminal TAP tag may also be used[12]. Additionally, since SNX-BARs only form 1:1 dimers, only one protein is required to be tagged for purification purposes. However, some SNX-BARs that normally form heterodimers in the cell have been shown to form homodimers under non-physiological concentrations[7]. Therefore, it is critical that upon purification of the heterodimer, the stoichiometric ratio of the two SNX-BARs should be 1:1, which can be verified by running an aliquot of the purified complex on an SDS-PAGE gel and performing Coomassie staining. Once engineered, these yeast strains can be saved in perpetuity as 15% (v/v) glycerol stock at -80 °C and/or additionally modified to query additional binding partners. Our workflow typically takes 2–3 weeks for strain construction and 3–4 days for expression and purification and 1 day for liposome binding assays. We believe that this workflow can help researchers further understand the lipid specificity of SNX-BAR proteins using native BAR proteins on synthetic liposomes or giant

unilamellar vesicles (GUVs) and reveal the precise makeup of lipids needed to drive membrane remodeling, thus revealing their mechanism of action.

Critical steps

During our first attempts at purifying SNX-BAR heterodimers from non-protease deficient cells, we often found reduced yields and degradation products. Therefore, during the initial steps of strain construction, we believe it is critical to begin with a parental yeast strain that is deficient for one or more of the major vacuolar proteinases. In particular, we found yeast strain TVY614, which is depleted for *pep4*, *prb1*, and *prc1,* to be the most optimal. Using the TVY614 strain, we routinely obtain >90% pure Snx4-Atg20 heterodimers (**Figure 4** and **Figure 5A**). However, the necessity for all three proteinases to be ablated may be SNX-BAR combination specific. For example, Vps5-Vps17 heterodimers have been successfully purified in non-protease deficient strains[10] and when we included the addition of a *PEP4* ablation, we observe modest increases in yield and purity (**Figure 5A**). Therefore, depending on the user's downstream applications and the need for purity or selectable markers, there may be flexibility when designing expression strains.

The order of gene construction is also important. We recommend C-terminally TAP tagging SNX-BAR ORF 1 first, in order to confirm expression by western blot without the need for galactose induction (**Figure 1**). During galactose induction, it is critical to pre-condition the cells overnight in 2% raffinose and 0.1% glucose. Failure to pre-condition the cells results in extremely slow growth or cell death. However, it is also critical for the cells to deplete the remaining glucose during overnight growth, otherwise galactose induction can be negatively impacted. It is also recommended to check multiple isolates by western blot to evaluate expression homogeneity of the TAP tagged SNX-BAR proteins. We typically screen 2–3 isolates and pick the most robustly expressing candidate.

Modification, alternative approaches, and future applications

In step 2.8, tandem affinity purification (TAP) typically requires a two-step affinity purification using IgG and calmodulin beads after TEV cleavage[12]. However, in this protocol, we elute SNX-BAR dimers by TEV protease with very high yield and purity. We find subsequent affinity purification using calmodulin beads produces inconsistent and reduced yields, thus we recommend stopping after TEV cleavage. The TEV eluate containing the SNX-BAR proteins and His(6)-tagged TEV protease can be further purified by Ni-NTA agarose beads. However, we also find this step can reduce overall SNX-BAR protein yield and is unnecessary, since TEV protease does not interfere with liposome binding or tubulation assays. Therefore, if the purified SNX-BARs will be used for any other application, we recommend the user assess the impact of TEV protease in their assays.

Thus far, we have been successful using this protocol and the described modifications to purify Snx4-Atg20 and Vps5-Vps17 heterodimers in yeast and have successfully assessed their lipid specificity on synthetic liposomes. However, we believe that the protocol can be successfully

adapted to any of the SNX-BARs in yeast. It is also possible to use the system to produce recombinant SNX-BAR proteins from any other organisms. However, this would require an additional step of strain construction to integrate an exogenous gene locus into the yeast genome. We also believe the system can be expanded to purify multimeric complexes including cargo proteins. Thus, we believe our expression system may extend beyond understanding the lipid specifies of SNX-BARs. Future applications will allow researchers to reconstitute whole cargo capture complexes on liposomes to understand how full assemblies can influence membrane remodeling.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

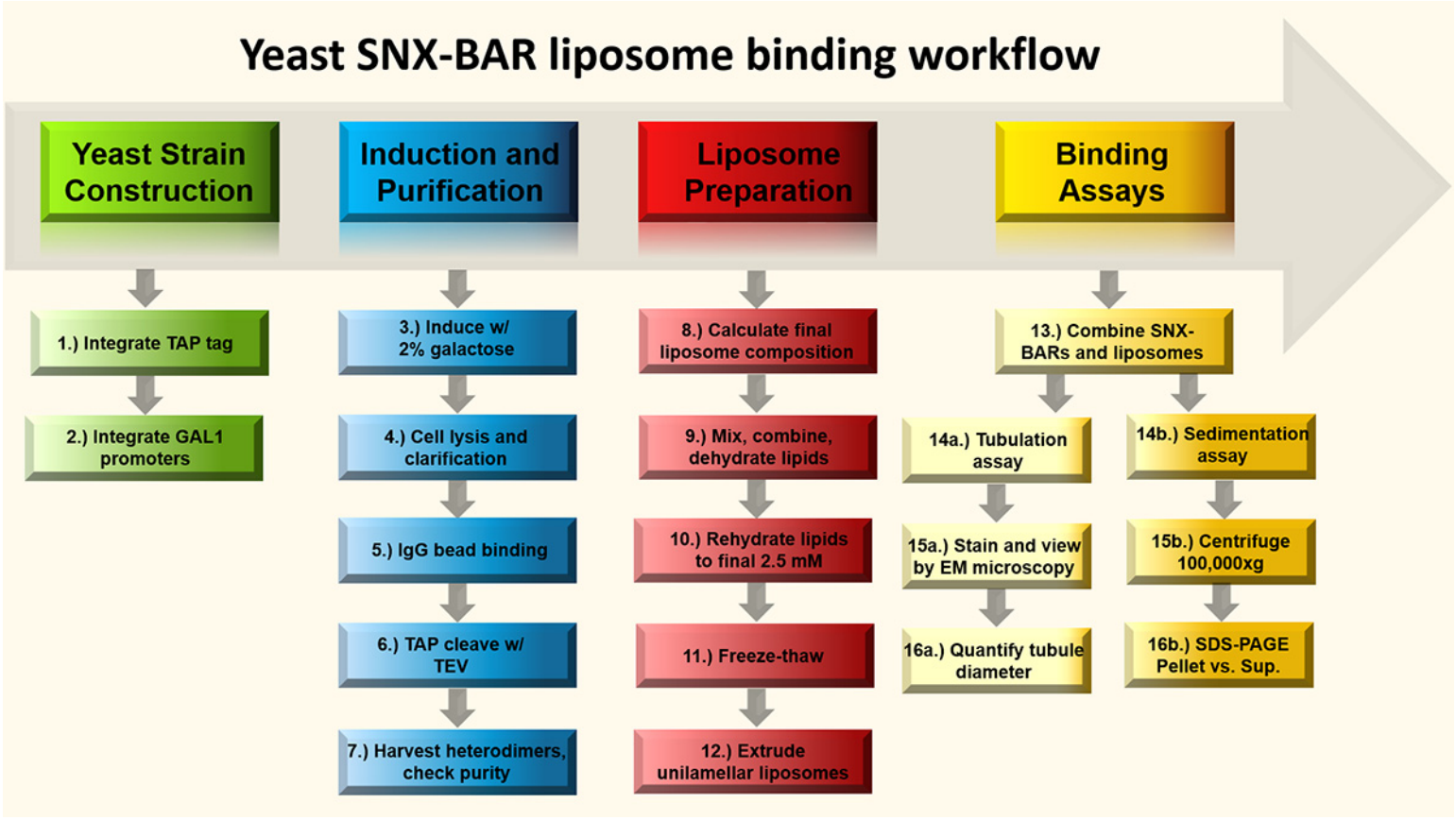


Figure 2

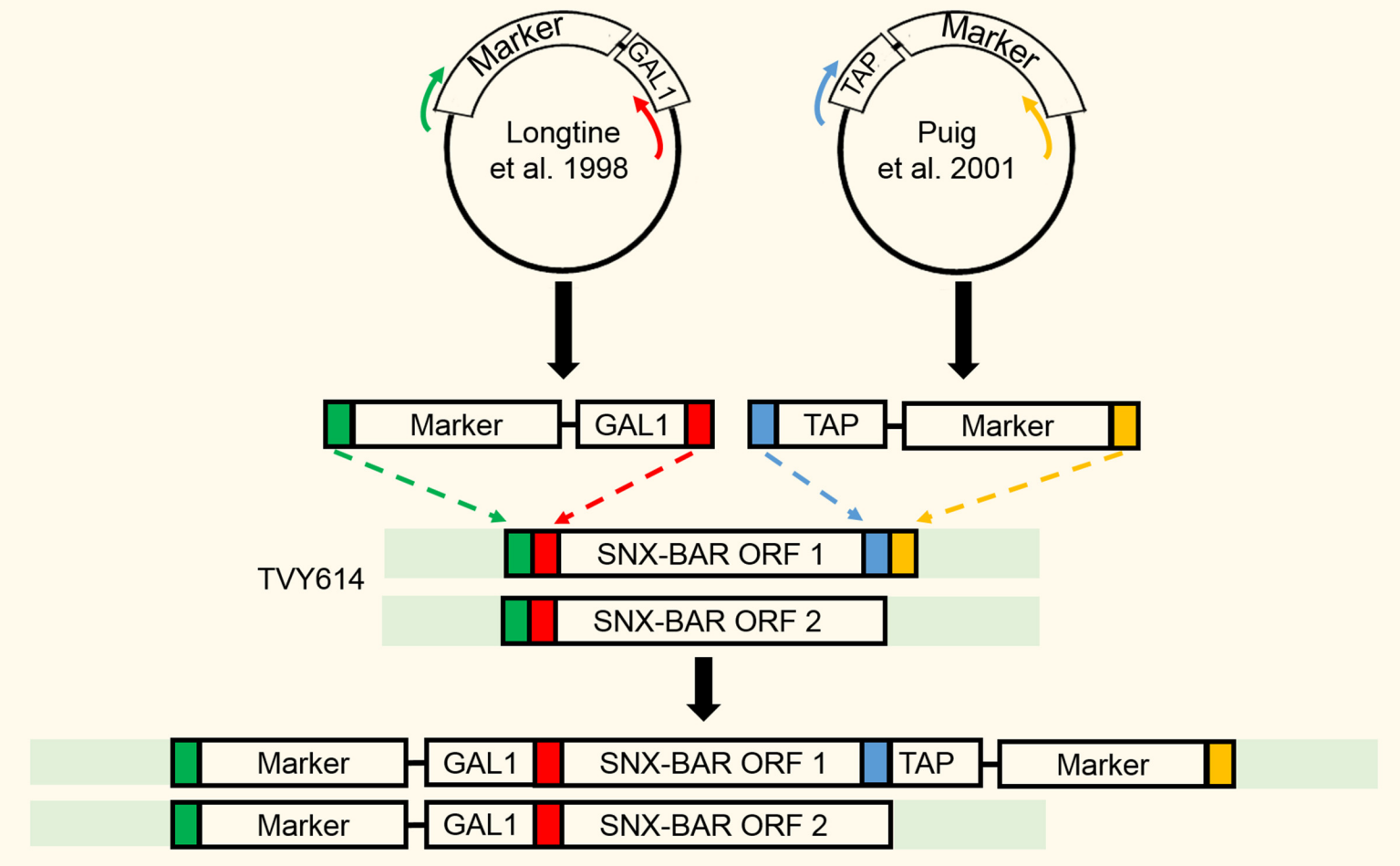
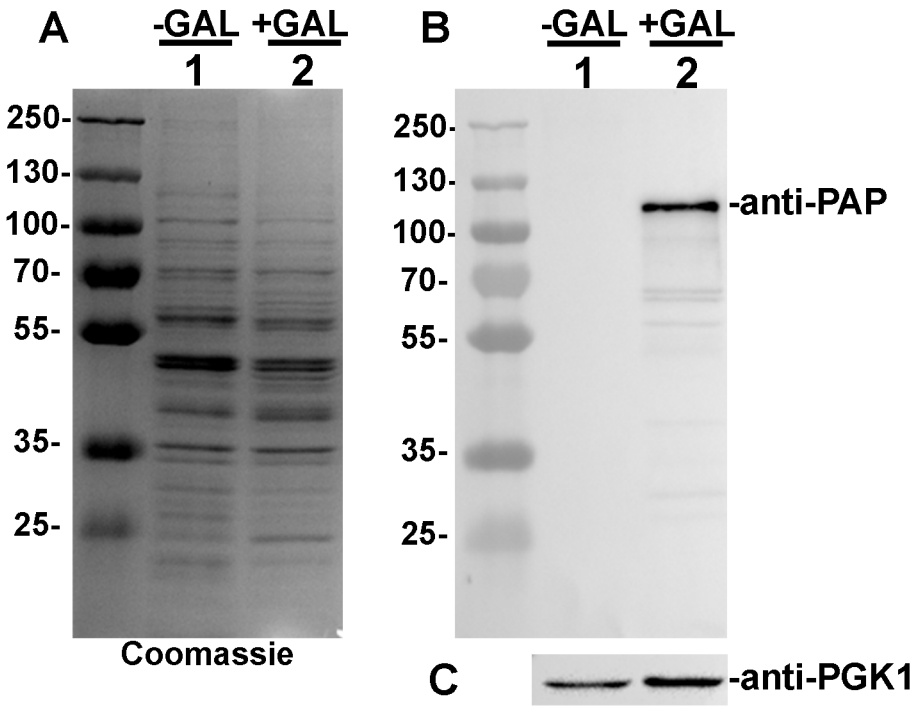


Figure 3



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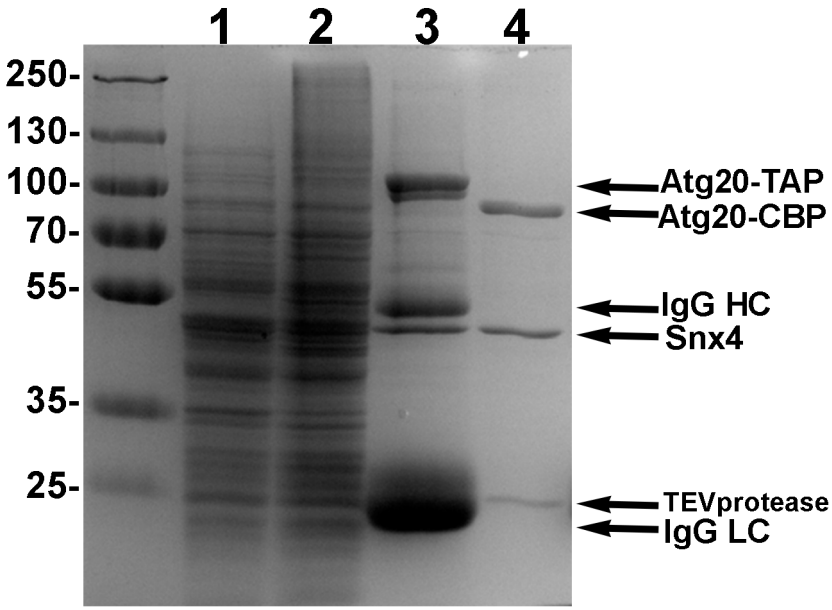

Figure 5

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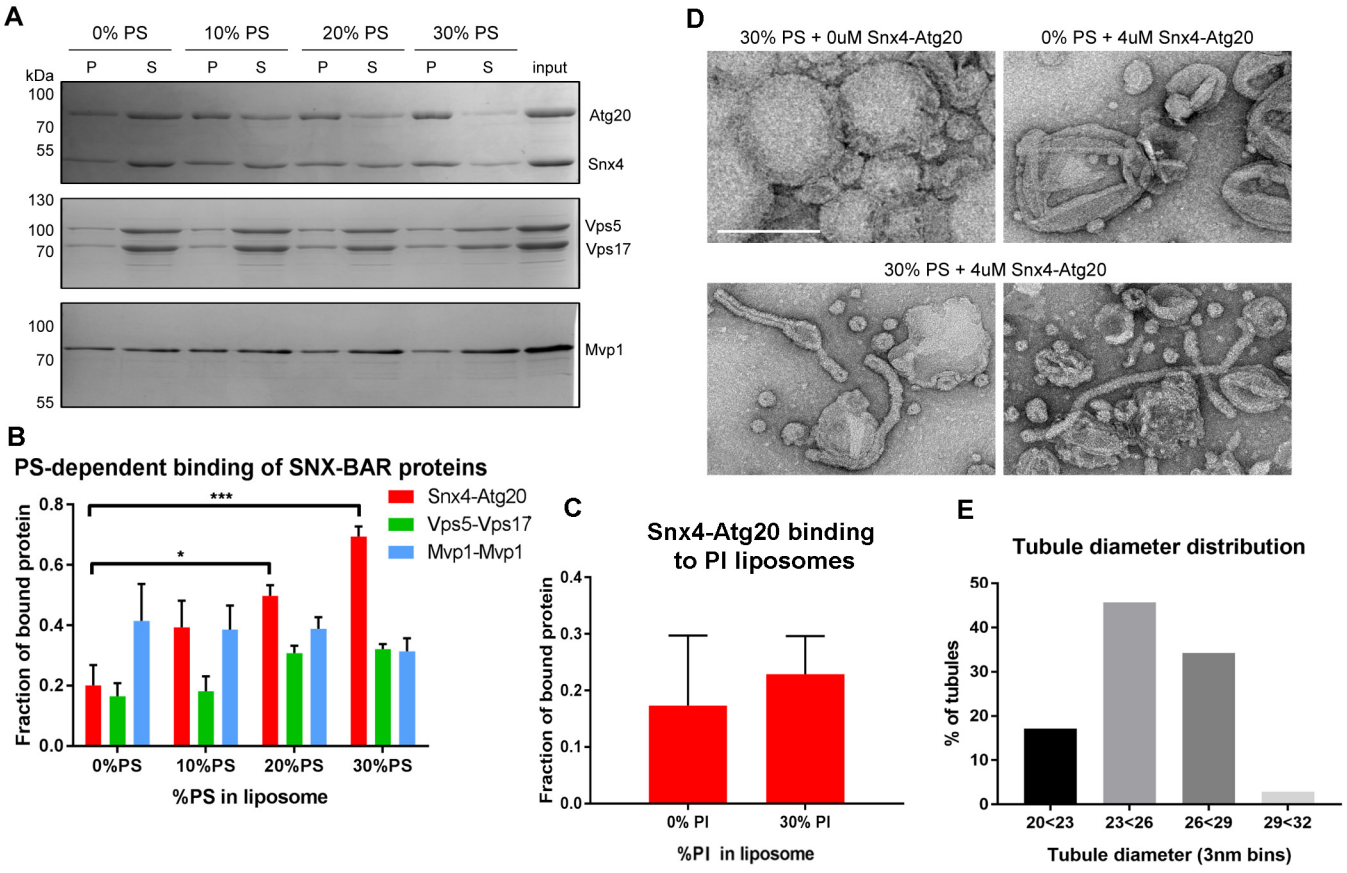

Table 1

Table 1 Typical Liposome Composition	DOPC	DOPS	Ergosterol	PI3P-C16
MW	786.1	810.0	396.7	957.0
mol fraction	49%	30%	20%	1%
Stock mM	32.0	12.0	25.0	1.0
Mass (μg)	385.2	243.0	79.3	9.6
Concentration (μg/μl)	25.2	9.7	9.9	1.0
Volume to RXN(μl)	15.3	25.0	8.0	10.0

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2 micrometer PC Membranes	Avanti	610006	
10 mL Poly-Prep Chromatography column (Bio-Rad)	Bio-Rad	731-1550	
27 Gauge needle	BD Biosciences	301629	
Amicon Ultra Centrifugal Filter with 10K cutoff	Amicon	UFC501024	
Avestin EmulsiFlex-C3 Homogenizer	Avestin	EF-C3	
BCA assay	Pierce	23225	
Beckman Optima MAX-XP Ultracentrifuge	Beckman Coulter	393315	
cOmplete Protease Inhibitor Cocktail	Roche	4693116001	
DOPC	Avanti	850375	
DOPS	Avanti	840035	
ergosterol (Sigma)	Sigma	47130-U	
Extruder Set with Block 0.2 microlter/1mL	Avanti	610000	
FEI Tecnai F20 transmission electron microscope (200 kV)			
Glass culture tubes	VWR	47729-570	
IgG sepharose beads (GE Healthcare)	GE Healthcare	17-0969-01	
Microlter glass syringes	Hamilton	7637-01	
New Brunswick Excella E25	Eppendorf	M1353-0000	or equivalent shaking 30 C
Ni-NTA Magnetic Agarose Beads	Pierce	78605	
Optima XE-90 Ultracentrifuge	Beckman Coulter	A94516	
Parafilm M	VWR	52858-076	
PI3P	Echelon	P-3016	or Echelon equivalent
Polycarbonate bottle assembly	Beckman Coulter	355622	
TLA-100 Fixed-Angle Rotor	Beckman Coulter	343840	
Type 45 Ti Rotor	Beckman Coulter		
Vacuum Desiccator, Bottom and Lid with Socket Valv	VWR	75871-436	
Vacuum Pump Alcatel (Pascal 2005 C1)	A&J Vacuum	PN07050	
Vortex with foam holder	VWR	10153-838	
VWR KIT MICROTUBE	VWR	12620-880	

Editorial Rebuttal

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have read though the manuscript and have not found any errors.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text on lines 72-77, 96-103, to avoid this overlap.

We have rewritten this portions of the manuscript.

• **Protocol Language:** Split up long steps (e.g. 2.2) into 2 or more steps.

We have expanded all long steps into more steps.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

We have highlighted 2.5 pages of filmable protocol.

• **Results:** Please add at least one paragraph of results text that explains your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures. You may include the figure captions under this heading but the captions and figure text must be separate entities.
We have included a "Representative Results section".

• **Discussion:** JoVE articles are focused on the s and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the

technique, 3) significance with respect to existing s, 4) future applications and 5) critical steps within the protocol.

Our discussion section includes all of the above details.

• **Figures:**
1) Please remove the text "Figure #.." from the figure files.
2) Please define all error bars.

We have modified each figure as requested and have defined the error bar in the figure 5 legend.

• **Tables:** Please upload each table as an individual Excel file.

We have uploaded the table excel file.

• **Figure/Table Legends:** *N/A*
• **References:** Please spell out journal names.

We have modified the references to spell out journal names.

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2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
3) Please avoid product names in Table 1 if possible.

We have modified the manuscript table of reagents and table 1 as requested,

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We have included re-print permission agreement for Figure 5 as a supplement.

Rebuttal to Reviewers

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:
The manuscript describes a recombinant expression system in S. cerevisiae that can be utilized to produce biochemically tractable amounts of functional SNX-BAR homo- and heterodimers. The authors demonstrate the utility of the system by producing three SNX-BAR complexes according to the protocol described and characterizing their phosphatidylserine-dependence for liposome binding. Overall, the manuscript is clearly written, easy to follow, and written at a level that should allow a novice to successfully complete the protocols (although see one minor comment below). After addressing two minor concerns, the manuscript will be suitable for publication.

Major Concerns: No major concerns were noted.

Minor Concerns:

1. An implicit assumption of the work as described is that the intended SNX-BAR heterodimer will be the sole species produced when two interacting SNX-BAR proteins are overproduced. However, as the authors point out, some SNX-BAR proteins homodimerize physiologically and several form non-physiological heterodimers when overproduced in E. coli. Thus, one concern for the described single-step purification scheme is that some contaminating homodimer of the tagged subunit may copurify with SNX-BAR heterodimer. Whereas the Reviewer suspects this would be a minor fraction of the overall yield, it could potentially confound some downstream assays and should be explicitly pointed out in the article (discussion section?) so that naive readers will be aware of this possible limitation.

We thank the reviewer for this suggestion. We have amended the discussion to include this point (page 10, line 434-438).

2. The protocols for integration of genetic elements into yeast, for EM of liposomes, and for liposome binding are appropriately brief as they are not the major focus of the manuscript. However, provision of references for basic negative staining and electron microscopy to direct a naive reader to more detailed protocols for these assays would be helpful.

We thank the reviewer for this suggestion. We have included a reference for negative staining that mirrors our protocol. (Zhang et al. 2011)

Minor comments to improve the manuscript:

1. Line 57: "expressions" should be "expression"

Thank you, we have corrected this.

2. Line 211: Indicating at this step that the proteins can also be adjusted to 15% glycerol and stored at -80*C would be helpful, rather than pointing this out in the discussion.
We thank the reviewer for this suggestion, however this description in the discussion is for storing the yeast strain used for purification. Unfortunately, the purified proteins cannot be stored in this way.

3. Line 222: At what temperature should resuspended lipids be stored?

We have added this detail.

4. Line 284: The amount of liposomes to be included here is a bit confusing because a concentration and a total reaction volume is listed, followed by instructions to vary the volume of liposomes. After reading this step, the Reviewer was unsure what the desired final concentration(s) of liposomes should be. Some rewording here would help the reader properly conduct this assay.

We thank the reviewer for pointing this out. We have expanded Steps 3.6 and 4.2 to better explain this point.

Reviewer #2:

Manuscript Summary:
The manuscript details a protocol to express and purify SNX-BAR proteins from yeast for in vitro studies including membrane binding and tubule formation. The protocol has the significant advantage that yeast proteins are expressed in yeast and therefore problems encountered with bacterial expression (proteolysis, aggregation etc) can be largely avoided. The use of yeast to express yeast proteins is also advantageous for the study of post-translational modifications - many of the SNX-BAR proteins are known to be phosphorylated. This is a well written manuscript providing the necessary information along with useful tips and suggestions for a successful outcome. Although there will probably not be a huge number of labs that will seek to adopt the methods described, this manuscript will occupy an important niche and deserves to be published.

Major Concerns:
I have no major concerns but only a couple of questions: does placing the SNX-BAR protein under Gal control mean that there is little to no expression in the absence of Galactose? Does this create a phenotype? i.e is there a lack of Vps5p when the VPS5 gene is modified with a Gal promoter? Similarly, could the position of the TAP tag (at the C-terminus) create any problems with respect to dimerisation of the SNX-BAR through the BAR domains which themselves are in the C-terminal region of the protein. Clearly with yeast there is the potential to establish that the modified gene is producing a functional protein which is another potential advantage to the use of yeast - I think a sentence or two added to the manuscript that addresses these points will suffice.

We thank the reviewer for this suggestion. We have included a discussion of these points in the discussion section. The reviewer is correct in that without galactose, there will be little to no

expression of the targeted SNX-BARs, resulting in a null phenotype. Since SNX-BARs tolerate C-terminal tags well, we generally use C-terminal TAP tags. However, depending on the protein of interest, an N-terminal TAP tag may also be used, as described in Puig et al.

Minor Concerns:
Some suggested changes and typos: Line 91 - I'd suggest changing "Mammalians" to "Mammals"; Line 122, don't need "a" at the end of the line; Line 123 - I'd suggest moving (Figure 1) to the beginning of the sentence so it reads. "In Figure 1, we present....."; Line 159, I think it would be helpful to define what 3 OD of cells is as non-yeast researchers may be confused by that terminology; Line 163, "cOmplete".

Thank you, we have amended these.

Reviewer #3:

Manuscript Summary:
In the manuscript "Expression, Purification and Liposome Binding of Budding Yeast SNX-BAR Heterodimers" by Ma et al. the authors describe a protocol for expressing and purifying SNX-BAR heterodimers from yeast rather than E. coli. They also describe the generation of liposomes and how to perform liposome binding assays with the purified protein. The intro and the protocol are very nicely written and easy to follow. The results of the purification are also very impressive resulting in a very pure sample as visualized by SDS-PAGE.

Major Concerns:

1. The overall protocol is broken up into four sections. The first section "Yeast Strain Construction" is very short in comparison to the other sections. Expanding this section with a couple more steps or more details regarding plasmid selection will help guide readers through generating the strains.

We agree that this section can be better clarified and have expanded this section. We thank the reviewer for this suggestion.

2. In the "Yeast Induction and SNX-BAR Dimer Purification" section it would be helpful to add more specifics about the cultures to help guide readers at each stage of the expression.
a. How many colonies are used to inoculate the 50 mL standard YP culture?
b. What is the expected OD600 after the 50 mL preculture grows overnight at 30 C?
c. What is the expected OD600 after growing the large volume culture in 2% galactose overnight?
d. 3 OD of uninduced and induced cells are recommended for SDS-PAGE and western blot. What percent of the total cells is this?
e. How many grams of cell pellet is used in 15 mL of purification buffer? What volume of cells were grown to achieve this? Just 1 L?

We have expanded on each of these points in the appropriate steps, thank you.

Minor Concerns:
1. In step 2.2 4500 rpm is listed for centrifugation. This should be converted to g.

We have changed this, thank you.

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