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Expression in *Saccharomyces cerevisiae* and Purification of a Human Phospholipid Flippase

Thibaud Dieudonné, Christine Jaxel, Maylis Lejeune, Guillaume Lenoir, and Cédric Montigny

Abstract

Membrane proteins (MPs) are challenging to study from a biochemical standpoint owing to the difficulties associated with the isolation of these proteins from the membranes they are embedded in. Even for the expression of closely-related homologues, protocols often require to be adjusted. Prominently, the solubilization step and the stabilization of recombinant proteins during the purification process are key issues, and remain a serious bottleneck. Here, we present a method for the expression and the purification of the human ATP8B1/CDC50A lipid flippase complex. Selection of the right *Saccharomyces cerevisiae* strain proved to be a critical step for the successful purification of this complex. Likewise, the use of cholesteryl hemisuccinate, a cholesterol analogue, contributed to significantly increase the yield of purification. We hope that the simple method described here can help researchers to succeed in the expression of other mammalian difficult-to-express lipid flippases and, by extension, help in the production of other membrane proteins whose isolation has so far proven difficult.

Key words ATP8B1/CDC50A, Cholesteryl hemisuccinate, Flippases, Heterologous expression, Membrane proteins, *Saccharomyces cerevisiae*, Yeast

Abbreviations

BAD	Biotin acceptor domain
BCA	Bicinchoninic acid
BSA	Bovin serum albumin
CHS	Cholesteryl hemisuccinate
DDM	n-dodecyl- β -D-maltopyranoside
MPs	Membrane proteins
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis

1 Introduction

Membrane proteins (MPs) are usually not naturally abundant and their production in recombinant systems and their purification remains very challenging. Despite the increasing number of innovative methods and the recent development of structural methods that do not require extensive amounts of purified protein, each MP can still be considered as a special case possibly requiring optimization of production and purification protocols. Among the available expression systems, yeast has proven to be a good compromise between simplicity of use and quality/quantity of target proteins produced [1–3]. Improvement of MP solubilization through, for example, the adaptation of the expression system or the development of new surfactant molecules, has also contributed greatly to recent advances in the functional and structural studies of MPs [4]. The use of natural lipids or lipid analogues during extraction and purification can for instance significantly improve the stability of the target protein all along the process from expression to functional and structural characterization [5–7]. Indeed, many MPs depend on specific lipid interactions to keep their function and to remain stable in a detergent solubilized state [8].

Like many other MPs, eukaryotic lipid flippases (P4-ATPases) are usually weakly expressed in native membranes. P4-ATPases are primary active transporters that catalyze lipid transport across membranes to create and to maintain transbilayer lipid asymmetry. They associate with transmembrane proteins from the CDC50 family that are essential for proper folding, addressing, and maturation of the complex [9, 10]. In the present chapter, we describe procedures for the expression and purification of the human ATP8B1/CDC50A lipid flippase complex, partly based on previously published methods [11–13]. We further observed that too large quantities of the heterologously expressed protein likely overload the cellular synthetic capacity or folding capacity, as using a *S. cerevisiae* host strain with decreased ability to express both ATP8B1 and CDC50A led to a dramatic improvement of its solubilization. In addition, we emphasize the contribution of a cholesterol analogue, cholesteryl hemisuccinate (CHS), to the extraction and purification processes, probably through stabilization of the ATP8B1/CDC50A complex.

2 Materials

2.1 Yeast Strains

Three *Saccharomyces cerevisiae* strains were used in this study. The parental W303-1B strain (MAT α , leu2–3, his3–11, ura3–1, ade2–1, can^r, cir⁺), the W303-1B/GAL4 (MAT α , leu2–3, his3–11, trp1–1::TRP1-GAL10-GAL4, ura3–1, ade2–1, can^r, cir⁺), which overexpresses the Gal4 transducer [12, 13], and the W303-1B/ Δ pep4 strain (MAT α , leu2–3, his3–11, ura3–1, ade2–1, Δ pep4, can^r, cir⁺) deleted for the vacuolar aspartyl protease Pep4p.

2.2 Yeast Transformation & Yeast Culture

Expression vectors, yeast transformation, and yeast culture were previously described in two former chapters [12, 13]. The co-expression strategy formerly developed for the yeast flippase Drs2/Cdc50 was used here and ATP8B1 and CDC50A genes were both cloned into a single pYeDP60 plasmids under the control of galactose-regulated promoters [13]. ATP8B1 was fused to a Biotin Acceptor Domain (BAD) followed by a TEV protease cleavage site in the N-terminus, while CDC50A was tagged with ten histidines at its N-terminus. Here, the original S6 media was replaced by SD minimal media to better control the selection conditions (*see Note 1*).

The media are prepared with distilled water.

1. SD minimal medium: 0.17% yeast nitrogen base without amino-acid and ammonium sulfate (w/v), 0.5% ammonium sulfate ((NH₄)₂SO₄) (w/v), 55 mg/L uracil, 55 mg/L adenine, and 2% dextrose (w/v). Prepare a 10% over-concentrated medium without dextrose. Sterilize for 20 min at 120 °C. After cooling (< 50 °C), add sterile dextrose and 1× essential amino-acid from a 100× concentrated drop-out solution.
2. SD agar minimal medium: prepare the medium as described above for liquid SD but with the addition of 2% agar (w/v) and one sodium hydroxide pellet per liter (*see Note 2*) before sterilization. Pour Petri dishes after dextrose and amino-acid addition.
3. SD-ura medium: prepare the medium as described above for SD minimal medium but leave out uracil.
4. SD-ura agar medium: prepare the medium as described above for SD agar minimal medium but leave out uracil.
5. Sterilize a 20% glucose (w/v) stock solution by filtration through a 0.22 μm filter.
6. 100× concentrated drop-out amino-acid solution: 1 mg/mL L-histidine, 6 mg/mL L-leucine, 4 mg/mL L-lysine, and 4 mg/mL L-tryptophane. Sterilize by filtration through a 0.22 μm filter.

2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Preparation of SDS-PAGE was previously described in two former chapters [12, 13]. Details for gel preparation and amount of protein loaded in the present chapter are indicated in figure legends.

2.4 Western-Blotting

The procedure for protein transfer to PVDF membranes was described in detail in a former chapter [13]. Below are only described the probes and antibodies used for protein detection with the appropriate buffers for incubation.

1. 10× concentrated PBS buffer: 27 mM KCl, 1.4 M NaCl, 0.13 M Na₂HPO₄, and 18 mM KH₂PO₄. Dissolve first in 800 mL water, adjust to pH 7.4 with NaOH, make up to 1 L with water, and filter-sterilize. Dilute tenfold before use.
2. PBS-T buffer: 1× concentrated PBS buffer supplemented with 0.2% Tween 20 (v/v).
3. Dried skimmed milk (Regilait or any other supplier available in supermarkets).
4. Albumin Fraction V, fatty acid-free.
5. Avidin peroxidase conjugate (commercial solution for single use). Store 2 μL aliquots at −70 °C. It will be referred to as biotin probe in the following protocol.
6. His-Probe-HRP peroxidase conjugate (commercial powder): dissolve with 0.4 mL of water to a final concentration of 5 mg/mL. Store aliquots at −20 °C.
7. ATP8B1 was detected using a mouse anti-ATP8B1 antibody (Epitope: 1161–1251, SC-134967 from Santa Cruz Biotechnology, no longer available). An anti-mouse HRP-coupled antibody was used as secondary antibody (1,706,516, Bio-Rad).

2.5 Solubilization of Yeast Membranes

1. n-Dodecyl-β-D-maltopyranoside stock solution (DDM): dissolve DDM powder at 100–200 mg/mL in ddH₂O. Store up to several days at 4 °C or up to several years at −20 °C. Add detergent to buffers just before use (*see Note 3*).
2. 1 M Tris-HCl pH 8.0 buffer. Filtered through 0.22 μm filters.
3. Cholesteryl hemisuccinate (CHS).
4. Beckman Coulter TL100 Benchtop ultracentrifuge with a TLA-45 rotor (1.5 mL Microfuge tubes).
5. Probe sonifier with a 13 mm diameter probe.
6. End-over-end shaker.
7. Solubilization and purification buffer (buffer A): 50 mM MOPS-Tris at pH 7, 100 mM NaCl, 1 mM DTT, 5 mM MgCl₂, and 20% glycerol (w/v). Filtered through 0.22 μm filters.
8. Sigma-fast EDTA-free protease inhibitor cocktail: as indicated by the supplier, dissolve one tablet in 10 mL of buffer A to obtain a tenfold concentrated solution. Store up to several days at 4 °C or up to several years at −20 °C.
9. 200 mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol (*see Note 4*). Store up to several days at −20 °C.

2.6 Purification of the Human ATP8B1/CDC50A Flippase Complex

Most of the required material is described in the previous section. In addition, large-scale purification also requires:

1. Streptavidin-sepharose resin.
2. Beckman Coulter Optima XPN 90 ultracentrifuge with a 45-Ti rotor (70 mL polycarbonate bottle assembly).
3. Beckman coulter Allegra benchtop centrifuge with a SW4400 swinging-bucket rotor for 15 mL or 50 mL conical tubes.
4. TEV protease expressed and purified according to Azouaoui et al. [14].
5. Wash and elution buffer (buffer B): 50 mM MOPS-Tris at pH 7, 100 mM KCl, 1 mM DTT, 5 mM MgCl₂, and 20% glycerol (w/v).

2.7 ATPase Activity of the Purified Complex

1. ATPase activity buffer (same as buffer B): 50 mM MOPS-Tris at pH 7, 100 mM KCl, 1 mM DTT, 5 mM MgCl₂, and 20% glycerol (w/v).
2. Agilent 8453 diode-array spectrophotometer with a thermostatic cell holder. The holder is connected to a circulating-water bath for ensuring both constant temperature and continuous stirring of the sample.
3. 2 mL quartz cuvette with a magnetic stirrer.
4. 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and L- α -phosphatidylinositol-4,5-bisphosphate (brain PI(4,5)P₂) (*see Note 5*).
5. DDM:POPC mixture at 50 mg/mL and 10 mg/mL in ddH₂O, respectively.
6. DDM:PI(4,5)P₂ mixture at 50 mg/mL and 5 mg/mL in ddH₂O, respectively.
7. Trypsin from bovine pancreas (6000 units/mg, Type XI): dissolve lyophilized powder at 10 mg/mL in ddH₂O.
8. 200 mM MgATP stock solution buffered at pH 7.5 (for recipe, *see Note 6*).
9. 50 mM phosphoenolpyruvate (PEP, phospho(enol)pyruvic acid cyclohexylammonium salt) in ddH₂O.
10. 15 mM Nicotinamide Adenine Dinucleotide (NADH) (*see Note 7*).
11. 50 mM BeCl₂ and 1 M KF.

3 Methods

We previously developed expression and purification strategies for integral MPs, in particular for P-type ATPases such as the rabbit sarco-endoplasmic reticulum calcium ATPase (SERCA1a) and the Drs2/Cdc50 yeast flippase complex [2]. Briefly, this system allows growing yeast cells at high densities with a high level of gene expression, thus resulting in recombinant MP amounts compatible with both functional and structural characterization.

The present protocol describes the procedure for expression and purification of the human ATP8B1/CDC50 flippase complex, amenable for further structural and functional studies [15]. In contrast to the expression and purification of the yeast homolog Drs2/Cdc50, both the choice of the yeast strain and the use of cholesteryl hemisuccinate all along the purification process were critical to achieve satisfactory purification yield.

3.1 Choice of the *Saccharomyces Cerevisiae* Strain for Co-Expression of ATP8B1 and CDC50A

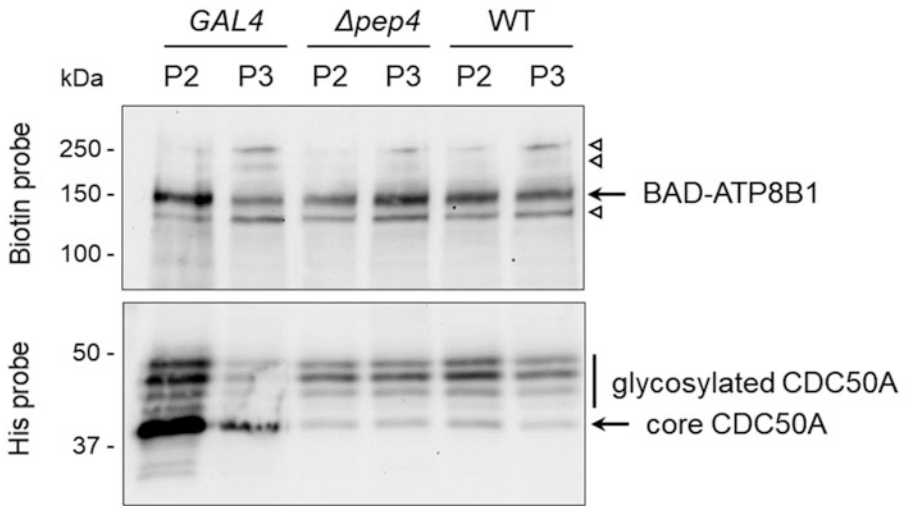
Yeasts were transformed with the pYeDP60 plasmid containing coding sequences for ATP8B1 and CDC50A using the lithium-acetate/single-stranded DNA/PEG procedure. Growth and expression were also done as previously described resulting in the recovery by differential centrifugation from the cell shred of two membrane fractions, namely, heavy membranes (P2) and light membranes (P3). Protein concentration was determined using the bicinchoninic acid assay (BCA, [16]). For a detailed procedure of all these preliminary steps, please refer to Azouaoui et al. [13]. The expression (*see* Fig. 1a) and solubilization levels (*see* Fig. 1b) were estimated by SDS-PAGE and western-blotting.

3.1.1 Western Blotting for ATP8B1 and CDC50A Detection

Proteins initially separated by SDS-PAGE were transferred onto a PVDF membrane for detection. Procedure for protein transfer to PVDF membranes was described in detail in a former chapter (please refer to Azouaoui et al. [13]).

1. Following transfer, prevent unspecific binding sites on the PVDF membranes by incubation for 1 h at room temperature with PBS-T supplemented with either 5% skimmed milk (w:v) for detection of biotinylated proteins and for specific detection of ATP8B1, or 2% BSA (w:v) for detection of histidine-tagged proteins.
2. For detection of biotinylated proteins, wash first membranes three times (10 min each) with PBS-T to remove any traces of milk (*see* Note 8). Add the biotin probe (coupled to HRP) at a 1:25,000 dilution in PBS-T and incubate for 1 h at 20 °C.

A



B

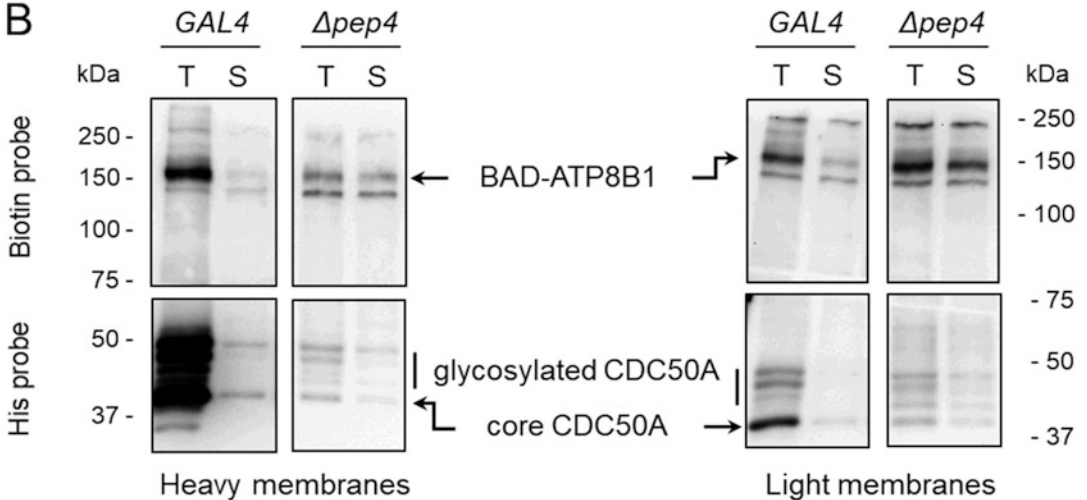


Fig. 1 Western-blot analysis of ATP8B1 and CDC50A expression in *S. cerevisiae* membranes. **(a)** Comparison of the content in ATP8B1 and CDC50A in heavy membranes (P2) and light membranes (P3) prepared after expression in W303-1B WT, W303-1B/GAL4, and W303-1B/ $\Delta pep4$ strains. **(b)** Solubilization by DDM of membranes prepared from W303-1B/GAL4 and W303-1B/ $\Delta pep4$ strains. T, total fraction before centrifugation; S, solubilized fraction. After SDS-PAGE, proteins were transferred onto PVDF membranes and revealed with either a biotin probe (*top gel*) or a histidine probe (*bottom gel*). 4 μ g of total protein were loaded on the gel. For the supernatants, a volume equivalent to the corresponding total fraction was loaded for a reliable comparison. The biotin probe also detects endogenously biotinylated proteins from yeast, namely, Acc1p (250 kDa), Dur1/2p (200 kDa), and Pyc1/2p (130 kDa) (*open arrowheads*). CDC50A appears as multiple bands corresponding to different glycosylation levels

3. For specific detection of ATP8B1, add the primary anti-ATP8B1 antibody at a 1:5,000 dilution in PBS-T supplemented with 2% BSA (w:v) and incubate for 1 h at 20 °C. Rinse once with PBS-T for 10 min. Add the secondary antibody at a 1:2000 dilution in PBS-T supplemented with 5% skimmed milk (w:v) and incubate for 1 h at 20 °C.
4. For detection of histidine-tagged proteins, add the histidine probe (coupled to HRP) at a 1:2,000 dilution in PBS-T and incubate for 1 h at 20 °C.
5. After incubation with probes or antibodies, wash membranes three times with PBS-T buffer (10 min each), and two times with PBS (10 min each). The bound probes are revealed using ECL reagents and emitted light is collected using a CCD cooled camera (*see* Fig. 1).

3.1.2 Small-Scale Solubilization of Yeast Membrane for Comparison of the Different Yeast Strains

Strain and detergent screening were initially performed on P2 and P3 membranes separately.

1. All steps are done at 4 °C or on ice.
2. Dilute P2 or P3 membranes at 2 mg/mL in buffer A supplemented with 1 mM PMSF, a 1× EDTA-free protease inhibitor mixture, and add DDM at 6 mg/mL (*see* Note 9). Mix on a vortex for a few seconds and incubate for 1 h on an end-over-end shaker. Final sample volume is 0.5–1 mL (*see* Note 10). Samples are prepared directly in 1.5 mL ultracentrifuge tubes.
3. Withdraw an aliquot of the total fraction (T) for SDS-PAGE and western blot analysis.
4. Centrifuge at 100,000 g for 45 min at 4 °C to pellet insoluble material. Collect carefully the supernatant (S).
5. Both the supernatant and the total fractions were loaded on SDS-PAGE for western blot analysis (*see* Fig. 1b).

3.2 Affinity Purification of the Human ATP8B1/CDC50A Lipid Flippase Complex

As depicted in Fig. 1a, the ATP8B1/CDC50A complex is expressed in the three strains tested. The wild-type strain was not considered for further use since the mass of yeast recovered at the end of the expression phase for this strain was much lower than for the two other strains, which grew the same way (about 20 g/L for WT versus about 35 g/L for the other two strains). Figure 1a also shows that the expression level and the distribution of both ATP8B1 and CDC50A between P2 and P3 membranes depends on the yeast strain. While the level of expression was significantly higher in P2 and P3 membranes prepared from the *GAL4* strain (T, total fraction) than from the *Δpep4* strain, we could hardly solubilize any ATP8B1 or CDC50A from these membranes (*see* Fig. 1b). We reasoned that the yeast ER translocation machinery could be overloaded by too large amounts of heterologous protein and therefore turned toward the parental W303-1B strain, which does

not contain an additional copy of the Gal4p activator and which is deleted for the major vacuolar protease Pep4p. Most of ATP8B1 and a substantial fraction of CDC50A could now be solubilized with DDM (*see* Fig. 1b), suggesting a better folding of both proteins in yeast membranes. Therefore, we decided to use the $\Delta pep4$ strain to express large amounts of ATP8B1/CDC50A for subsequent purification, functional and structural characterization.

The strategy for purification of ATP8B1/CDC50A was similar to that used for the yeast Drs2p/Cdc50p flippase complex [17]. It is based on the fusion of a biotin-acceptor domain (BAD) to the target protein (here ATP8B1), which is biotinylated *in vivo* by the Bpl1p yeast biotin ligase during the expression phase [2]. Briefly, the biotinylated target proteins recovered from the DDM-solubilized membranes are fished out thanks to a streptavidin resin. After washing, the resin is incubated with a TEV protease that cleaves between the BAD and ATP8B1, releasing ATP8B1 from the resin. CDC50A, which is intimately associated to ATP8B1, is co-purified [15]. As indicated below, addition of cholesteryl hemisuccinate during both solubilization and affinity purification significantly improves the yield of purification.

3.2.1 Incorporation of Cholesteryl Hemisuccinate into DDM Stock Solutions

1. Prepare 40 mL of a 250 mM Tris-HCl pH 8 buffer by mixing 10 mL of 1 M Tris-Cl pH 8 with 30 mL of ddH₂O.
2. Add 5 g of DDM powder and mix on an end-over-end shaker at 20 °C until complete dissolution of the powder. Final solution should be transparent.
3. Add 1 g of CHS powder. Sonicate continuously with a probe sonifier with intensity at 0% until solution is warm to the touch (<50 °C) and become translucent.
4. Adjust volume to 50 mL and mix again on an end-over-end shaker until solution becomes see-through. Depending on the temperature of the room (18–23 °C), an overnight incubation could be necessary.
5. The final solution contains 100 mg/mL DDM together with 20 mg/mL CHS. Flash-freeze and store at –20 °C (*see Note 11*).

3.2.2 Affinity Purification on Streptavidin-Sephrose Resin

All steps were done at 4 °C or on ice. P2 and P3 membranes were now mixed together (P2 + P3) for purification. The final yield of purification is about 75–100 µg purified ATP8B1/CDC50A per liter of yeast culture.

1. Dilute yeast membranes (P2 + P3) at 5 mg/mL in ice-cold buffer A supplemented with 1 mM PMSF, a 1× EDTA-free protease inhibitor mixture, directly in the 45Ti polycarbonate centrifuge tube. Mix the suspension gently on an end-over-end shaker for 5 min (*see Note 12*).

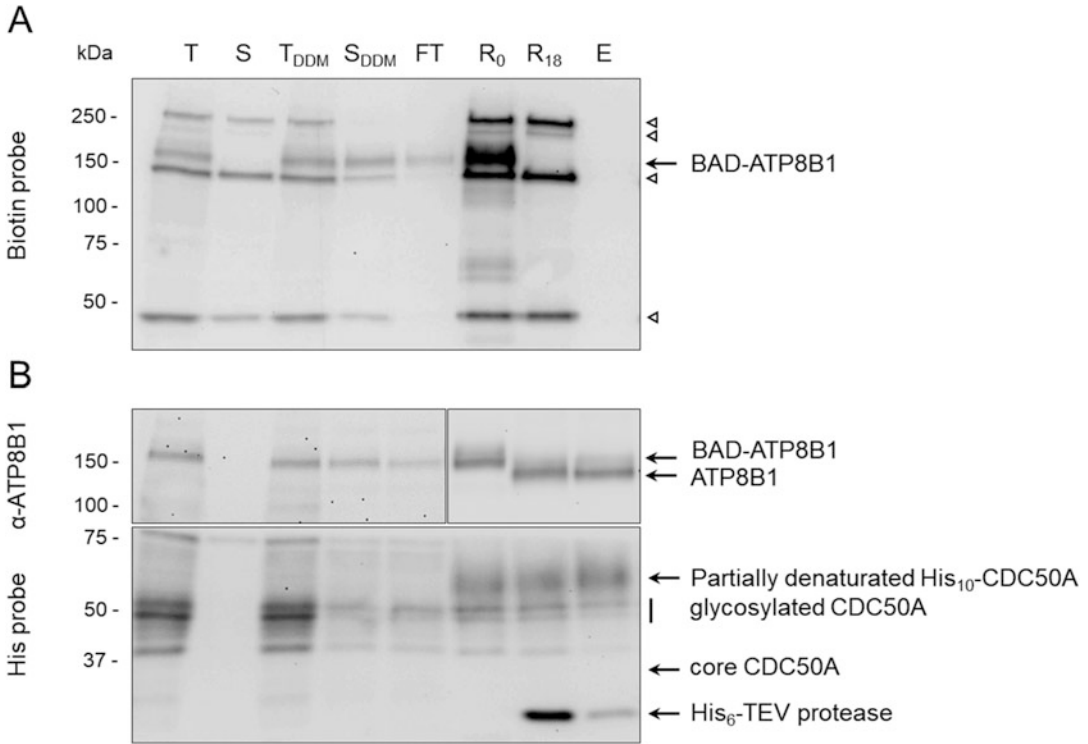


Fig. 2 Affinity purification of the ATP8B1/CDC50A complex by affinity chromatography on a streptavidin-sepharose resin. Western-blot analysis of the samples withdrawn during purification of ATP8B1/CDC50A expressed in the W303-1B/ Δ pep4 strain. Proteins were detected using either a biotin probe (panel **a**, to detect the biotinylated proteins) or an antibody directed against ATP8B1 (panel **b**, top) or a probe against the His-tagged proteins such as CDC50A (panel **b**, bottom). Open arrowheads indicate endogenous biotinylated proteins, namely, Acc1p (250 kDa), Dur1,2p (200 kDa), Pyc1,2p (130 kDa), and Arc1p (42 kDa). T, total membranes; S, supernatant recovered after membrane washing; T_{DDM}, washed membranes supplemented with DDM; S_{DDM}, DDM-solubilized fraction; FT, flow-through fraction corresponding to the unbound material; R₀, resin before incubation with TEV protease; R₁₈, resin after incubation with TEV protease for 18 h at 4 °C; E, eluted fraction corresponding here to a E1 + E2 mix

2. Withdraw an aliquot of the total fraction (*see* Fig. 2, T) for western-blot analysis.
3. Equilibrate centrifuge tubes carefully and centrifuge at 100,000 g for 45 min at 4 °C to pellet the washed membranes. Withdraw an aliquot of the supernatant (*see* Fig. 2, S) for western-blot analysis.
4. Resuspend the pellet at 5 mg/mL in ice-cold buffer A supplemented with 1 mM PMSF, a 1× EDTA-free protease inhibitor mixture, and at last DDM at 15 mg/mL (DDM:prot, 3:1, w:w) and CHS at 3 mg/mL from the DDM/CHS stock solution. Mix on an end-over-end shaker for 1 h. Sample for solubilization are prepared directly in 45Ti polycarbonate centrifuge tubes.

5. Withdraw an aliquot of the total fraction (*see* Fig. 2, T_{DDM}) for western-blot analysis.
6. Equilibrate centrifuge tubes carefully and centrifuge at 100,000 g for 45 min at 4 °C to pellet the insoluble material. Carefully recover the supernatant corresponding to the solubilized fraction (*see* Fig. 2, S_{DDM}).
7. In the meantime, prepare the streptavidin-sepharose resin. We set the resin/ATP8B1 ratio to 0.3–0.33 mL resin per mg of ATP8B1 in the solubilized fraction (the expression level of ATP8B1 is about 0.5% (w:w) of the total protein in the P2 + P3 membranes, and there is about 60% of ATP8B1 recovered after solubilization). This starting amount of resin is defined as 1 volume (1 V) for the rest of the protocol (*see* **Note 13**). The resin is centrifuged for 3 min at 500 g (SW4400 rotor in an Allegra X30 Beckman centrifuge) and the ethanol is discarded. The resin is then resuspended with 5 V ddH₂O, mixed gently on an end-over-end shaker for 5 min, and then centrifuged again for 3 min at 500 g. Supernatant is discarded. The resin is then washed with 3.3 V of buffer A supplemented with 0.5 mg/mL DDM, 0.1 mg/mL CHS and 1 mM DTT, and left on an end-over-end shaker again for 5 min, before centrifugation for 3 min at 500 g again and supernatant removal. This step was repeated twice more. The total volume for equilibration of the resin is about 10 V.
8. Incubate the solubilized membranes recovered at **step 6** for 2 h with equilibrated streptavidin-sepharose resin to allow binding of the biotinylated BAD-tagged ATP8B1 to the resin.
9. Centrifuge for 3 min at 500 g to pellet the resin. Remove carefully the supernatant containing the unbound material (*see* Fig. 2, Flow-through, FT).
10. Resuspend the resin with 3 V of buffer B supplemented with 0.5 mg/mL DDM and 0.1 mg/mL CHS in the presence of 1 mM PMSF and a 1× EDTA-free protease inhibitor cocktail, stirred on an end-over-end shaker for 5 min, and then centrifuge for 3 min at 500 g before removal of the supernatant.
11. Repeat this washing step thrice. Final washing volume is therefore 12 V.
12. Resuspend the resin in 1 V of buffer B supplemented with 0.5 mg/mL DDM and 0.1 mg/mL CHS in the presence of 1 mM PMSF and a 1× EDTA-free protease inhibitor cocktail. Withdraw an aliquot of the resin (*see* Fig. 2, R₀) for western-blot analysis. Add 60 µg of purified TEV per mL of resin. Incubate overnight (~15–18 h) on the end-over-end shaker.

13. At the end of this overnight incubation, withdraw an aliquot of the resin (*see* Fig. 2, R₁₈) for western-blot analysis.
14. Add 1 V of buffer B supplemented with 0.5 mg/mL DDM and 0.1 mg/mL CHS in the presence of 1 mM PMSF and a 1× EDTA-free protease inhibitor cocktail (*see* Note 14). Incubate for 10 min on an end-over-end shaker.
15. Centrifuge for 3 min at 500 g to pellet the resin. Carefully withdraw the first elution fraction (E1, ~2 V).
16. Add 2 V of buffer B supplemented with 0.5 mg/mL DDM and 0.1 mg/mL CHS in the presence of 1 mM PMSF, and a 1× EDTA-free protease inhibitor cocktail. Incubate for 10 min on an end-over-end shaker.
17. Centrifuge for 3 min at 500 g to pellet the resin. Carefully withdraw the second elution (E2, ~2 V).
18. Flash-freeze the purified ATP8B1-CDC50A complex (*see* Fig. 2, E) and store it at -80 °C.
19. ATP8B1 protein concentrations are calculated based on Coomassie-blue stained SDS-PAGE using known amounts of purified Drs2p as standard (*see* Fig. 3a, b).

3.3 ATPase Activity of the Purified Complex

To assess the quality of the purified complex, the ATPase activity of ATP8B1/CDC50A is monitored continuously at 340 nm using an assay, which couples ATP hydrolysis to NADH oxidation [7, 18] (*see* Fig. 3c). Conversion from NADH oxidation rates expressed in mAU/s to ATPase activities expressed in $\mu\text{mol}/\text{min}/\text{mg}$ is based on the extinction coefficient of NADH at 340 nm ($\sim 6.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) (*see* Note 15).

ATPase activity is measured at 37 °C in buffer B supplemented with 1 mM MgATP, 1 mM phosphoenolpyruvate, 0.4 mg/mL pyruvate kinase, 0.1 mg/mL lactate dehydrogenase, 250 μM NADH, 1 mM NaN_3 , 1 mg/mL DDM (2mM), 100 $\mu\text{g}/\text{mL}$ POPC ($\sim 57 \mu\text{M}$), 25 $\mu\text{g}/\text{mL}$ PI(4.5)P₂ ($\sim 23 \mu\text{M}$), residual CHS at 0.01 mg/mL (from the purified protein), and 60 $\mu\text{g}/\text{mL}$ trypsin to remove N- and C-terminal autoinhibitory extensions of ATP8B1 [15].

In these experiments, 50–200 μL of the purified ATP8B1-CDC50A complex (final protein concentration of 1–5 $\mu\text{g}/\text{mL}$) was added to a total volume of 1.8 mL. NADH consumption was recorded for 300–500 s after addition of all components in the cuvette. 1 mM BeCl_2 and 5 mM KF were added to inhibit ATP8B1 and to evaluate the background level of NADH oxidation (*see* Note 16).

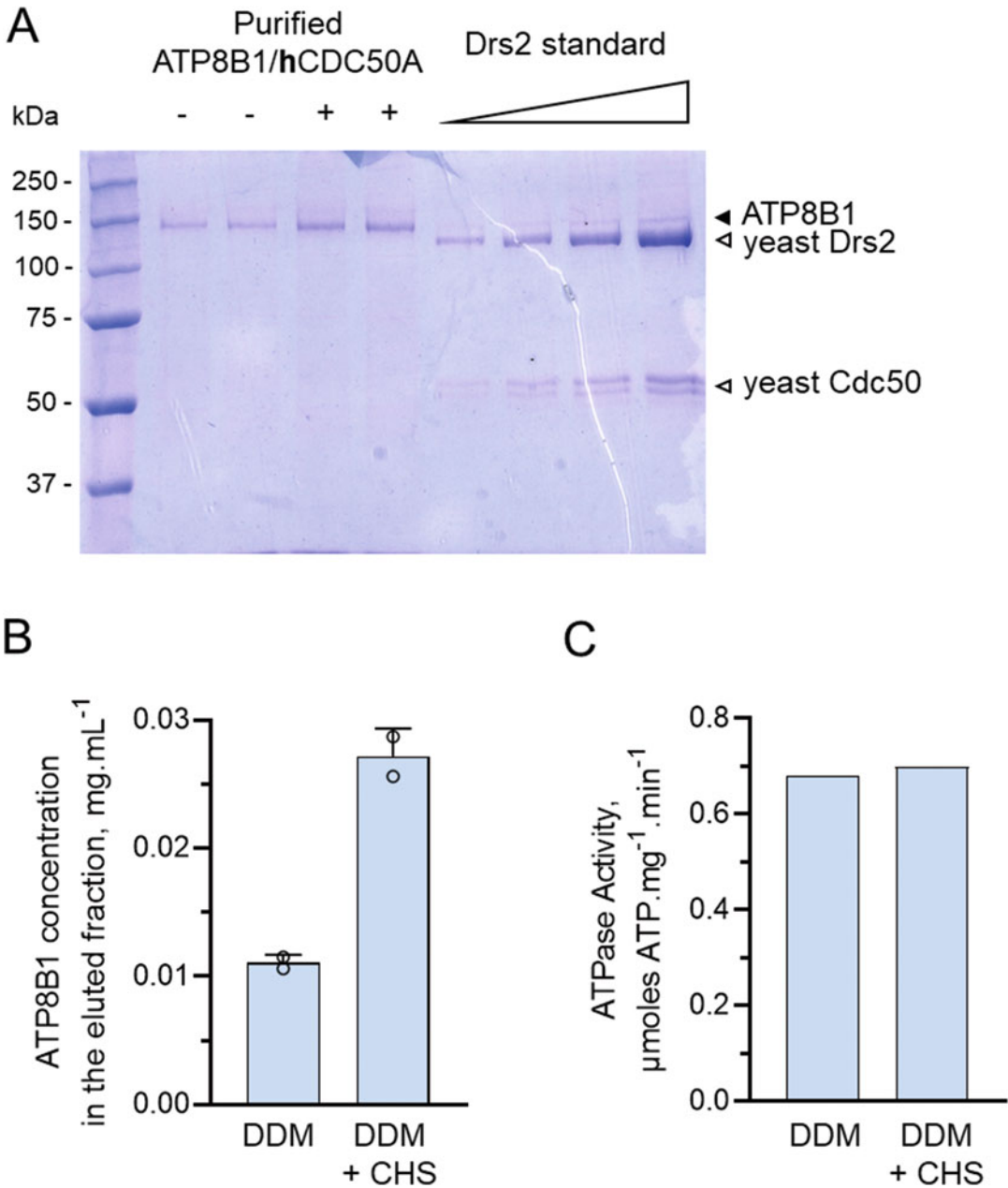


Fig. 3 Effect of the additional presence of CHS during purification of ATP8B1/CDC50A. (a) Coomassie-blue stained streptavidin-purified fractions, obtained either in the absence (–) or the presence (+) of CHS during solubilization and purification were compared to known amounts of purified yeast flippase Drs2/Cdc50 [17]. From left to right, increasing concentrations of purified Drs2p/Cdc50p correspond to 0.0125, 0.025, 0.05, and 0.1 mg/mL. 10 μ L of both purified ATP8B1/CDC50A and standards were loaded (see **Note 17**). (b) Quantification of the concentration of purified protein by densitometry analysis of the gel in panel a. (c) Specific ATPase activity of the purified complex, whether purified in DDM alone or supplemented with CHS

4 Notes

1. S6 medium contains a bactocasamino acid mix as a complete source of amino-acids while a drop-out amino-acid solution is prepared for SD medium, the latter containing only essential amino-acids. Therefore, the composition in amino-acid of SD medium may be easily adapted for auxotrophy selection.
2. NaOH is essential for agar to properly solidify. A pellet corresponds to about 250 mg of NaOH.
3. Store detergent powders up to several years at $-20\text{ }^{\circ}\text{C}$ under an argon or nitrogen atmosphere. Condensation may cause oxidation or partial hydrolysis of detergent. To avoid condensation a desiccator can be used, if available. At least, remove the flacon from the freezer and warm at room temperature for 10 min before opening. After taking the required amount of powder, fill the flacon with dry nitrogen or argon before closing and returning to the freezer.
4. Absolute ethanol is fine too.
5. Lipids powders can be stored up to several years at $-20\text{ }^{\circ}\text{C}$ under nitrogen atmosphere. Powders vials are taken out from the freezer to the bench at room temperature some tenth of minutes before opening to avoid condensation. Condensation may cause oxidation or partial hydrolysis of lipids. Use a desiccator if available. After taking the necessary amount, the vial is filled with dry nitrogen before closing and storage in the freezer. Incorporate lipids to buffers just before use.
6. Prepare a 220 mM solution of $\text{Na}_2\text{-ATP}$ in ddH_2O . Adjust to pH 7.5 with NaOH. Mix 9 volumes with 1 volume of 2 M MgCl_2 hexahydrate. Store up to several years at $-20\text{ }^{\circ}\text{C}$. The molar extinction coefficient of Mg-ATP at 260 nm is $15.4\text{ mM}^{-1}\cdot\text{cm}^{-1}$.
7. Freshly prepared NADH solution will be yellowish. NADH is light sensitive and is spontaneously oxidized to NAD^+ if exposed to light. Cover your tube with aluminum foil to protect the solution from light or use a suitable smoked-glass made bottle.
8. As milk contains biotin in large amount, it may prevent biotin probe binding to the biotinylated proteins. Therefore, milk must be completely removed before hybridization.
9. A large range of detergent to protein ratios can be also explored. However, we recommend to work with detergent: protein ratios from 1:1 to 5:1 (w:w) to limit the amount of detergent and thus to prevent as much as possible detergent-induced irreversible inactivation of the target membrane protein. Note that for detergents with high CMC, solubilization may occur at a total detergent concentration below the CMC.

Therefore, detergent concentrations below the CMC also could be investigated [7].

10. According to our own experience, working with concentrations of protein from 1 to 5 mg/mL improves centrifugation and solubilized fraction recovery.
11. Pay attention to thawing when removing DDM:CHS stock solution from the freezer, as phase transition can occur with temperature changes. Mix vigorously after reaching room temperature to obtain a clear solution.
12. This initial washing of pellets eliminates most of endogenous biotinylated proteins of the yeast, which, although soluble, remain associated with the membranes during the preparation of the P2 and P3 fractions. These biotinylated proteins compete with the target protein for binding to the streptavidin-sepharose resin.
13. The resin is supplied as a ~1:1 resin:ethanol suspension. The volume of resin indicated in the present protocol corresponds to the resin after removal of the ethanol.
14. Although TEV protease is a cysteine protease, it is not affected by the presence of the antiprotease mix and in particular by leupeptin.
15. For all experiments, photobleaching of NADH was reduced by inserting an MTO J310A filter that eliminates short wavelength UV exciting light. This setup reduced the spontaneous rate of NADH absorption changes down to about 0.01 mAU/s.
16. BeCl_2 and KF can form a BeF_x complex, an inhibitor of P-type ATPases. It mimics the inorganic phosphate and therefore blocks the flippase in a state analogous to the phosphorylated state.
17. Yeast Cdc50p and human CDC50A are glycosylated. While glycosylated forms of Cdc50 are clearly visible after Coomassie-blue staining, CDC50A remains very faint, probably because of the heterogenous nature of CDC50A glycosylation. Treatment of ATP8B1/CDC50A with endoglycosidase H results in consolidation of the different glycosylated forms to a single band [15].

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