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Characterization of Protein–Protein Interactions

Application to the Understanding of Peroxisome Biogenesis

Sebastien Leon, Ivet Suriapranata, Mingda Yan, Naganand Rayapuram, Amar Patel, and Suresh Subramani

Abstract

With the approaching completion of the *Pichia pastoris* genome, a greater emphasis will have to be placed on the proteome and the protein–protein interactions between its constituents. This chapter discusses methods that have been used for the study of such interactions among both soluble and membrane-associated proteins in peroxisome biogenesis. The procedures are equally applicable to other cellular processes.

Key Words: Protein complexes; protein interactions; yeast two-hybrid, co-immunoprecipitation; tandem affinity purification; TAP-tag; mass spectrometry; membrane protein complexes; soluble protein complexes.

1. Description of Techniques

1.1. Biochemical Methods Using Homologous Systems

Several biochemical methods, including co-immunoprecipitation and the recently described tandem affinity purification (TAP) method, are available and have been used successfully in *Pichia pastoris*. These biochemical methods allow the extraction of proteins from their in vivo context together with their interacting polypeptides. It should be noted that these procedures may yield proteins that interact directly or indirectly with the protein under study.

1.1.1. Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) allows the identification of physical interactions between proteins both in vivo and in vitro, independently of the fact that this interaction is direct or bridged by another protein (second-order interaction).

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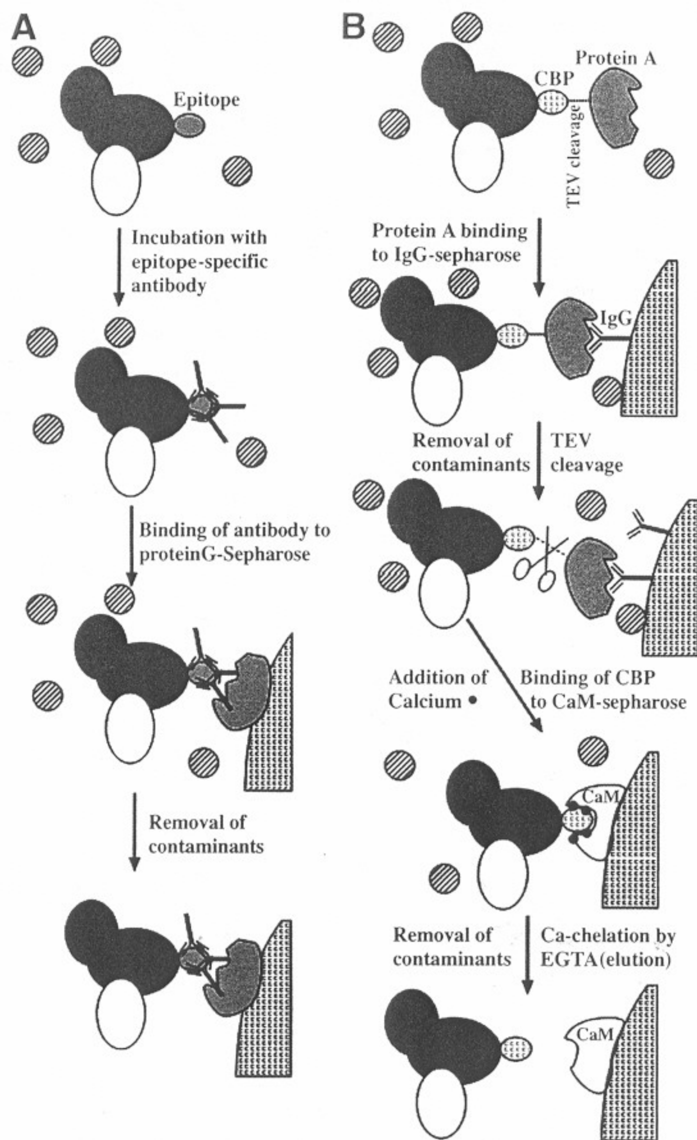

Fig. 1. Biochemical methods to detect protein–protein interactions. **(A)** Immunoprecipitation. The extract containing the protein of interest (black ellipse) is incubated with its corresponding antibody and the mixture is then bound to IgG-sepharose beads. Washes allow the removal of contaminants (hatched circles) and retain intact protein–protein complexes bound to the beads. **(B)** TAP-tag procedure. The extract containing the di-tagged protein is bound to IgG-sepharose beads through interaction with the Protein A moiety of the tag. After several washes, the beads are treated with TEV

Co-IP can be performed on a native protein provided that a good antibody is available, or on an epitope-tagged version of this protein if the tag does not alter the function, location or level of expression of the protein. Depending on the stability of the interaction between the protein of interest and its potential partners, the experiment can be performed with or without the addition of crosslinkers. Both approaches have been used successfully in *P. pastoris* to understand the composition and organization of protein complexes involved in the biogenesis of peroxisomes *(1–3)* (*see* **Fig. 1A**).

The overall protocol includes lysis of cells, solubilization of the protein in the event it is in membranes, incubation of the protein extract with the primary antibody, and then incubation of this mixture with beads (agarose- or sepharose) coupled to Protein A or Protein G depending on the source of the secondary antibody *(4)*. The ternary complex (epitope/primary antibody/secondary antibody-beads) is pelleted, washed, repelleted and the protein content associated with the beads is analyzed by immunoblotting using antibodies against the putative interacting protein(s) (which may or may not be epitope-tagged).

1.1.2. Tandem Affinity Purification

The tandem affinity purification (TAP) tag has been used for the systematic analysis of protein complexes in the proteome of *S. cerevisiae (5)*. It can also be used to study the composition of complexes in which a given protein is found *(6)*. The TAP tag consists of two affinity-purification modules separated by a cleavage site for tobacco etch virus (TEV) protease, and fused to the C-terminus of the protein under study *(7)*. The first tag is the calmodulin-binding peptide (CBP) of the kfc (kemptide-factor Xa cleavage site-calmodulin binding peptide) cassette (GenBank X66255) followed by the TEV protease site, and an IgG-binding domain based on Staphylococcal protein A as the second tag. This protein fusion with the dual tag can be introduced into the chromosome at the locus of the wild-type gene in *P. pastoris* using homologous recombination *(8)*. Care should be taken that the addition of this tag does not alter the function, location or level of expression of the protein.

After lysis of the cells, two rounds of affinity purification are performed, as opposed to co-IP which uses only a single purification (*see* **Fig. 1B**). In the first round, the protein is bound to an IgG-sepharose resin via the protein A moiety of the tag. Any contaminating, or non-specifically bound, proteins are left

Fig. 1. *(Continued)* protease to release the singly-tagged (CBP) protein. The supernatant is then incubated with calmodulin-sepharose (CaM-sepharose) beads in the presence of calcium. Washes remove other contaminants. The protein and its interacting partners (*dark grey ellipses*), are eluted from the beads by chelation of calcium using EGTA.

behind on the IgG column by releasing the tagged protein from the beads by TEV protease cleavage. This treatment removes the protein A domain on the fusion. The released fusion with its interacting proteins is then passed over a column of calmodulin-sepharose beads in the presence of calcium. The bound proteins are washed to remove the TEV protease and other nonspecific proteins, and the tagged protein is eluted using EGTA to chelate calcium and therefore releasing CBP from calmodulin. This final eluate contains a relatively pure fraction of the protein–CBP fusion together with its interacting partners. This fraction can be analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (for polypeptide composition), immunoblotting (for identification of predicted interacting proteins) and mass spectrometry (for identification of new proteins).

1.2. Reporter-Based Methods in Heterologous Systems

These methods are in common use and are not performed in *P. pastoris*, so they are reviewed briefly but the methods are not described here.

1.2.1. Yeast Two-Hybrid

Other methods routinely used for the analysis of protein–protein interactions, such as the yeast two-hybrid method (methods recently reviewed in *[9]*) are based on the addition, to the proposed interacting proteins, of tags which will perform a biological function when these tags are brought together spatially as a consequence of protein interactions *(10)*. In a given engineered system, this leads to a scorable phenotype (e.g., growth, resistance) that allows one to conclude whether the proteins interact or not (*see* **Fig. 2A**).

The most common system, referred to as the *GAL4* system, is based on the modular nature of the *S. cerevisiae* Gal4p transcriptional regulator: whereas residues 1–147 have the ability to bind DNA, residues 768–881 can activate transcription of basically any gene provided it is placed under the control of the adequate *cis* regulatory element (Upstream Activating Sequence) bound by Gal4p. The other major yeast two-hybrid system, the LexA system, utilizes in a similar manner the DNA-binding domain of the *Escherichia coli* LexA protein and the transcriptional activation domain of the herpes simplex virus VP16 protein.

Several *S. cerevisiae* host strains differing in the number and nature of the reporter(s) used are available for the two-hybrid interaction test. This technique has allowed tremendous progress in the identification of novel protein components of a given complex, and provided clues regarding the function of proteins following the understanding of the protein networks they are involved in.

1.2.2. Bacterial Two-Hybrid

The bacterial two-hybrid system was recently developed as an alternative method for the study of protein–protein interaction in vivo in a heterologous

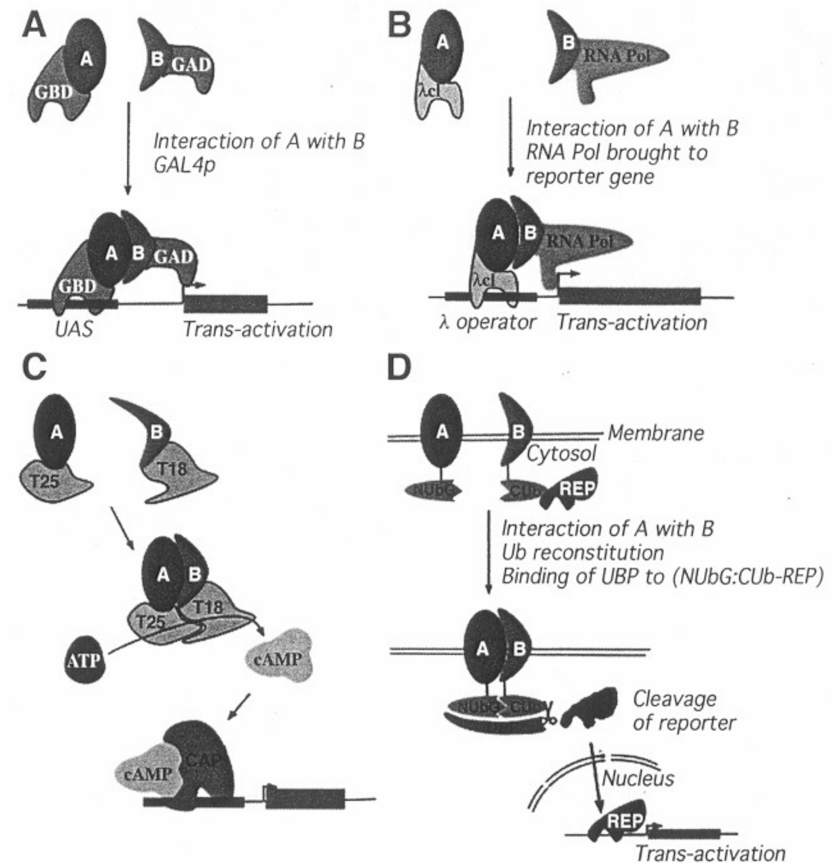

Fig. 2. Study of protein–protein interactions in heterologous systems. **(A)** The yeast two-hybrid GAL4 system is based on the reconstitution of the transcription factor, Gal4p, upon interaction of the proteins under study. **(B,C)** Transcription- and nontranscription-based bacterial two-hybrid assays. **(B)** Interaction of the studied proteins will bring the RNA polymerase to the λ operator and activate transcription at this locus, or **(C)** reconstitute adenylate cyclase activity. **(D)** The split-ubiquitin system is based on the release of a reporter after cleavage by a ubiquitin-specific protease. This occurs only when two proteins, each fused to one half of the ubiquitin molecule, interact and therefore reconstitute ubiquitin.

system (reviewed in *[11]*). These methods are of two types, either based on the reconstitution of (i) a transcription factor (in a similar way as described above for the yeast two-hybrid *[12,13]*), or (ii) of the *Bordetella pertussis* adenylate cyclase protein *(14)* in an engineered *E. coli* strain (*see* **Fig. 2B,C**).

In the first case, the studied protein is fused to the DNA-binding domain of the bacteriophage λ repressor protein (cI), while the second protein is fused to

the N-terminal domain of the α subunit of *E. coli* RNA polymerase. The bacterial strain used possesses the *HIS3* reporter gene under the control of the λ operator. Interaction of the two proteins will stabilize the RNA polymerase at the λ operator, thus allowing transcription of the *HIS3* reporter gene. This system is now commercially available under the name BacterioMatch® (trademark of Stratagene, La Jolla, CA).

The nontranscription-based bacterial two-hybrid makes use of the CyaA protein (calmodulin-dependent adenylate cyclase toxin) from *Bordetella pertussis*. The catalytic domain of this protein (residues 1–399, out of 1706) is cleaved into two fragments (namely: T25, residues 1–224, and T18, residues 225–399) that cannot interact with each other. However, upon fusion of each domain to interacting proteins, the catalytic domain is reconstituted and cAMP is synthesized. This is characterized, in an *E. coli cya* strain, by restoration of the cAMP level, which in turn triggers the expression of a reporter gene placed under the control of a cAMP-dependent promoter giving rise to a selectable phenotype.

Although these methods have essentially been used for the study of bacterial proteins, they have been used in several instances for mammalian ***(15–18)***, and even viral, proteins ***(19)***. This method could therefore be used for the study of in vivo protein–protein interactions. Interestingly, it can be also used as a complementary approach, as reported in a study that compares the traditional yeast two-hybrid to the nontranscription-based bacterial two-hybrid system ***(18)***.

1.2.3. Split-Ubiquitin (Ubiquitin-Based Split-Protein Sensor [USPS])

The split-ubiquitin method ***(20)*** is based on expressing one protein (A) as a fusion to a modified N-terminal domain (residues 1–34 with mutation of I13→G, denoted NUbG) of ubiquitin (Ub), and expressing another protein (B) as a fusion to the Ub C-terminal domain (residues 35–76, CUb) followed by a stable reporter protein. In vivo, NUbG and CUb-reporter have a weak affinity for each other but can assemble and form split-ubiquitin: (NUbG:CUb)-reporter.

In eukaryotes, Ub-protein fusions are cleaved by the action of Ub-specific proteases (UBPs), a process that releases the attached protein. This cleavage step requires Ub to be properly folded (i.e. the N- and the C-terminal regions must interact with each other).

Therefore, upon interaction of the two tagged proteins (A and B) under study, reconstitution of a full-length-equivalent of the Ub molecule occurs, which allows recognition and cleavage by UBP and release of the reporter protein (*see* **Fig. 2C**). The phenotype observed due to protein A-protein B interaction is based on the gain of activity of the released reporter, a transcription factor that activates reporter genes in the nucleus in the suitable strain (methods reviewed in ***[21,22]***).

2. Materials

2.1. Co-Immunoprecipitation

Most of the methods for studying protein–protein interactions were developed initially with the analysis of soluble, and often stable rather than transient, protein complexes in mind. However, the application of such procedures to the study of interactions between soluble and membrane proteins, or between membrane proteins, has been more difficult because the procedures for extracting and solubilizing proteins from their membranes of origin require harsher conditions, such as the use of detergents, which might also disrupt the interactions with partner proteins. We present below two different protocols for co-immunoprecipitations. The first is with cleavable crosslinkers to stabilize interactions between membrane proteins prior to their solubilization from membranes ***(1)***. This method also has the advantage of being able to capture transient and dynamic interactions between proteins. The second co-IP procedure is without the use of crosslinkers and generally works better for interactions between soluble proteins.

For co-IPs in the presence of crosslinker, the general strategy involves breaking the cells open after spheroplasting, while keeping subcellular organelles intact. The addition of a membrane-permeable crosslinker stabilizes interactions between proteins that are in close contact (determined by the length of the crosslinker). The proteins are then resuspended in a buffer that is partially denaturing, but compatible with antibody–antigen interactions. The protein of interest is immunoprecipitated. After reduction of the crosslinker to release the interacting proteins, the polypeptides are separated by SDS-PAGE and detected by immunoblotting. Controls are necessary to rule out nonspecific crosslinking of proteins, for example, resulting from the use of excessive amounts of the crosslinker.

2.1.1. Materials for Co-Immunoprecipitation of Crosslinked Proteins

1. *P. pastoris* strains PPY12 (*arg4 his4*), SMD1163 (*his4 pep4 prb1*).
2. Yeast culture medium: YPD: 1% yeast extract, 2% peptone, 2% glucose), or peroxisome induction medium such as methanol medium (yeast nitrogen base + 0.7% *[w/v]* ammonium sulfate, 0.05% *[w/v]* yeast extract, 0.5% *[v/v]* methanol, plus required amino acids).
3. Reducing buffer: 100 m*M* Tris-HCl (pH 7.5), 50 m*M* ethylene diamine tetraacetic acid (EDTA), 100 m*M* β-mercaptoethanol.
4. Spheroplasting buffer: 10 m*M* K_2HPO_4, 10 m*M* KH_2PO_4, and 1.2 *M* sorbitol (pH 7.4).
5. Lytic enzyme: Zymolyase 20T (Seikagaku Corp).
6. Lysis buffer: 20 m*M* K_2HPO_4, 20 m*M* KH_2PO_4, and 1 m*M* EDTA (pH 7.5).
7. Protease inhibitors: phenylmethylsulfonylfluoride (PMSF) 0.1 *M* in ethanol, Protease Inhibitor Cocktail for yeast (Sigma; cat. no. P8215).

8. Dithiobis[succinimidylpropionate] (DSP) (Pierce Chemicals) in dimethyl sulfoxide (DMSO) at concentration of 20 mg/mL. Use freshly prepared solution each time. For mock treatment, add only DMSO to the reaction.
9. Hydroxylamine (Sigma): 1 *M* solution.
10. TCA (trichloracetic acid) (Fisher Scientific): 100% *(w/v)* solution.
11. Cracking buffer: 50 m*M* Tris-HCl, 1 m*M* EDTA (pH 7.5), 1% SDS, and 6*M* Urea.
12. Tween-20 IP buffer: 50 m*M* Tris-HCl (pH 7.5), 0.1 m*M* EDTA, 150 m*M* NaCl, and 0.% Tween-20.
13. Antiserum against protein of interest.
14. Gamma-bind G Sepharose (Amersham Pharmacia Biotech) or Protein A Sepharose (Amersham Pharmacia Biotech) (*see* **Note 1**).
15. Tween-20 urea buffer: 100 m*M* Tris-HCl (pH 7.5), 200 m*M* NaCl, 2 *M* Urea, and 0.5% Tween-20.
16. TBS buffer: 25 m*M* Tris-HCl (pH 7.5) and 140 m*M* NaCl.
17. Urea sample buffer: 6 *M* Urea, 125 m*M* Tris (pH 6.8), 6% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, and 0.01% bromophenol blue.

2.1.2. Materials for Co-Immunoprecipitation Without the Use of Crosslinker

1. Yeast strain: this protocol has been used with *P. pastoris* strains PPY12 (*arg4 his4*) and SMD1163 (*his4 pep4 prb1*). The strain of interest is either a wild-type (when an antibody against the native form is available [*see* **Note 2**]), or one expressing an epitope-tagged protein. Also, a negative control strain must be used: either a deletion strain or the same strain lacking the tagged protein. This allows the assessment of the specificity of the co-immunoprecipitation for the protein studied, and shows that the presence of an interacting protein in the final extract depends on the presence of the protein studied.
2. Yeast culture medium (*see* **Subheading 2.1.1.**).
3. Gamma-bind G Sepharose (Amersham Pharmacia Biotech) or Protein A-Sepharose (Amersham Pharmacia Biotech) (*see* **Note 1**).
4. Protease inhibitors stock solutions: Leupeptin 1.25 mg/mL, Aprotinin 5 mg/mL, Protease Inhibitor Cocktail solution (Sigma; cat. no. P8215), and PMSF 0.1 *M* in ethanol.
5. Acid-washed glass beads: diameter 425 µm to 600 µm (Sigma).
6. IP lysis buffer: 50 m*M* HEPES-KOH (pH 7.5), 0.5 *M* NaCl, 0.5% *(w/v)* NP-40, 10% *(v/v)* glycerol, and 1 m*M* EDTA.
7. Wash buffer: 50 m*M* HEPES-KOH (pH 7.5), 150 m*M* NaCl, and 1 m*M* EDTA.
8. Sample buffer, 5X stock: 250 m*M* Tris-HCl (pH 6.8), 10% *(w/v)* SDS, 0.5 *M* dithiothreitol (DTT), 0.5% *(w/v)* bromphenol blue, and 50% *(v/v)* glycerol.
9. Nondyed sample buffer: 50 m*M* Tris-HCl (pH 6.8), 2% *(w/v)* SDS, 0.1*M* DTT, and 10% *(v/v)* glycerol.
10. Bromphenol blue 100X stock solution: 100 mg/mL.

2.2. Materials for TAP-Tagging

1. Plasmid pMY62 (or pMY63/64 [*see* **Note 3**]).
2. TAP primers: **OMY69:** 5′-TCTGACGCTCAGTGGAACGAA-3′, **OMY70:** 5′-TGCCCCGGAGGATGAGATT-3′.
3. Primers 1, 2, and 3 (*see* **Subheading 3.3.1.** for primer design).
4. *P. pastoris* genomic DNA.
5. LB + zeocin plates: 25 g/L Luria broth (LB) (Sigma; cat. no.L-3522), 15 g/L agar, and 100 µg/mL zeocin.
6. YPD + zeocin plates: 10 g/L yeast extract, 20 g/L peptone, 2% dextrose, 15 g/L agar, and 100 µg/mL zeocin.
7. French Press.
8. Buffer A: 10 m*M* HEPES-KOH (pH 7.9), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, and 0.5 m*M* DTT, 0.5 m*M* PMSF, 1 m*M* leupeptin, protease inhibitor cocktail (use following manufacturer's instructions), and 5 µg/mL aprotinin.
9. Buffer D: 20 m*M* K-HEPES (pH 7.9), 50 m*M* KCl, 0.2 m*M* EDTA, 0.5 m*M* DTT, 25% *(w/v)* glycerol, 0.5 m*M* PMSF, 1 m*M* leupeptin, protease inhibitor cocktail (use as per manufacturer's instructions), and 55 µg/mL aprotinin.
10. Dialysis tubing: Molecular weight cut-off 12–14,000.
11. IgG-agarose beads (Sigma; cat. no. A2909).
12. IPP150 buffer: 10 m*M* Tris-Cl (pH 8.0), 150 m*M* NaCl, and 0.1% NP40.
13. TEV cleavage buffer: 10 m*M* Tris-Cl (pH 8.0), 150 m*M* NaCl, 0.1% NP40, 0.5 m*M* EDTA, and 1 m*M* DTT.
14. TEV protease, recombinant (Invitrogen; cat. no. 10127-017).
15. Calmodulin beads (Stratagene; cat. no. 214303).
16. IPP150 calmodulin-binding buffer: 10 m*M* Tris-Cl (pH 8.0), 150 m*M* NaCl, 0.1% NP40, 10 m*M* β-mercaptoethanol, 1 m*M* Mg-acetate, 1 m*M* imidazole, and 2 m*M* $CaCl_2$.
17. IPP150 calmodulin-elution buffer: 10 m*M* Tris-Cl (pH 8.0), 150 m*M* NaCl, 0.1% NP40, 10 m*M* β-mercaptoethanol, 1 m*M* Mg-acetate, 1 m*M* imidazole, and 5 m*M* EGTA.

3. Methods

3.1. Methods for Co-Immunoprecipitation of Crosslinked Proteins

3.1.1. Lysis of Cells and Crosslinking of Proteins

1. Measure the OD_{600} of a 50 mL yeast culture and when it is approx OD_{600} of 1/mL, collect cells by centrifugation at 2000*g* for 10 min at room temperature.
2. Resuspend cells in 15 mL of reducing buffer, transfer the suspension into a 50 mL centrifuge tube and incubate them at room temperature for 20 min with gentle shaking.
3. Pellet cells by centrifugation (2000*g* for 10 min) at room temperature, wash once in the same amount of Spheroplasting buffer.

4. Resuspend pellet in Spheroplasting buffer to give an OD_{600} of 5/mL, add Zymolyase 20T, usually 6 mg/1000 ODs (*see* manufacturer product information for amount to be used).
5. Incubate suspension for 30 to 45 min at 30°C with gentle rotation (*see* **Note 4**).
6. For each cross-linking reaction, transfer 1 mL of spheroplasts to a 1.5 mL reaction tube. For each co-IP, two reactions are required, one with addition of crosslinker agent and one without crosslinker addition as a control. In most cases, detergent-solubilized membrane proteins should not interact without crosslinker.
7. Pellet spheroplasts by centrifugation at low speed (400*g* for 10 s). Resuspend pellet in 1 mL lysis buffer containing protease inhibitors (PMSF to a final concentration of 1 m*M* and 2.5 µL of Protease Inhibitor Cocktail).
8. Immediately add the cross-linking agent, DSP, to a final concentration of 200 µg/mL to stabilize transiently-interacting proteins or detect complexes between membrane proteins (*see* **Note 5**).
9. Incubate at room temperature for 30 min with occasional shaking.
10. Quench the crosslinking reaction by adding 1 *M* hydroxylamine to a final concentration of 20 m*M*.
11. Precipitate protein by adding 100% TCA to a final concentration of 5%. Mix well by inversion, incubate on ice for at least 30 min.
12. Collect TCA precipitates by centrifugation (14,000 rpm; 5 min). Discard supernatant and wash pellet twice with cold acetone (*see* **Note 6**).
13. Dry the washed pellets using a speed-vac concentrator (Savant Instruments).

3.1.2. Co-Immunoprecipitation

1. Dissolve pellets, from **Subheading 3.1.1.**, **step 13** in 100 µL Cracking Buffer using a water-bath sonicator (*see* **Note 7**).
2. Incubate the samples at 65°C for 5 min.
3. Add 1 mL Tween-20 IP Buffer and 10 µL of 100 mg/mL BSA. Mix well by inversion.
4. Centrifuge the mixture at 4°C (14,000*g* for 10 min). Transfer 950 µL of the supernatant to a new 1.5-mL reaction tube.
5. Add antiserum and incubate at 4°C for at least 2 h or overnight with rocking.
6. Add 75 to 100 µL of prehydrated/prewashed Protein A-sepharose 50% slurry for polyclonal or Protein G-sepharose for monoclonal antiserum used for co-IP. Incubate at 4°C for at least 1 h with rocking.
7. Wash beads twice with 1 mL Tween-20 urea buffer, twice with 1 mL Tween-20 IP buffer, and once with 1 mL TBS. Centrifuge at 2000*g* for 10 s each time.
8. Extract co-IP proteins from the sepharose beads by adding 100 µL urea sample buffer and incubating at 65°C for 5 min.
9. Run SDS-PAGE using the samples. The β-mercaptoethanol in the SDS-PAGE sample buffer will reduce the crosslink between the interacting proteins. Use immunoblots with appropriate antibodies to detect the immunoprecipitated protein and other partners it might interact with.

3.2. Method for Co-Immunoprecipitation of Proteins Without Use of Crosslinkers

3.2.1. Cultures

1. Inoculate cells (from a fresh preculture) at A_{600} = 0.2/mL. Grow cells to A_{600} = 0.6–1/mL (10–50 mL, depending on the expression level of the protein) in the required medium.
2. Spin down between 6 and 30 ODs of cells, by spinning at 1000*g* for 3 min.

3.2.2. Lysis

1. The following protocol is for 10 mL culture (6 to 10 ODs). Keep all samples in ice, all steps must be done on ice or in a cold room.
2. Give the cells a quick wash with 1.5 mL of cold phosphate buffered saline (PBS) and transfer cells to a 1.5-mL microcentrifuge tube.
3. Centrifuge and remove as much as PBS as possible. Resuspend the cell pellet in 200 µL of IP lysis buffer containing the following volumes of protease inhibitor stock solutions: 2 µL aprotinin, 2 µL leupeptin, 2 µL PMSF (add right before use), 2 µL protease inhibitor cocktail.
4. Add glass beads up to the meniscus and vortex for 1 min in cold room, 10× with 1 min incubation on ice in between.
5. Punch a small hole on the bottom of the tube, small enough to prevents beads from going through. Quickly put this tube into a new, cold microcentrifuge tube, and spin 5 s at 2000*g*. Repeat until the beads from the upper tube are dry (i.e., until all the extract has been transferred to the bottom tube).

3.2.3. Solubilization: Clearance

1. Spin down the cell debris. If the protein under study (or predicted interacting proteins) is membrane associated, follow **step a** (solubilization) and then **step b**. If the protein is soluble, go directly to **step b**.
   a. In the case of a membrane protein, first spin down intact yeasts cells and cell debris by centrifuging for1 min at 1000*g* at 4°C. Transfer the supernatant into a clean, cold microcentrifuge tube. Add detergent to solubilize membrane proteins of interest. Proper solubilization of a given protein depends of (i) the nature of the detergent, (ii) its concentration, (iii) the protein/detergent ratio for solubilization, and (iv) time and temperature at which solubilization occurs. This depends mainly on the protein and the characteristics of the membrane and therefore has to be optimized.
   b. Spin down cell debris/unsolubilized material for 10 min at 14,000*g*, at 4°C.
2. Transfer the clear extract to a new tube and accurately measure the volume. Determine the volume per OD of cell equivalent (e.g., if the intial 10 ODs end up in a final volume of 200 µL, 1 OD of cells = 20 µL).
3. Remove 1 OD equivalent of cell extract and keep this in a separate tube labeled input. Add 5X sample buffer and store at –20°C.

3.2.4. Immunoprecipitation

1. Add the 1st antibody to the rest of the extract. Dilutions at which the antibody should be used depend on the antibody. For instance, 1 μL of monoclonal α-HA antibody can be used per 200 μL reaction.
2. Mix overnight at 4°C. This time depends on the stability of the interaction and the quality of the antibody. For unstable interactions, this time can be shortened to between 3 and 4 h.
3. Add 100 μL of Protein-A- or Protein G-coupled sepharose beads (previously hydrated and washed in lysis buffer) (*see* **Note 1**).
4. Mix at 4°C for 1 h. A longer incubation (up to 6 h) usually brings more protein, but can also increase the background.
5. Spin beads at 500*g* for 10 s. It is important to spin at low speed to prevent possible membrane vesicles from being pelleted.
6. Transfer 1 OD of cell equivalents to a new cold microcentrifuge tube labeled "unbound." Add 5X sample buffer and keep at –20°C. The rest of the unbound fraction can also be saved.
7. Wash with 1 mL IP lysis buffer: mix at 4°C for 5 min and spin at 500*g* for 10 s, remove supernatant.
8. Repeat twice with 1 mL wash buffer.
9. After the final wash, remove buffer from beads completely. This can be done by aspirating the beads with a syringe and a 30 gage, 1/2 inch needle.
10. Add 50 μL of 1X non-dyed sample buffer to the dried beads and boil for 5 min.
11. Punch a small hole at the bottom of the tube, proceed as in **step 6** (except this time the beads are smaller).
12. Add 100X bromophenol blue stock solution (1X final concentration) to the eluate. Use or freeze at –20°C.

3.2.5. Immunoblot Analysis

1. Denature samples before loading.
2. Load on SDS-PAGE the equivalent of 0.2 OD of "input" and 0.2 OD of "unbound" samples. This can be adjusted in case the protein has a high/low expression. In the unbound fraction, the immunoprecipitated protein is sometimes depleted relative to the input. Usually, the immunoprecipitated protein can be seen when the equivalent of 1 OD of the final extract is loaded. For co-IP proteins, load from 1 to 5–7 OD equivalents depending on the affinity of the antibody, and the strength of the interaction (as long as unrelated marker proteins do not appear).
3. Even after denaturation, there is still a strong reactivity of the antibody present in the extract (and therefore loaded on the gel) with the secondary antibody used in the immunoblotting process. Accordingly, the acrylamide concentration can be adjusted to prevent overlapping of signals (*see* **Note 2**).

3.3. Methods for Long-Homology TAP-Tagging of P. pastoris Proteins

3.3.1. Tagging of the Strain

The TAP-tag sequence has been amplified from CellZome's pBS1479 *(6)*, and subcloned into the pPICZ-B (Invitrogen, La Jolla, CA) backbone (leaving behind the bacterial origin of replication, the AOX1 (alcohol oxidase 1) transcription terminator and, most importantly, the prokaryotic/eukaryotic dual functional zeocin resistance cassette), to obtain pMY62, a new TAP-tagging vector with a relatively small size (*see* **Fig. 3A**).

1. Design three primers corresponding to the target gene's coding sequence (*see* **Fig. 3B**). Primer 1 (reverse direction): contains four nucleotides at the 5′-end (for better restriction enzyme digestion), a site for the restriction enzyme *Afl*II, and the region that is complementary to the sequence of the target gene coding sequence preceding, but not including, the stop codon. The reading frame must be maintained with the *Afl*II site CTT.AAG. (If there is an *Afl*II site in the amplification region, use a blunt-end restriction enzyme site instead.) Primer 2 (forward direction): contains four nucleotides at the 5′-end, a site for the restriction enzyme *Cla*I, and the region corresponding to the internal sequence of the gene. Amplify a region longer than 500 bp for better homologous recombination in the later step. (If there is a *Cla*I site in the amplification region, use a blunt-end restriction enzyme site instead.) Primer 1 and Primer 2 should be designed so that the amplified region possesses a unique restriction site located in the middle region of the sequence, which is not present in pMY62, and which will be used for plasmid linearization required before transformation (*see* **Fig. 3A**). Primer 3 (forward direction) is chosen upstream of Primer 2, for verification of the TAP-tag cassette integration at the appropriate locus (*see* **step 4** and **Fig. 3C**).
2. Amplification of the target gene fragment by PCR and insertion of this fragment into pMY62.
   a. Use Primers 1, 2, and the *P. pastoris* genomic DNA (you may use Promega's Wizard Genomic DNA purification kit) as template to amplify the fragment by a high-fidelity thermo-polymerase such as PfuTurbo (Stratagene; cat. no. 600250).
   b. Digest the PCR fragment with *Afl*II and *Cla*I (or *Sca*I/other blunt-end restriction enzyme), clone into appropriately-digested pMY62. Transform *E. coli* and isolate zeocin-resistant colonies on LB + zeocin plate.
   c. The inserted fragment can be sequenced using OMY69 and OMY70 primers to confirm the sequence and the reading frame.
3. Linearize construct to transform *P. pastoris* cells.
   a. Linearize the pMY62 plasmid within the insert by digesting once (as close to the middle of the insert as possible) in the target gene fragment (the vector should not be cut; its sequence is available for computer analysis). Avoid

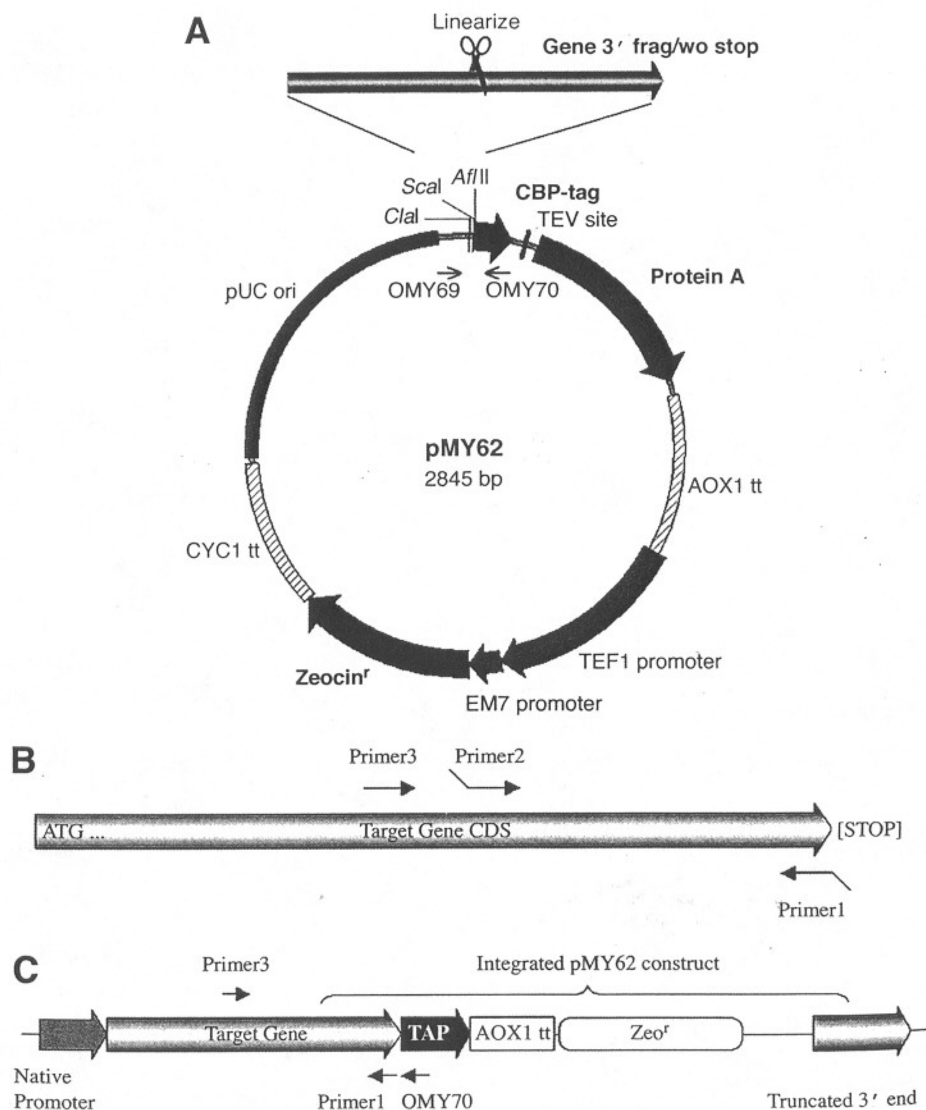

Fig. 3. TAP-tagging strategy in *Pichia pastoris*. (**A**) Map of the TAP-tagging vector (pMY62). The insert is cloned (without its STOP codon for translation) using the restriction sites *ClaI*/*AflII* or *ScaI*, in-frame with the CBP tag, the TEV protease cleavage site and the Protein A tag. A unique restriction site, in the insert, is used to linearize the vector for transformation by homologous recombination in *P. pastoris*. (**B**) Strategy for primer design (*see* text for details). (**C**) Overall structure of the TAP-tagged locus in its chromosomal context.

regions of less than 100-bp homology with the target gene, on either side of the site of linearization.

b. Transform *P. pastoris* (by electroporation or chemical methods such as LiAc/polyethylene glycol (PEG) *(8)*, plate on YPD + zeocin and isolate zeocin-resistant colonies.

4. PCR identification of correct transformants.
   a. Pick some colonies from the YPD + Zeocin plate and resuspend each of them in 100 μL H_2O.
   b. Prepare raw genomic DNA by taking 50 μL of the resuspended colony and adding Zymolase 20T to a final concentration of 1 mg/mL. Incubate the reaction at 30°C for 30 min. Next, freeze reaction in liquid nitrogen, then thaw in a water bath at room temperature. Repeat freeze-thaw three or more times. Spin down to pellet debris; the raw genomic DNA is in the supernatant.
   c. Run two PCR reactions on each raw genomic DNA sample. A control reaction using Primer3 + Primer1 to check Primer3 and raw genomic DNA quality, and a test reaction using Primer3 + OMY70 to validate the location of the integrated TAP tag. Also do control and test reactions using genomic DNA from a wild-type strain.
   d. PCR results: Wild-type genomic DNA will yield one band in the control reaction and no band in the test reaction. Transformants with incorrect integration should yield the same results. Transformants with the correct integration should produce a band in the control reaction and a band that is slightly larger in the test reaction.
   e. Correct integration of the TAP-tagged segment into the chromosomal locus of the target gene should yield the following layout (*see* **Fig. 3C**).
5. Western blot confirmation of the TAP-fusion protein. The strain may be further confirmed by Western blots with an antibody raised against the target protein (add 20 kDa to the native protein size for the whole TAP-tag, or 5 kDa for the CBP tag remaining after TEV protease cleavage). Alternatively, anti-CBP (Upstate; cat. no. 07-482) at a dilution of 1:5,000) or Protein A antibodies can also be used to detect the fusion protein.

3.3.2. Large-Scale TAP Purification (for Integral-Membrane Peroxins)

(The TAP purification is done essentially following the Séraphin Lab's protocol *[6]*).

1. Inoculate 500 mL of YPD medium in a 2 L flask and incubate overnight at 30°C with vigourous shaking.
2. Harvest the cells by centrifugation at 3000*g* for 10 min and wash them with 500 mL sterile distilled water.
3. Resuspend the cells in 2 L of peroxisome inducing medium (such as methanol medium (*see* **Subheading 2.1.1., step 2**) and incubate overnight at 30°C with vigorous shaking. This step induces peroxisome biogenesis.

4. Harvest the cells by centrifugation at 3000*g* for 10 min and wash them with 500 mL ice-cold water twice, then pellet the cells.
5. Resuspend the cell pellet in 10 mL of buffer A at room temperature.
6. All the following procedures must be carried out at 4°C.
7. Mechanically disrupt the cells using a French Press at 11,000 psi. Add KCl to a final concentration of 0.2 *M* to the lysate.
8. Centrifuge the lysate at 27,000*g* for 30 min at 4°C and add digitonin (A.G. Scientific Inc., cat. no. D-1029) to the supernatant to the desired concentration (0.25–1%) to extract membrane protein complexes. Addition of excessive amount of detergent can destabilize protein complexes. Incubate for 1 h at 4°C.
9. Centrifuge at 100,000*g* for 1 h at 4°C and dialyse the supernatant in buffer D overnight.
10. To the dialysed lysate, add Tris-Cl (pH 8.0) to a final concentration of 10 m*M*, NaCl to 150 m*M*, and NP40 to 0.1%.
11. Wash 200 μL of IgG agarose beads with IPP150 buffer and incubate the dialyzed lysate with the beads for 2 h at 4°C.
12. Wash the beads once with three volumes of IPP150 buffer followed by three volumes of TEV cleavage buffer.
13. Resuspend the IgG agarose beads in 1 mL of TEV cleavage buffer and incubate with 100 units of recombinant TEV protease at 25°C for 90 min.
14. Recover the supernatant and add three volumes of calmodulin-binding buffer and 3 μL of 2 *M* $CaCl_2$/mL of eluate to titrate the EDTA.
15. Wash 200 μL of calmodulin beads with IPP150 calmodulin-binding buffer and incubate the eluate with the beads for 1 h at 4°C.
16. Allow the column to drain by gravity flow and wash the column with 30 mL of IPP150 calmodulin-binding buffer.
17. Elute four fractions of 100 μL each with IPP150 calmodulin-elution buffer.

3.3.3. Analysis of Interacting Proteins

After the two-step TAP procedure, the eluted proteins may be concentrated by TCA precipitation (add TCA to a final concentration of 20%, keep overnight on ice, and centrifuge for 15 min at 14,000*g* at 4°C) and subjected to SDS-PAGE and immunoblotting with antibodies to the target protein and to its putative interacting partners. Alternatively, the proteins in the sample may be subjected to mass spectrometry for identification.

4. Notes

1. It is advisable to use protein G except when the source of the antibody is guinea pig. Neither Protein A nor Protein G bind chicken IgY.
2. Care should be taken in the choice of the epitope and the animal of origin of the primary antibodies used for the detection of interacting protein(s) in the final extract. For instance, if the protein of interest is immunoprecipitated with a monoclonal (mouse) antibody and the interacting protein is also detected using a mouse antibody, the IgG used for the procedure and present in the extract will react strongly with the secondary anti-mouse antibody used during the immunoblotting (between 50 - and 60 kDa and 20 and 25 kDa). In this case, it is advised to use a different antibody source for (i) immunoprecipitation, or (ii) the detection of interacting proteins. Another alternative consists of using secondary antibody raised against native primary antibodies (TrueBlot™, eBioscience, La Jolla, CA) that will therefore not bind to the denatured primary antibodies present in the SDS-PAGE.
3. TAP-tag vectors with different drug-resistance markers are available. pMY62, as described in the text, has Zeocin resistance; pMY63 and pMY64, carrying neomycin–kanamycin phosphotransferase type I and II, respectively, are essentially the same as pMY62 except that the drugs used for selection are kanamycin in bacteria and G418 in yeast. Their full-length sequences, which are useful for finding an appropriate site for linearization, are available upon request.
4. Spheroplasting is an important step of this procedure. Spheroplasts lyse when diluted in water and this can be easily observed with a light microscope or by the decrease of turbidity at 600 nm after addition of water to spheroplasts.
5. It is important to titrate the amount of cross-linker used so that only specific interactions are detected, but not nonspecific ones. It is a good idea to confirm that the co-IP does not contain non-specific partners due to the addition of too much crosslinker.
6. Resuspend pellet thoroughly for the acetone wash. Use of a water-bath sonicator helps.
7. Solubilize pellet completely in Urea Cracking Buffer until suspension becomes clear.

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