TITLE:

Fluorescence-based measurements of phosphatidylserine/phosphatidylinositol 4-phosphate exchange between membranes

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SUMMARY: (Instructions)

We describe protocols to measure whether a protein extracts and transports PS or PI(4)P *in vitro* using fluorescent lipid sensors and liposomes.

ABSTRACT:

Several members of the evolutionarily conserved Oxysterol-Binding Protein (OSBP)-related proteins (ORP)/OSBP homologs (Osh) family have recently been found to represent a novel lipid transfer protein (LTP) group in yeast and human cells. They are able to transfer phosphatidylserine (PS) from the endoplasmic reticulum (ER) to the plasma membrane (PM) by PS/phosphatidylinositol 4-phosphate (PI(4)P) exchange cycles. This finding allows us to better understand how PS, which is critical for signaling processes, is distributed throughout the cell and to investigate the link between this process and phosphoinositide (PIP) metabolism. The development of new fluorescence-based protocols has been instrumental in the discovery and characterization of this new cellular mechanism *in vitro* at the molecular level. Here, we describe the production and the use of two fluorescently-labelled lipid sensors, NBD-C2_{Lact} and NBD-PH_{FAPP} to measure the ability of a protein to extract PS or PI(4)P and to transfer these lipids between artificial membranes. First, we detail how to produce, label and obtain high purity samples of these two constructs. Secondly, we explain how to use these sensors with a fluorescence microplate reader to determine whether a protein is able to extract PS or PI(4)P from liposomes, using Osh6p as a case study. Finally, we show how to accurately measure the kinetics of PS/PI(4)P exchange between liposomes of defined lipid composition and to determine lipid transfer rates by FRET using a standard fluorometer.

INTRODUCTION:

Lipids are precisely distributed between different membranes and within the membranes of eukaryotic cells^{1,2}, which has profound biological implications. Decrypting how LTPs function is an important issue in cell biology³⁻⁶ and *in vitro* approaches are of great value in addressing this issue (*e.g.*,⁷⁻¹¹). Here, we present an *in vitro*, fluorescence-based strategy that has been instrumental in establishing that several ORP/Osh proteins execute PS/PI(4)P exchange between cell membranes¹², and thereby constitute a new class of LTPs.

PS is an anionic glycerophospholipid that represents 2-10 % of total membrane lipids in eukaryotic cells¹³⁻¹⁶. It is distributed along a gradient between the ER and the PM, where it represents 5-

7 % and up to 30 % of glycerophospholipids, respectively¹⁷⁻¹⁹. Moreover, PS is essentially concentrated in the cytosolic leaflet of the PM. This build-up and the uneven partition of PS in the PM are critical for cellular signaling processes¹⁹. Due to the negative charge of PS molecules, the cytosolic leaflet of the PM is much more anionic than the cytosolic leaflet of other organelles^{1,2,19,20}. This enables the recruitment, *via* electrostatic forces, of signaling proteins such as MARCKS²¹, Src²², K-Ras²³ and Rac1²⁴ that contain a stretch of positively-charged amino-acids and a lipidic tail. PS is also recognized by conventional PKC in a stereo-selective manner *via* a C2 domain²⁵.

However, PS is synthesized in the ER²⁶, indicating that it must be exported to the PM before it can play its role. It was not known how this was accomplished¹⁹ until the finding that, in yeast, Osh6p and Osh7p transfer PS from the ER to the PM²⁷. These LTPs belong to an evolutionarily conserved family in eukaryotes whose founding member is OSBP and which contain proteins (ORPs in human, Osh proteins in yeast) that all contain an OSBP-related domain (ORD) with a pocket to host a lipid molecule. Osh6p and Osh7p consist only of an ORD whose structural features are adapted to specifically bind PS and transfer it between membranes. Nevertheless, how these proteins directionally transferred PS from the ER to the PM was unclear.

We found that Osh6p and Osh7p can trap PI(4)P as an alternative lipid ligand¹². In yeast, PI(4)P is prominent in the Golgi and plasma membranes where it is synthesized from phosphatidylinositol (PI) by PI 4-kinases called Pik1p and Stt4p, respectively. In contrast, there is no PI(4)P in the ER membrane, as this lipid is hydrolyzed into PI by the Sac1p phosphatase. As a consequence, a PI(4)P gradient exists at both the ER/Golgi and ER/PM interfaces. We established that Osh6p and Osh7p transfer PS from the ER to the PM through PS/PI(4)P exchange cycles using the PI(4)P gradient that exists between these two membranes¹². Within one cycle, Osh6p extracts PS from the ER, exchanges PS for PI(4)P at the PM and transfers PI(4)P backward to the ER to extract another PS molecule. Osh6p/Osh7p interacts with Ist2p²⁸, one of the few proteins that connect and bring the ER membrane and the PM into close proximity with each other to create ER-PM contact sites in yeast²⁹⁻³¹. In addition, we uncovered that the association of Osh6p with negatively-charged membranes becomes weak as soon as the protein extracts one of its lipid ligands due to a conformational change that modifies its electrostatic features³². This aids Osh6p by shortening its membrane dwell time and thereby maintaining the efficiency of its lipid transfer activity. Combined with the binding to Ist2p, this mechanism could allow Osh6p/7p to both quickly and accurately execute lipid exchange at the ER/PM interface.

In human cells, ORP5 and ORP8 proteins execute PS/PI(4)P exchange at ER-PM contact sites but have distinct mechanisms³³. They have a central ORD, akin to Osh6p, but are directly anchored to the ER

via a C-terminal transmembrane segment³³ and dock to the PM via an N-terminal PH domain that recognizes PI(4)P and PI(4,5)P₂³³⁻³⁵. ORP5/8 use PI(4)P to transfer PS, and it has been shown that ORP5/8 additionally regulate PM PI(4,5)P₂ levels and presumably modulate signaling pathways. In turn, a diminution of PI(4)P and PI(4,5)P₂ levels downregulates ORP5/ORP8 since these proteins associate with the PM in a PIP-dependent fashion. Abnormally high PS synthesis, which leads to Lenz-Majewski syndrome, impacts PI(4)P levels through ORP5/8³⁶. When the activity of both proteins is blocked, PS becomes less abundant at the PM, lowering the oncogenic capability of signaling proteins³⁷. Conversely, ORP5 overexpression seems to promote cancer cell invasion and metastatic processes³⁸. Thus, alterations to ORP5/8 activity can severely modify cellular behavior through changes in lipid homeostasis. ORP5 and ORP8 also occupy ER-mitochondria contact sites and preserve some mitochondrial functions, possibly by supplying PS³⁹. ORP5 also localizes to ER-lipid droplet contact sites and might deliver PS to lipid droplets by PS/PI(4)P exchange⁴⁰.

Our strategy to measure (i) PS and PI(4)P extraction from liposomes and (ii) PS and PI(4)P transport between liposomes has been devised to establish and analyze the PS/PI(4)P exchange activity of Osh6p/Osh7p^{12,32} and used by other groups to analyze the activity of ORP5/ORP8³⁵ and other LTPs^{10,41}. It is based on the use of a fluorescence plate reader and a standard L-format spectrofluorometer and two fluorescent sensors, NBD-C2_{Lact} and NBD-PH_{FAPP} that are able to detect PS and PI(4)P, respectively. NBD-C2_{Lact} corresponds to the C2 domain of the glycoprotein, Lactadherin, that we reengineered to include a unique solvent-exposed cysteine near the presumed PS binding site; a polarity-sensitive NBD (7-nitrobenz-2-oxa-1,3-diazol) fluorophore is covalently linked to this residue (**Fig.1A**)¹². A major advantage is that this domain selectively recognizes PS in a Ca²⁺-independent manner contrary to other known C2 domains or Annexin A5⁴². NBD-PH_{FAPP} is derived from the pleckstrin homology (PH) domain of the human PI(4)P adapter protein (FAPP1). We reengineered this domain to include a single solvent-exposed cysteine, which can be labeled with an NBD group, near the PI(4)P binding site (**Fig.1A**)⁴³.

To measure PS extraction from liposomes, NBD-C2_{Lact} is mixed with liposomes made of phosphatidylcholine (PC) containing trace amounts of PS. Due to its affinity for PS, this construct binds to the liposomes and the NBD fluorophore experiences a change in polarity as it comes into contact with the hydrophobic environment of the membrane, which elicits a blue-shift and an increase in fluorescence. If PS is nearly completely extracted by a stoichiometric amount of LTP, the probe does not associate with liposomes and the NBD signal is lower (**Fig.1B**)³². This difference in signal is used to determine whether an LTP (*e.g.*, Osh6p) extracts PS. A similar strategy is used with NBD-PH_{FAPP} to measure PI(4)P extraction (**Fig.1B**), as described previously^{12,32}.

We also designed two FRET-based assays to (i) measure PS transport from L_A to L_B liposomes, which mimic the ER membrane and the PM, respectively, and (ii) PI(4)P transport in the backward direction. These assays are performed under the same conditions (*i.e.*, same buffer and temperature, identical lipids concentration) to measure PS/PI(4)P exchange. To measure PS transport, NBD-C2_{Lact} is mixed with L_A liposomes, made of PC and doped with 5 mol% PS and 2 mol% of a fluorescent lipid called Rhodamine-PE (Rhod-PE), and L_B liposomes, which incorporate 5 mol% PI(4)P. At time zero, the NBD fluorescence is quenched due to a FRET process with Rhod-PE. If PS is transported from L_A to L_B liposomes (*e.g.*, upon injecting Osh6p), a fast dequenching occurs due to the translocation of NBD-C2_{Lact} molecules from L_A to L_B liposomes (**Fig. 1C**). Given the amount of accessible PS, NBD-C2_{Lact} remains essentially in a membrane-bound state over the course of the experiment¹². Thus, the intensity of the NBD signal directly reflects the distribution of NBD-C2_{Lact} between L_A and L_B liposomes and can be easily normalized to determine how much PS is transferred.

To measure the transfer of PI(4)P in the opposite direction, NBD-PH_{FAPP} is mixed with L_A and L_B liposomes and, given that it only binds to L_B liposomes that contain PI(4)P but not Rhod-PE, its fluorescence is high. If PI(4)P is transferred to L_A liposomes, it translocates to these liposomes and the signal decreases due to FRET with Rhod-PE (**Fig. 1C**). The signal is normalized to determine how much PI(4)P is transferred⁴³.

PROTOCOL

1. Purification of NBD-C2_{Lact}

The C2 domain of Lactadherin (*Bos. Taurus*, Uniprot: Q95114, residue 270-427) was cloned into a pGEX-4T3 vector to be expressed in fusion with GST. The C2_{Lact} sequence was then mutated to substitute two solvent-accessible cysteines (C270, C427) with alanines and to introduce a cysteine into a region near the putative PS-binding site (H352C mutation)¹². A cleavage site for thrombin is present between the GST protein and the N-terminus of the C2 domain. Here, we detail a protocol relying on a Cell Disruptor to break bacteria but this protocol can be modified to use other lysis strategies (*e.g.*, a French press). At the beginning of the purification, it is mandatory to use buffer that is freshly degassed, filtered and supplemented with 2 mM DTT to prevent the oxidation of cysteine. However, for the protein labelling step, it is crucial to completely remove DTT. Many steps must be carried out on ice or in a cold room, to avoid any protein degradation. One must collect 30 μ L samples at different steps of the protocol to perform an analysis on a 15 % acrylamide SDS-PAGE gel to check the progress of the

purification. Mix a sufficient amount of denaturing Laemmli sample buffer with each aliquot and heat the mixture at 95 °C. Freeze and store the tubes at -20 °C until analysis.

1.1 Expression of GST-C2_{Lact} in *E. coli*

1. Mix 20 µL of BL21 Gold Competent Cells with 18 µL of sterilized water. Then, mix 2 µL of pGEX-C2_{Lact} plasmid (at ~ 65 ng/µL) with the bacteria and transform them by electroporation. Resuspend the bacteria with 150 µL of Lennox LB Broth medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl in deionized water, glucose-free) that has been previously autoclaved. Let the bacteria grow at 37 °C for 1 h in a 2 mL snap-cap microcentrifuge tube.
2. Inoculate 25 mL of LB medium, supplemented with 50 µg/mL ampicillin, with 150 µL of bacterial suspension in a 125 mL sterile Erlenmeyer flask.
3. Put the flask in an orbital shaker at 37 °C at 185 rpm and let the bacteria grow overnight.
4. Fill two sterile 2 L Erlenmeyer flasks with 500 mL of LB medium supplemented with 50 µg/mL ampicillin, and add 5 mL of pre-culture suspension. Let the bacteria grow at 37 °C under agitation at 220 rpm.
5. Measure the optical density of the suspension at a wavelength (λ) of 600 nm from time to time. When it reaches a value of around 0.6-0.7, add 500 µL of a stock solution of 1 M Isopropyl β -D-1-thiogalactopyranoside (IPTG) to each flask to initiate the expression of GST-C2_{Lact}. Shake the flasks at 185 rpm for 4 h at 37 °C.
6. Transfer the content of each flask in a polypropylene centrifuge bottle. Centrifuge the two bottles for 30 min at 3000 $\times g$ at 4 °C to pellet the bacteria cells.
7. Discard the supernatant and resuspend each pellet with 50 mL of cold PBS buffer. Transfer the bacterial suspension contained in each bottle in a 50 mL conical centrifuge tube.
8. Centrifuge the two tubes for 30 min at 3500 $\times g$ at 4 °C. Remove the supernatant and store the tubes, which each contains a bacterial pellet, at -20 °C.

1.2 Purification of C2_{Lact}

1. On ice, fill two 50 mL conical centrifugal tubes with 50 mL of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl buffer (thereafter called TN buffer), previously filtered and degassed by membrane vacuum filtration.
2. To prepare the lysis buffer in each tube, dissolve a tablet of cComplete EDTA-free protease

- inhibitor cocktail in the TN buffer by mild sonication or vortexing. Add other antiproteases, (10 μ M bestatin, 1 μ g/mL pepstatin A and 10 μ M mM phosphoramidon). Importantly, supplement the buffer with 2 mM DTT.
3. Fill the two tubes that contain the bacterial pellets prepared in step 8 of section 1.1, with lysis buffer, to reach a final volume of 30 mL in each tube. Slowly defrost the pellets on ice for 10 min. Crush each pellet with a stainless steel spatula and resuspend them by vortexing the tubes and/or by pipetting the suspension back and forth with a pipette controller and a 25 mL pipette until homogeneity.
 4. Lysis is performed using a pre-cooled Cell Disruptor (Dynamic System). Load 30 mL of sample inside the reservoir and run a breaking cycle in continuous mode with a pressure of 1.6 bar. Collect the lysate in the same tube, keep the tube on ice and immediately add 250 μ L from a stock solution of 200 mM PMSF (Phenylmethylsulfonyl fluoride, prepared in isopropanol).
 5. Lyse the other sample following the same procedure.
 6. The rest of the lysis buffer is used to wash the Cell Disruptor and is collected to adjust the volume of each lysate ($\sim$ 30 mL) to a final volume of 50 mL.
 7. Supplement each lysate with 5 mM MgCl_2 and add a pinch of DNase I in order to fragment the DNA and therefore to reduce the viscosity of the sample. Incubate on ice for 30 min. Collect a sample for gel analysis.
 8. Transfer each 50 mL lysate to a pre-chilled polycarbonate ultracentrifuge tube (two in total) suited for a Beckman Ti45 rotor. Centrifuge at $186,000 \times g$ at 4 $^{\circ}\text{C}$ for at least 1 h using a Beckman ultracentrifuge.
 9. In parallel to the centrifugation step, dispense 1.4 mL of Glutathione Sepharose 4B bead slurry into two 50 mL conical centrifugal tubes, add 20 mL of TN buffer supplemented with 1 mM DTT (TND buffer) in each tube, centrifuge at $500 \times g$ for 5 min and discard the supernatant. Repeat this washing step twice.
 10. Once the centrifugation of the bacterial lysate is completed, take a 30 μ L sample from the supernatant and transfer the supernatant from each ultracentrifuge tube into a corresponding 50 mL conical centrifuge tube that contains clean beads. For gel analysis, resuspend the debris pellet in one of the ultracentrifuge tubes with 50 mL of TND buffer and collect a 30 μ L sample.
 11. Place the tubes on a rotator to continually homogenize the beads suspension for 3-4 h at 4

220 °C.

221 12. Pool the bead suspensions in an empty 25 mL chromatography column. Let the beads

222 decant and remove the buffer and unbound proteins by gravity flow. Take a sample from

223 the eluate for analysis.

224 13. Resuspend the beads with 20 mL of TND buffer and collect the eluate by gravity flow.

225 Repeat this step twice to completely wash them. Pool the collected eluates and keep a 30 µL

226 sample for further analysis. After a short decantation, a volume of ~ 2 mL of bead

227 suspension, to which GST-C2_{Lact} is attached, sediments at the bottom of the column.

228 14. Add 1 mL of bead suspension into two 2 mL snap-cap microcentrifuge tube. Fill each tube

229 with TND buffer up to 1.970 mL. Take a 30 µL sample from one tube for further analysis (B1

230 sample). Add 10 µL of 10 mM CaCl₂ solution and 25 µL from a stock solution of human

231 thrombin protease solution at 0.02 U/µL.

232 15. Place the two tubes on a rotator at 4 °C overnight. The GST tag is cleaved off the C2_{Lact}

233 domain by the thrombin.

234 16. The next day, in each tube, mix 10 µL of 200 mM PMSF solution with the bead suspension to

235 inhibit the thrombin action.

236 17. Centrifuge the tubes at 700 × *g* for 5 min and collect the supernatant, which contains

237 soluble C2_{Lact} domain, from each tube, without taking beads. Pool the supernatants into a 2

238 mL snap-cap microcentrifuge tube (E1 eluate) that is kept on ice.

239 18. Add 1 mL of TN buffer to each tube to resuspend the beads and wash them, and repeat the

240 step 17. Perform this three times more to recover a maximum amount of protein. Each

241 time, pool the collected supernatants into a new 2 mL tube (E2, E3, E4 and E5 eluates) and

242 take an aliquot for further analysis. At the end of washing steps, take an aliquot from the

243 bead suspension (aliquot B2).

244 19. Analyze the 30 µL samples that were collected at the different steps of the purification

245 protocol by SDS-PAGE separation on a 15 % acrylamide gel.

246 20. Remove potential contaminating beads by pooling all the supernatants (*i.e.*, ~ 10 mL)

247 collected during the step 17 and 18 into a 10 mL chromatography column. The eluate is

248 collected by gravity flow and the beads are retained at the bottom of the column.

249 21. Concentrate the C2_{Lact} sample using an Ultra-4 centrifugal filter unit with a molecular weight

250 cut-off (MWCO) of 3 kDa and a centrifugation speed of 1500 × *g*. Stop the concentration

251 procedure when the volume of the protein sample is ~ 1 mL.

1.3 Preparation and purification of NBD-C2_{Lact}

1. Equilibrate a desalting column (*e.g.*, Illustra NAP 10) with TN buffer. Load the column with 1 mL of concentrated C2_{Lact} sample. Allow the sample to enter the Sephadex gel bed completely. Then, add 1.5 mL of freshly degassed DTT-free TN buffer to the column and collect the eluate by gravity flow into a 2 mL snap-cap microcentrifuge tube.
2. Dilute 50 µL of eluate in a final volume of 300 µL TN buffer and record an absorbance spectrum from 230 to 450 nm using pure TN buffer as a blank. Determine the C2_{Lact} concentration based on the absorbance measured at 280 nm, considering an extinction coefficient ϵ equal to $44,920 \text{ M}^{-1} \cdot \text{cm}^{-1}$
3. To label the C2_{Lact} construct with a NBD fluorophore, mix the protein with a ten-fold molar excess of IANBD Amide (N,N'-Dimethyl-N-(Iodoacetyl)-N'-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Ethylenediamine). Dissolve 1 mg of IANBD in anhydrous DMF (>99 % pure), keeping in mind that the final volume of DMF used for labeling the C2_{Lact} construct must not exceed 5% (v/v) of protein sample volume. To determine the volume of DMF (V_{DMF}) to dissolve IANBD, first calculate the required amount of IANBD (m , expressed in mg) to label the protein, by considering $m = 10,000 \times C \times V \times \text{MW}_{\text{IANBD}}$. C is the concentration of C2_{Lact} measured in step 2, V is the volume of the C2_{Lact} sample and MW_{IANBD} is the molecular weight of the fluorophore (420 g/mol). Then calculate V_{DMF} considering that $V_{\text{DMF}} = (m_0/m) \times V_{\text{IANBD}}$ with $m_0 = 1$ and $V_{\text{IANBD}} = 0.05 \times V$. m_0 is the quantity of IANBD powder in mg and V_{IANBD} is the volume of IANBD solution to be added to the C2_{Lact} sample.
4. Add a volume V_{IANBD} of the freshly-prepared IANBD solution to C2_{Lact} sample and shake the reaction mixture at 800-900 rpm for 30 min at 25 °C using a thermomixer protected from light. Let the reaction complete for 90 min on ice. In the meantime, clean an Ultra-4 centrifugal filter unit (MWCO= 3 kDa) with 10 mL of TN buffer.
5. Add L-cysteine to the reaction mixture to inactivate free IANBD. L-cysteine must be in a 10-fold molar excess relative to IANBD.
6. Add 15 mL of TN buffer to NBD-C2_{Lact} solution.
7. Transfer the NBD-C2_{Lact} solution into the Ultra-4 centrifugal filter unit. Concentrate the sample to 2 mL to separate most of the free NBD from the protein by centrifugation at $1500 \times g$. Repeat this washing step two times.
8. Centrifuge the sample in a 2 mL snap-cap centrifuge tube for 10 min at $25,000 \times g$ at 4 °C to

- pellet potential aggregates and collect the supernatant.
9. Dilute 50 μL of eluate in a final volume of 300 μL of TN buffer. Record the absorbance spectrum from 230 to 650 nm using the eluate collected during the concentration procedure as a blank. Determine the NBD-C2_{Lact} concentration using the maximal absorbance at $\lambda=280$ and 495 nm, and extinction coefficients $\epsilon= 44,920 \text{ M}^{-1}.\text{cm}^{-1}$ (protein) and $25,000 \text{ M}^{-1}.\text{cm}^{-1}$ (NBD fluorophore). If the two concentration values are alike, this means that the C2_{Lact} construct is labeled at a 1:1 ratio with the NBD group.
 10. If the NBD-C2_{Lact} concentration estimated from the measurement of NBD absorbance exceeds the concentration estimated from Trp absorbance, repeat steps 6 and 7 to further remove free NBD.
 11. Add glycerol to the sample at a final concentration of 10 % (v/v) to cryo-protect the NBD-C2_{Lact} construct during flash-freezing. Measure the final protein concentration.
 12. Prepare 50 μL aliquots of protein in 0.5 mL snap-cap microcentrifuge tubes. Flash-freeze the tubes in liquid nitrogen and store them at -80°C .

2. Purification of NBD-PH_{FAPP}

The nucleotide sequence of the PH domain of the human FAPP protein (Uniprot: Q9HB20, segment [1-100]) has been cloned in a pGEX-4T3 vector to be expressed in tandem with a GST tag in *E.Coli*. PH_{FAPP} sequence has been modified to insert a unique cysteine within the membrane-binding interface of the protein that can be subsequently labeled with IANBD⁴³. Moreover, a nine-residue linker has been introduced between the thrombin cleavage site and the N-terminus of the PH domain to ensure accessibility to the protease. The procedure to produce and label PH_{FAPP} is identical to that of NBD-C2_{Lact} until step 7 of Section 1.3. From this step, follow the protocol that is described below:

1. After the concentration step, keep the 2 mL NBD-PH_{FAPP} sample at 4°C in the dark, before performing size-exclusion chromatography. This storage stage should not last more than one day.
2. Prior to size-exclusion chromatography, verify that there is no orange deposit at the bottom of the tube. Indeed, some aggregation can occur during the concentration step. If this is the case, centrifuge the sample at $540,000 \times g$ for 10 min at 4°C . The supernatant can be then purified by size-exclusion chromatography.
3. Size-exclusion chromatography is performed on a HR XK16–70 column (GE Healthcare) packed

with Sephacryl S200 HR (120 mL, GE Healthcare), which has been previously equilibrated with TND buffer, using a FPLC system (*e.g.*, ÄKTA Purifier). The column must be protected from light. A flow rate of 1 mL/min is used and the elution of NBD-PH_{FAPP} construct is followed by recording the absorbance at $\lambda=280$ (protein) and 480 nm (NBD) at the column exit.

4. Inject the NBD-PH_{FAPP} sample, loaded in a 2 mL injection loop, on the column and immediately collect 2.5 mL fractions of eluate. A main peak, which is simultaneously detected at $\lambda=280$ and 480 nm, appears once a volume of ~ 150 mL buffer is passed through the column. Analyze all the fractions that correspond to this peak on a 15 % SDS-PAGE gel: mix 25 μ L sample of each fraction with 15 μ L of Laemmli sample buffer prior to heating and loading onto the gel.
5. Pool the fractions that exclusively contain NBD-PH_{FAPP} protein (~ 12.2 kDa) and add glycerol at a final concentration of 10 % (v/v). Concentrate the sample using an Ultra-15 centrifugal filter unit with a MWCO of 3 kDa to a final volume of 1 mL using a centrifugation speed of $1500 \times g$.
6. Then prepare aliquots and record an absorbance spectrum as described for NBD-C2_{Lact}. Use an extinction coefficient $\epsilon = 29,450 \text{ M}^{-1} \cdot \text{cm}^{-1}$ to determine the concentration of the protein based on the absorbance measured at $\lambda=280$ nm.

3. Preparation of liposomes for PS and PI(4)P extraction or transfer assays

Perform all the steps at room temperature unless otherwise specified. Handle organic solvents, rotavapor and liquid nitrogen with caution.

1. Prepare fresh, filtered and degassed 50 mM HEPES-KOH, pH 7.4, 120 mM K-Acetate (HK) buffer.
2. For each sort of liposomes, take a precise amount of different lipids from stock solutions and mix them in a 25 mL pear shape glass (see **Table 1**). Add pure CHCl₃ to adjust the volume of each mixture to 1 mL.
3. Label each flask with the liposome name. Wrap the flasks containing a lipid mixture doped with Rhod-PE with aluminum foil.
4. Place the flask on a rotary evaporator. Dry the lipids under vacuum and at 25 °C for a minimal time of 30 min at a rotation speed of 500 rpm. For lipid films that contain PI(4)P, pre-warm the flask at 32-34 °C for 5 min, under a gentle rotation, in order to properly mix PI(4)P with the other lipids before creating vacuum in the flask to remove the solvent.
5. A film of dry lipids will appear on the flask wall. Disconnect the flask from the evaporator and put it in a vacuum chamber for 45 min. This allows for the removal of any remaining trace of solvent.

6. Fill the flask with 2 mL of HK buffer and add a few 4 mm-diameter glass beads to the solution. Gently vortex the flask for 2 min to resuspend the lipids and make multilamellar lipid vesicles (MLVs). The lipid concentration is equal to 4 mM. Prepare 0.5 mL aliquots of MLVs in 1.5 mL screw-cap microcentrifuge tubes.
7. Freeze and thaw the tubes 5 times (using liquid nitrogen and a water bath at 37 °C, respectively). Liposomes can then be extruded or stored at -20 °C.
8. Use a mini extruder to prepare liposomes (*i.e.*, large unilamellar vesicles (LUVs)) from MLVs according to the manufacturer's guidelines. Use a polycarbonate filter with uniform cylindrical pores of 200-nm diameter. To prepare each type of liposome, extrude at least 250 µL of the corresponding suspension of MLVs. Store extruded liposomes at 4 °C and in the dark if they contain Rhod-PE. Liposomes can be used within 2 days.

[Insert Table 1 here]

4. Measurement of PS or PI(4)P extraction

Measurements are conducted using a black 96-well plate and a fluorescence plate reader (*e.g.*, TECAN Infinite M1000Pro) equipped with monochromators: one for fluorescence excitation, and one for emission, with a variable bandwidth.

1. Prepare fresh, filtered and degassed HK buffer supplemented with 1 mM MgCl₂ (HKM buffer).
2. Prepare pure PC liposomes and PC liposomes doped with 2 mol% PS or 2 mol% PI(4)P (4 mM final lipid concentration, see **Table 1**).
3. Maintain the tubes filled with the suspension of extruded liposomes at room temperature throughout the experiment. Keep the proteins on ice. Additionally, protect the lipid sensors from light.
4. For the PS extraction assay, in one well, mix liposomes containing 2 mol% PS (80 µM final lipid concentration, 0.8 µM accessible PS concentration) with NBD-C2_{Lact} (250 nM final concentration) in a final volume of 100 µL.
5. Fill a second well with the same amount of liposome (80 µM, 2 mol% PS) and NBD-C2_{Lact} (250 nM) mixed with 3 µM LTP (Osh6p as a positive control or a protein of interest). An incubation time of 5 min is sufficient for Osh6p to achieve lipid extraction.
6. Fill a third well with NBD-C2_{Lact} (250 nM) mixed with pure PC liposomes (80 µM).
7. Fill a fourth well only with pure PC liposomes (80 µM).
8. Repeat the step 4-7 to prepare three additional series of four wells.

9. For each well, record a NBD spectrum from 505 to 650 nm (bandwidth 5 nm) upon excitation at 490 nm (bandwidth 5 nm) at 25 °C.
10. For each series, subtract the spectrum recorded with liposomes only from the other spectra.
11. The intensity at 536 nm measured with PS-containing liposomes in the presence or absence of LTP corresponds to F and $F_{\max}$, respectively, whereas the intensity measured at the same wavelength with pure PC liposomes corresponds to F_0 . For each series, the percentage of accessible PS that is extracted by the protein is given by using the formula $100 \times (1 - ((F - F_0)/(F_{\max} - F_0)))$.
12. For the PI(4)P extraction assay, liposomes doped with 2 mol% PI(4)P are prepared and measurements are done with NBD-PH_{FAPP} probe. Liposome and protein concentration are identical to those used in the PS extraction assay. Control experiments and determination of extraction percentage are performed in the same way.

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366 **5. Real-time measurement of PS transport**

367 A standard fluorimeter (90° format) equipped with a temperature-controlled cell holder and a
 368 magnetic stirrer is used to record lipid transfer kinetics (*e.g.*, a Jasco FP-8300). To accurately acquire
 369 data, it is key to permanently maintain the sample at the same temperature (set between 25 and 37 °C
 370 depending on the origin of the protein (*e.g.*, yeast or human)) and to constantly stir it. The protocol
 371 described below is for the measurement of lipid transport in a 600 µL sample contained in a cylindrical
 372 quartz cell.

- 373 1. Prepare freshly degassed and filtered HKM buffer.
- 374 2. Keep the tubes containing extruded liposomes at room temperature. Tubes that contain
 375 liposomes with Rhod-PE must be wrapped with aluminum foil and/or stored in an opaque box to
 376 prevent any photobleaching.
- 377 3. Adjust the excitation and emission monochromators at $\lambda = 460$ nm (with a short bandwidth (1-3
 378 nm)) and at $\lambda = 530$ nm (with a large bandwidth (≥ 10 nm)), respectively. The acquisition time
 379 must be set at 25 min with a time resolution ≤ 1 s.
- 380 4. In the quartz cuvette, dilute 30 µL of the L_A liposome suspension and a volume of NBD-C2_{Lact}
 381 stock solution in pre-warmed HKM buffer to prepare a 570 µL sample that contains 200 µM total
 382 lipids and 250 nM NBD-C2_{Lact}. Add a small magnetic stirring bar and position the cuvette in the
 383 fluorometer holder.
- 384 5. Once the sample is thermally equilibrated (after 3-5 min), trigger the measurement. After 1 min,

add 30 μ L of L_B liposomes suspension (200 μ M total lipids, final concentration) to the sample.

- After 3 min, inject LTP into the sample so that the final concentration of the LTP is 200 nM. Acquire the signal for the remaining 21 minutes.

- Carry out a parallel experiment in order to normalize the NBD signal. Mix 30 μ L of L_{A-Eq} liposome suspension with 250 nM NBD-C2_{Lact} in HKM buffer (final volume 570 μ L). After 1 min, inject 30 μ L of L_{B-Eq} liposome suspension. The lipid composition of L_{A-Eq} and L_{B-Eq} liposomes are similar to that of L_A and L_B liposomes used in the transfer assay, except that each of them contains 2.5 mol% PS and 2.5 mol% PI(4)P. As a result, the NBD signal that is measured, referred to as F_{Eq} , corresponds to the signal that should be measured if PS was fully equilibrated between L_A and L_B liposomes by a transfer process.

- Convert the kinetic curves measured with an LTP of interest to determine the amount of PS (in μ M) transferred from L_A to L_B liposomes over time. Normalize each data point (F) of the curve by using the formula $F_{Norm} = (F - F_0) / (F_{Eq} - F_0)$ in which F_0 corresponds to the NBD signal just before the addition of an LTP and F_{Eq} is the signal measured in step 7. The amount of PS (in μ M) transferred from L_A to L_B liposomes corresponds to $2.5 \times F_{Norm}$ considering that the equilibrium corresponds to a situation where one half of accessible PS molecules, contained in the outer leaflet of the L_A liposomes, (*i.e.*, corresponding to 5 mol% of $0.5 \times 200 \mu$ M total lipids) has been transferred into L_B liposomes.

5. Real-time measurement of PI(4)P transport

- Set the fluorimeter (excitation and emission wavelength, bandwidth, acquisition time, time resolution) as for PS transfer assay. Likewise, use the same buffer, cuvette and liposomes and perform the experiments under constant stirring at the same temperature.
- In the cuvette, mix 30 μ L of L_B liposome suspension and NBD-PH_{FAPP} with pre-warmed HKM buffer to a final volume of 570 μ L (200 μ M total lipids, 250 nM NBD- PH_{FAPP}).
- Once the thermal equilibration of the sample is reached, start the measurement and after 1 min, inject 30 μ L of L_A liposome suspension. After 3 min, inject the LTP of interest (200 nM final concentration) and record the signal.
- Perform a second experiment to normalize the NBD signal. Mix 30 μ L of L_{B-Eq} liposome suspension with 250 nM NBD-PH_{FAPP} in 570 μ L of HKM buffer. After 1 min, inject 30 μ L of L_{A-Eq} liposome suspension. Here, the NBD signal, referred to as F_{Eq} , corresponds to the one which should be measured if PI(4)P was fully equilibrated between L_A and L_B liposomes.

5. Convert the kinetic curves to determine the amount of PI(4)P (in μM) transferred from L_B to L_A liposomes over time. Each data point (F) is normalized by using the formula $F_{\text{Norm}} = (F - F_0)/(F_{\text{Eq}} - F_0)$ in which F_0 corresponds to the NBD signal prior to the addition of an LTP and F_{Eq} is the signal measured in step 4. The amount of PI(4)P (in μM) transferred from L_A to L_B liposomes corresponds to $2.5 \times F_{\text{Norm}}$ considering that the equilibrium corresponds to a situation where one half of the PI(4)P contained in the outer leaflet of the L_B liposomes (*i.e.*, $0.5 \times 5 \mu\text{M}$) has been transferred in L_A liposomes.

5. Analysis of kinetics curves

A convenient approach to quantify the extent to which an LTP is efficient is to determine the speed at which this LTP transfers lipids from one liposome population to the other one in the very first seconds following its injection to the cuvette. This initial transfer rate is given (i) by performing a linear regression of the first data points of the transfer kinetics to obtain a slope (ii) then by dividing the slope value by the LTP concentration in the reaction mixture in order to determine the number of lipid molecules transferred *per protein per* time unit (min or s).

REPRESENTATIVE RESULTS

Figure 2A shows an SDS-PAGE analysis of the different steps leading to the purification of $C2_{\text{Lact}}$. Lane 1 shows the protein profile of lysed bacteria expressing GST- $C2_{\text{Lact}}$ ($\sim 44.8 \text{ kDa}$) whereas lane 2 and 3 respectively show the protein profile of the supernatant and bacterial debris after ultracentrifugation. The comparison of these lanes shows that GST- $C2_{\text{Lact}}$ has been recovered in the supernatant, and thus can be isolated on Glutathione Sepharose beads. Lane 4 and 5 show the protein profile of the supernatant, once incubated with beads, and washing buffers, which have been all recovered by gravity flow, whereas lane 6 shows the profile of proteins that have been retained on the beads. Analyzing these lanes indicates that almost all GST- $C2_{\text{Lact}}$ has been isolated on the beads. Lanes 8-12 show the presence of a major band corresponding to $C2_{\text{Lact}}$ ($\sim 17.9 \text{ kDa}$) in the supernatants recovered through successive washes of the beads after the thrombin treatment. Lane 13 indicates that non-cleaved GST- $C2_{\text{Lact}}$, along with GST ($\sim 26.9 \text{ kDa}$), remained bound to beads after this treatment. The comparison of these lanes indicates that the cleavage procedure, albeit not 100 % efficient, has led to the formation of $C2_{\text{Lact}}$ that can be then fluorescently-labelled. **Figure 2B** shows an UV-visible absorbance spectrum of $C2_{\text{Lact}}$ labelled with NBD. As the construct is 100 % pure, one can establish that all $C2_{\text{Lact}}$ molecules are labelled with a NBD group based on the

optical density measured at 280 nm (Trp) and 495 nm (NBD). The purity of NBD-C2_{Lact} and its fluorescence are revealed by an SDS-PAGE analysis (**Figure 2C**).

[Insert Figure 2]

Figure 3 shows results from PS and PI(4)P extraction assays using Osh6p. When only incubated with liposomes containing 2 mol% PS, the fluorescence of NBD-C2_{Lact} is maximal, as the NBD fluorophore is inserted in the membrane (*i.e.*, a hydrophobic context), indicating that the sensor is membrane-bound. In the presence of Osh6p, the fluorescence must be lower and comparable to that measured with pure PC liposomes (**Figure 3A**). The normalization of intensity values at 536 nm indicate that ~75 % of accessible PS is extracted. In the second assay, NBD-PH_{FAPP} is mixed with liposomes containing 2 mol% PI(4)P. Without Osh6p, the NBD signal is high whereas it must be low when Osh6p is present, and similar to that measured with PI(4)P-free liposomes (**Figure 3B**). Intensity analysis reveals that ~ 100 % of accessible PI(4)P is extracted by the LTP.

[Insert Figure 3]

Figure 4A shows typical results from a PS transfer assay using Osh6p as an LTP. At time zero, NBD-C2_{Lact} is mixed with L_A liposomes containing 5 mol% 16:0/18:1-PS (POPS) and 2 mol% Rhod-PE in a volume of 570 µL of HKM buffer at 30 °C. As the probe is bound to L_A liposomes, its signal is quenched due to FRET with Rhod-PE that is contained in these liposomes. After one minute, Rhod-PE-free L_B liposomes (30 µL) are added: this should only provoke a slight change in the signal due to light diffusion by this second liposome population and/or a dilution effect. The signal intensity after the addition of the L_B liposomes corresponds to F₀. The injection of a few µL of a stock solution of Osh6p (typically 40 µM) to dilute the protein at 200 nM final concentration in the reaction mix elicits a slow increase of the NBD signal, due to the dequenching of the fluorophore as PS is transported from L_A to L_B liposomes, thereby promoting the translocation of NBD-C2_{Lact}. When L_B liposomes contain 5 mol% PI(4)P, the dequenching is much faster, as PS is transferred more rapidly by Osh6p to L_B liposomes due to the counter-exchange of PS with PI(4)P by the LTP (second curve). The third curve corresponds to an experiment in which NBD-C2_{Lact} is mixed with an equal amount of L_{A-Eq} and L_{B-Eq} liposomes. The signal is higher than F₀ and corresponds to a situation where the probe is evenly bound to L_A and L_B liposomes, thus reflecting a situation where PS is fully equilibrated between the two populations of liposomes. F_{Eq} is calculated by averaging the value of the signal measured in the last 5 min of the experiment.

[Insert Figure 4]

Figure 4B shows average kinetic curves of PS transfer from L_A liposome to L_B liposomes doped or not with PI(4)P after the normalization of F data using F_0 and F_{Eq} as reference values. The initial transport rate for each experiment is calculated by fitting the first data points measured after the injection of the protein with a linear function. **Figure 4C** shows the mean initial PS transport rate determined from three distinct experiments using L_B liposomes with or without PI(4)P. When L_B liposomes contain 0 and 5 mol% PI(4)P, the rates are respectively equal to 1.4 and 15.8 $PS \cdot min^{-1}$ per Osh6p.

Figure 5A shows typical results from a PI(4)P transfer assay, using Osh6p as an LTP, which is carried out with the same material and conditions as those used for the PS transport assay. At time zero, NBD-PH_{FAPP} is mixed with L_B liposomes containing 5 mol% PI(4)P in a volume of 570 μL of HKM buffer. Because NBD-PH_{FAPP} is bound to L_B liposomes, its signal is high. After one minute, L_A liposomes (30 μL) are added: this should only provoke a slight change in the signal. The intensity then corresponds to F_0 . Injecting Osh6p (200 nM final concentration) into the reaction mix triggers a quenching of the NBD signal, due to the translocation of NBD-PH_{FAPP} molecules to L_A liposomes as PI(4)P is transferred from L_B to L_A liposomes. When L_A liposomes contain 5 mol% POPS, the dequenching is much faster, due to a faster PI(4)P transfer resulting from PS/PI(4)P exchange. The third curve corresponds to an experiment in which NBD-PH_{FAPP} is mixed with an equal amount of L_{A-Eq} and L_{B-Eq} liposomes. The signal must be lower than F_0 as it corresponds to a situation where the probe is uniformly bound to L_A and L_B liposomes, thus indicative of a full equilibration of PI(4)P between the liposomes. F_{Eq} is calculated by averaging the value of the signal measured in the last 5 min of the experiment. **Figure 5B and 5C** respectively show averaged kinetics curves obtained after signal normalization and mean PI(4)P initial transfer rates measured with L_A liposomes that are doped or not with 5 mol% PS.

[Insert Figure 5]

Here, it is worth noting that the two lipid ligands are transported faster, with similar velocities, when each lipid is initially present in L_A and L_B membranes, respectively, which indicates that Osh6p is a PS/PI(4)P exchanger.

DISCUSSION:

The outcome of our assays directly relies on the signal of the fluorescent lipid sensors. Thus, a

critical step is to purify these probes labelled at a 1:1 ratio with NBD and without free NBD fluorophore contamination. It is also mandatory to check whether the LTP under examination is properly folded and not aggregated.

The amount of LTP tested in the extraction assays must be equal or superior to that of accessible PS or PI(4)P molecules to properly measure whether this LTP efficiently extracts these lipids. Indeed, NBD-C2_{Lact} and NBD-PH_{FAPP} bind to PS and PI(4)P, respectively, according to a classical saturation binding curve. Given their respective affinity for PS and PI(4)P, these probes remain largely bound to the liposomes even if the liposomes contain residual traces of these ligands. This can lead one to erroneously conclude that an LTP does not efficiently extract these lipids. The protocol relies on standard and commercially available PC, PS and PI(4)P subspecies that are 18:1/18:1-PC, 16:0/18:1-PS and 16:0/16:0-PI(4)P, respectively. Performing experiments using lipid species with other acyl chains can give different results, as previously seen^{12,35}. In the transfer assays, if the bulk lipid composition (not considering PS or PI(4)P) of L_A and L_B liposomes has to be modified, it is key to also perform the control experiments using L_{A-Eq} and L_{B-Eq} liposomes with a bulk composition similar to that of L_A and L_B liposomes, respectively. The same principle applies for the extraction assays.

Genetically-encoded fluorescent lipid-binding domains are broadly used to analyze lipid distribution inside cells⁴⁴. Caution is taken when analyzing such experiments as the association of these lipid probes with membrane, although primarily driven by the presence of the targeted lipid, can be biased by other parameters (density of anionic lipids, lipid-packing) that differ between cell compartments. In our assays, the composition of liposomes is much simpler than that of cellular membranes: they are mostly made of PC, a zwitterionic lipid, and thereby expose a rather inert surface that does not impact how PS and PI(4)P are recognized by their corresponding sensor. We ascertained that the PH_{FAPP} can be used in the presence of anionic lipids like PS, phosphatidic acid (PA) and PI³² and that C2_{Lact} does not recognize PI(4)P¹², allowing unbiased measurements of PS/PI(4)P exchange. NBD-PH_{FAPP} is not influenced by sterol⁴³. However, we do not know whether these probes, in particular NBD-C2_{Lact}, can be properly used with liposomes with extreme features (*e.g.*, very low or high lipid-packing, highly negatively-charged surface, presence of lipid domains).

Despite this potential limitation in respect to liposome composition, our method based on fluorescent sensors has tremendous advantages compared to other methods. First, it does not rely on fluorescently-labeled PS and PI(4)P that bear an extra bulky group and are likely not properly accommodated by the binding pocket of LTPs. Second, our transport assays offer far better time resolution than methods based on liposome separation (radioactivity-based assays⁴⁵ or mass

spectrometry³³), and it is of note that radiolabeled PI(4)P and PS are not commercially available. The capacity of a protein to transfer PS or PI(4)P is not impacted by the association of the fluorescent sensors with liposomes. Indeed, if we consider the amount of NBD-C2_{Lact} or NBD-PH_{FAPP} and that of PS and PI(4)P in the outer leaflet of liposomes, accessible to the LTP, only 5 % of PS or PI(4)P are associated with probe during a kinetics measurement. Moreover, only 0.55-0.86 % of the membrane surface is covered by probe, considering the total surface of liposomes (L_A+L_B liposomes; $5.1 \times 10^{16} \text{ nm}^2$ with an area of 0.7 nm^2 per lipid), the membrane surface that one individual C2 or PH molecule can occupy (≈ 3.1 and 4.8 nm^2 , respectively, estimated from references⁴⁶⁻⁴⁸) and their concentration (250 nM). Thus, we consider that the principles underlying our assays are adapted to properly analyze kinetic and mechanistic aspects of LTPs.

As mentioned in the Introduction, alterations in the activity of ORP5/8 can lead to cellular dysfunctions⁴⁹. For example, invasiveness of pancreatic cancer cells seems to rely on the level of ORP5 expression. Moreover, a causality exists between high levels of ORP5 expression and the poor prognosis of human pancreatic cancer. A high level of ORP5 expression is also detected in lung tumor tissues, and more particularly in metastatic cases. Interestingly, the ability of ORP5 to transfer lipids might explain why it promotes cell proliferation and migration. ORP5 upregulates the mTORC1 complex, which plays a central role in cell survival and proliferation, likely as it enhances the activity of Akt, an upstream activator of mTORC1, by supplying the PM with PS. Overall, these observations suggest that ORP5 might be an interesting pharmacological target and our *in vitro* assays might serve to screen molecules that are able to inhibit its activity.

Our assay can also help to better define whether other members of the ORP/Osh family are PS/PI(4)P exchangers. Notably, ORP10 was found to encapsulate PS *in vitro* and transfer PS in ER-Golgi contact sites^{27,50}, but it is still unknown whether it acts as a PS/PI(4)P exchanger. Furthermore, our protocols can serve to explore the ability of LTPs belonging to other families to transport PI(4)P, as was recently shown with START-like proteins¹⁰, or PS. NBD-PH_{FAPP} has also been used to follow the ability of LTP to transport PI(4,5)P₂ between liposomes, as it recognizes this PIP^{10,35}. Finally, our strategy could be adapted to measure other extraction or transport processes *in vitro* by using other lipid-binding domains (*e.g.*, a non-catalytic PI-PLC to detect PI⁵¹, Spo20 fragment to detect PA⁵², domain 4 (D4) of Perfringolysin O to detect sterol⁵³).

FIGURE AND TABLE LEGENDS:

Figure 1. Description of the fluorescent lipid sensors and *in vitro* assays. (A) Tridimensional model of

NBD-C2_{Lact} and NBD-PH_{FAPP} respectively based on the crystal structure of the C2 domain of bovine Lactadherin (PDB ID: 3BN6⁴⁸) and the NMR structure of the PH domain of the human FAPP1 protein (PDB ID: 2KCJ⁴⁶). An N,N'-dimethyl-N-(thioacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine moiety, built manually and energetically minimized, is grafted to the thiol function of C352 (NBD-C2_{Lact}) and C13 (NBD-PH_{FAPP}) residues (represented in sphere, with carbon in green, nitrogen in blue, oxygen in red, sulfur in yellow, hydrogen in white). The surface of the lipid-binding site of each probe is colored in blue. The figure is prepared with PyMOL (<http://pymol.org/>). **(B)** Extraction assays. In the PS extraction assay, PC/PS liposomes (98/2 mol/mol) are incubated with 250 nM NBD-C2_{Lact}. In the absence of extraction, the probe strongly binds to liposomes, resulting in a blue-shift of NBD fluorescence and an increase of its emission intensity. If PS extraction occurs in the presence of an LTP (*e.g.*, Osh6p), the probe dissociates from the liposomes and its fluorescence is lower. In the PI(4)P extraction assay, liposomes are doped with 2 mol% PI(4)P and 250 nM NBD-PH_{FAPP} is used. **(C)** FRET-based lipid transport assays. In the PS transport assay, PC/PS/Rhod-PE liposomes (93/5/2 mol/mol, L_A) are incubated with 250 nM NBD-C2_{Lact}. PC liposomes, doped or not with 5 mol% PI(4)P, and Osh6p are added at t= 1 min and t= 4 min, respectively. If PS transport occurs, this elicits a dequenching of the NBD signal corresponding to the translocation of NBD-C2_{Lact} from L_A to L_B liposomes. In the PI(4)P transport assay, L_B liposomes doped with 5 mol% PI(4)P are incubated with 250 nM NBD-PH_{FAPP}. PC liposomes (L_A) doped or not with 5 mol% PS are added. If PI(4)P transport occurs, this provokes a quenching of the NBD signal due to the translocation of NBD-PH_{FAPP} from L_B to L_A liposomes.

Figure 2. NBD-C2_{Lact} purification. **(A)** SDS-PAGE analysis is used to check the presence of the protein at different steps of the purification procedure before labeling. The arrowheads indicate the position of the C2_{Lact} domain (red arrowhead), of the GST alone (grey arrowhead) and of the GST-C2_{Lact} construct (black arrowhead). MW: molecular-weight size marker **(B)** UV-visible absorbance spectrum of NBD-C2_{Lact}. **(C)** SDS-PAGE analysis of the purified NBD-C2_{Lact}. The first image is acquired under UV illumination without staining and reveals the presence of the NBD-C2_{Lact} construct since it emits fluorescence. The second picture shows the same gel after a SYPRO Orange staining procedure.

Figure 3. Extraction assays. **(A)** Fluorescence spectra of NBD-C2_{Lact} (250 nM) measured upon excitation at 460 nm in the presence of liposomes (80 μM, 2 mol% PS) in the absence or presence of 3 μM Osh6p; reference spectra are recorded with pure PC liposomes incubated or not with NBD-C2_{Lact} (left panel). Several spectra are recorded from different series of wells, corrected by subtracting the background

scattering signal from DOPC liposomes alone and averaged ($n=4$, $\pm$ s.e.m). The percentage of accessible PS that is extracted is indicated (right panel). **(B)** Fluorescence spectra of NBD-PH_{FAPP} (250 nM) mixed with liposomes (80 μ M, 2 mol% PI(4)P) in the absence or presence of 3 μ M Osh6p. Reference spectra recorded with PC liposomes in the presence and absence of the sensor are represented (left panel). Several spectra are recorded from different series of wells, corrected by subtracting the background scattering signal from DOPC liposomes alone and averaged ($n=4$, $\pm$ s.e.m.). The percentage of accessible PI(4)P that is extracted is indicated (right panel).

Figure 4. Typical PS transport kinetics measured with Osh6p and reference curve. (A) L_B liposomes devoid of PI(4)P (200 μ M total lipids) and Osh6p (200 nM) are sequentially added to a cuvette containing NBD-C2_{Lact} (250 nM) and L_A liposomes (200 μ M) doped with 5 mol% PS and 2 mol% Rhod-PE (left curve). The same experiment is done with L_B liposomes doped with 5 mol% PI(4)P (middle curve). To determine F_{Eq}, NBD-C2_{Lact} (250 nM) is pre-mixed with L_{A-Eq} liposomes, then L_{B-Eq} liposomes are added (right curve). **(B)** Averaged PS transport curves determined after the normalization of several measurements done using L_B liposomes with or without PI(4)P (mean $\pm$ s.e.m., $n=3$). **(E)** Initial PS transfer rates (mean $\pm$ s.e.m., $n=3$).

Figure 5. Typical PI(4)P transport kinetics measured with Osh6p and reference curve. (A) PS-free liposomes containing 2 mol% Rhod-PE (L_A, 200 μ M total lipids) and Osh6p (200 nM) are sequentially added to a cuvette containing NBD-PH_{FAPP} (250 nM) and L_B liposomes (200 μ M) doped with 5 mol% PI(4)P (left curve). The same experiment is done with L_A liposomes doped with 5 mol% PS (middle curve). To determine F_{Eq}, NBD-PH_{FAPP} (250 nM) is pre-mixed with L_{B-Eq} liposomes, then L_{A-Eq} liposomes are added (right curve). **(B)** PI(4)P transfer kinetics determined after a normalization of several measurements of L_A liposomes with or without PS (mean $\pm$ s.e.m., $n=3$). **(C)** Initial PI(4)P transfer rates (mean $\pm$ s.e.m., $n=3$).

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DISCLOSURES:

The authors declare there is no conflicts of interest.

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Table 1. Volume of lipid stock solutions to be mixed for liposome preparation

	Liposome name	Lipid composition (mol/mol)	Lipid			
			DOPC (25 mg/mL)	POPS (10 mg/mL)	16:0 Liss Rhod-PE (1 mg/mL)	C16:0/C16:0- PI(4)P (1 mg/mL)
Extraction assays	Liposome 2 mol% PS	PC/PS 98/2	247 µL	12.5 µL		
	Liposome 2 mol% PI(4)P	PC/PI(4)P 98/2	247 µL			153 µL
	DOPC liposome	PC 100	252 µL			
Transport assays	L _A	PC/PS/Rhod-PE 93/5/2	234 µL	31.4 µL	200 µL	
	L _A without PS	PC/Rhod-PE 98/2	247 µL		200 µL	
	L _B	DOPC/PI(4)P 95/5	237 µL			383 µL
	L _B without PI(4)P	PC 100	252 µL			
	L _A -Eq	DOPC/PS/PI(4)P/Rhod- PE 93/2.5/2.5/2	234 µL	15.7 µL	200 µL	191 µL
	L _B -Eq	DOPC/PS/PI(4)P 95/2.5/2.5	239 µL	15.7 µL		191 µL

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