Published in final edited form as:

Science. 2015 July 24; 349(6246): 428–432. doi:10.1126/science.aab1370.

PI4P/phosphatidylserine countertransport at ORP5- and ORP8-mediated ER–plasma membrane contacts

Jeeyun Chung¹, Federico Torta², Kaori Masai¹, Louise Lucast¹, Heather Czapl¹, Lukas B. Tanner^{2,*}, Pradeep Narayanaswamy², Markus R. Wenk², Fubito Nakatsu^{1,†}, and Pietro De Camilli¹

Fubito Nakatsu: pietro.decamilli@yale.edu; Pietro De Camilli: nakatsu@med.niigata-u.ac.jp

¹Department of Cell Biology, Howard Hughes Medical Institute, Kavli Institute for Neuroscience, and Program for Cellular Neuroscience, Neurodegeneration, and Repair, Yale School of Medicine, New Haven, CT 06520, USA

²Department of Cell Biology, Howard Hughes Medical Institute, Kavli Institute for Neuroscience, and Program for Cellular Neuroscience, Neurodegeneration, and Repair, Yale School of Medicine, New Haven, CT 06520, USA

Abstract

Lipid transfer between cell membrane bilayers at contacts between the endoplasmic reticulum (ER) and other membranes help to maintain membrane lipid homeostasis. We found that two similar ER integral membrane proteins, oxysterol-binding protein (OSBP)–related protein 5 (ORP5) and ORP8, tethered the ER to the plasma membrane (PM) via the interaction of their pleckstrin homology domains with phosphatidylinositol 4-phosphate (PI4P) in this membrane. Their OSBP-related domains (ORDs) harbored either PI4P or phosphatidylserine (PS) and exchanged these lipids between bilayers. Gain- and loss-of-function experiments showed that ORP5 and ORP8 could mediate PI4P/PS counter transport between the ER and the PM, thus delivering PI4P to the ER-localized PI4P phosphatase Sac1 for degradation and PS from the ER to the PM. This exchange helps to control plasma membrane PI4P levels and selectively enrich PS in the PM.

Membrane lipids can be exchanged between bilayers at contact sites between the endoplasmic reticulum (ER) and other membranes (1–7). One class of molecules mediating these contacts are oxysterol-binding proteins (OSBP) and the closely related OSBP-related

Correspondence to: Fubito Nakatsu, pietro.decamilli@yale.edu; Pietro De Camilli, nakatsu@med.niigata-u.ac.jp.

*Present address: Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA.

†Present address: Department of Neurochemistry and Molecular Cell Biology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8510, Japan.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/349/6246/428/suppl/DC1

Materials and Methods

Figs. S1 to S16

Table S1

References (34–39)

Movies S1 to S3

proteins (ORPs) (Osh proteins in yeast), which harbor lipids in a hydrophobic cavity of their OSBP-related domain (ORD) (fig. S1) (3, 7–13). Members of this protein family (more than 10 in mammals) have been thought to function selectively as sterol sensors or transport proteins (12, 13), but recent studies show that they can also harbor different lipids (3, 7, 9–11). OSBP and Osh4/Kes1 function in a lipid countertransport between the Golgi complex and membranes of the ER by delivering cholesterol to the Golgi in exchange for phosphatidylinositol 4-phosphate (PI4P), which is degraded by the Sac1 phosphatase in the ER (9, 14). Whether other ORPs also function in lipid countertransport reactions to help maintain membrane heterogeneity, such as a selective concentration of phosphatidylserine (PS) in the plasma membrane (PM) (15), is unclear. We focused on two very similar mammalian ORPs, ORP5 and ORP8, which are anchored to the ER membrane, where they reside via a hydrophobic tail sequence (12, 16, 17). Their ORDs are the mammalian ORDs most closely related to the ORDs of Osh6 and Osh7, which transport PS to the PM in yeast, although ORP5/8 and Osh6/7 are otherwise different in domain organization (fig. S2A) (3, 8, 11, 12).

Green fluorescent protein (GFP)–ORP5 and GFP-fusions of the two splice variants of ORP8 (17), which differ by the inclusion (ORP8L) or exclusion (ORP8S) of an N-terminal 42 amino acids sequence (fig. S5), were independently expressed in HeLa cells and analyzed by means of confocal microscopy. ORP5 predominantly accumulated in small patches at the cell periphery in a pattern reminiscent of ER-PM contacts (18, 19): a row of peripheral dots in mid-cell optical sections and tightly apposed patches in optical sections of the flat base of the cell (Fig. 1A). More numerous and longer ER-PM contacts were detected as a result of excess ORP5 expression (Fig. 1C to E). In contrast, GFP-ORP8L had a broad reticular distribution throughout the cell (Fig. 1A) that overlapped with that of the ER marker Sec61 β (fig. S3), with only a faint puncta at the cell periphery. GFP-ORP8S had a somewhat intermediate localization pattern (Fig. 1A). Coexpressed GFP-ORP5 and mCherry-ORP8L partially colocalized at the cell cortex (Fig. 1F), and ORP8L co-immunoprecipitated with ORP5 (Fig. 1G), indicating that ORP5 may help mediate ORP8 recruitment to ER-PM contacts via heteromerization.

Because both ORP5 and ORP8 contain a PH domain, we next investigated whether their tethering function depended on phosphoinositides in the PM. The cortical pool of GFP-ORP5 and GFP-ORP8S, and even the very weak cortical accumulation of GFP-ORP8L, increased upon overexpression of phosphatidylinositol 4-kinase III α (PI4KIII α) and its associated factors [the enzyme complex responsible for PI4P synthesis in the PM (20–23)] (Fig. 1A and B). In cells treated with the PI4KIII α inhibitor A1 (25), both ORP5 and ORP8S dissociated from the PM and dispersed throughout the ER (Fig. 1H and movies S1 and S2). This redistribution correlated with the dissociation from the PM of near-infrared fluorescent protein (iRFP)–P4M, a PI4P reporter (24), but not of PH_{PLC5}, a phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] reporter (movie S3) (25). Thus, PI4P is required for the binding of ORP5 and ORP8 to the PM.

ORP5 and ORP8L constructs lacking their PH domains were localized throughout the ER even upon coexpression of PI4KIII α (Fig. 1I and J, and fig. S4). Constructs lacking the transmembrane region or comprising the PH domain and upstream N-terminal sequences

mimicked the properties of the full-length proteins (cortical localization of ORP5 constructs and strong dependence on PI4KIII α overexpression for the cortical accumulation of ORP8L constructs) (Fig. 1I and J). Both PH domain-only constructs were similarly targeted to the PM and more prominently upon PI4KIII α overexpression (Fig. 1I and J). Thus, differences in the PM recruitment of ORP5 and ORP8L are dictated by their N-terminal amino acid sequences that differ substantially in the two proteins and confer an overall more negative charge to ORP8L. Most of this negative charge is accounted for by the ORP8L-specific extension (fig. S5), which explains the greater accumulation of ORP8S at ER-PM contacts. The ORD is dispensable for cortical localization (fig. S4).

The PI4P-dependent cortical accumulation of ORP5 was supported by studies of tamoxifen-inducible PI4KIII α -conditional knockout embryonic fibroblasts (MEFs) (20). In sham-treated MEFs, GFP-ORP5 was concentrated at ER-PM contacts, but in knockout (tamoxifen-treated) cells, in which global PI4P levels are 70% lower than in controls with dramatic loss of PMPI4P (20), GFP-ORP5 was dispersed throughout the ER (Fig. 2A). ORP5 and ORP8 expression was up-regulated in knockout MEFs, pointing to a functional link between ORP5/ ORP8 function and PI4KIII α (Fig. 2B). This phenotype, as well as the loss of cortical localization of ORP5, was rescued by expression of PI4KIII α in the knockout MEFs (Fig. 2A and B). PI4KIII α knockout MEFs also had lower PS levels (about 50% reduction) (Fig. 2C and fig. S6) but no major changes in levels of phosphatidylethanolamine (PE) and phosphatidylcholine (PC), the precursors of PS (Fig. 2C) (15, 26).

If ORP5 and ORP8 operate in a countertransport mechanism, they should contain more than one lipid in their ORD domains, which are the most similar to each other in their portion defining the lipid-harboring cavity (fig. S2A). Because only the ORD of ORP8 (amino acids 370 to 809) could be purified in sufficient yield (Fig. 3A), we focused on this domain. Mass spectrometry comparison between the denatured ORD [apo form, molecular weight (MW) 53,865 daltons] and the native ORD (mixture of native apo and holo forms) revealed two main ORD-lipid complexes: MW 54,654 daltons (apo form + 789 daltons) and MW 54,808 daltons (apo form + 943 daltons) (Fig. 3B). These mass increments correspond well to the mass of PS 36:1 (789.55 daltons) and phosphatidylinositol phosphate (PIP) 36:1 or 36:2 (944.5 or 942.5), respectively. PS and PIP were also found in a quantitative analysis of the lipid fraction extracted from the purified ORD construct (Fig. 3C and D). The PIP species presented in ORD_{ORP8} is most likely PI4P because the cluster of amino acids that mediate specific binding of PI4P in other ORDs (His¹⁴³, His¹⁴⁴, Lys³³⁶, and Arg³⁴⁴ in Osh4) (9, 10) is conserved in this ORD as well as in ORD_{ORP5} (fig. S2B and C). Other phospholipids, sphingolipids, and cholesterol were not detected in appreciable amounts, indicating that reported effects of ORP5 and ORP8 on cholesterol dynamics (16, 17) result from indirect actions of these proteins.

On the basis of these findings, we examined whether ORD_{ORP8} could exchange PS and PI4P between artificial bilayers in vitro. “Heavy” and “light” liposomes composed of 90% PC and either 10% PI4P or 10% PS, respectively, were incubated (27) with wild-type (WT) ORD_{ORP8}- and ORD_{ORP8}-harboring mutations in the PI4P binding motif (H514A, H515A) (fig. S2B) (9, 10) (or with no proteins as a control) and then separated by centrifugation.

(Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. In the mutants, other amino acids were substituted at certain locations; for example, H514A indicates that histidine at position 514 was replaced by alanine.) The supernatant (light liposomes) contained no PI4P in the absence of protein, 20% PI4P in samples incubated with WT ORD_{ORP8}, and much reduced PI4P levels in samples incubated with mutant ORD_{ORP8} (Fig. 3E). Conversely, a greater specific amount of PS was recovered in the heavy liposomes pellets when WT ORD_{ORP8} was used instead of H514A, H515A ORD_{ORP8} (Fig. 3E). A similar assay in which transfer of either PI4P or PS from heavy liposomes to light liposomes was tested in the presence or absence of the other lipid in the light liposomes demonstrated that presence of the other lipid accelerated transfer (fig. S7). This effect was more prominent for PS transport (fig. S7). Thus, PI4P/PS countertransport occurs in vitro.

We next investigated a potential role of ORP5/ ORP8 in a countertransport of PI4P and PS between the PM and the ER aimed at delivering PM PI4P to the ER for degradation by Sac1 (28) and PS from its site of synthesis in ER (15) to the PM. Overexpression of ORP5 reduced PI4P in the PM, as detected by the dissociation of the PI4P biosensor P4M (Fig. 4A and B). Residual PI4P in this membrane was implied by the PI4P-dependent cortical localization of ORP5 (Figs. 1A and 4A, inset) (binding of multiple ER-localized ORP5 molecules to PM PI4P likely accounts for increased avidity) and by the PM localization of a tandem PH_{ORP5} (fig. S8). Conversely, ORP5 overexpression also resulted in increase of PM-localized PS, as revealed by two PS biosensors, evt2-2XPH (tandem PH domains of evectin-2) (29) or LactC2 (C2 domain of lactadherin) (30), respectively (Fig. 4C and D, and fig. S9). These effects were not observed upon expression of ORP5-harboring mutations that impair the lipid-binding properties of its ORD domain: the double H478A, H479A mutation (impaired PI4 binding) (fig. S2B) (9) and the L to D mutation at position 389 (L389D) (11), which corresponds to a leucine critical for PS binding in yeast Osh6 (Leu⁶⁹) and Osh7 (fig. S2D) (11). In fact, these mutant constructs were even more enriched in the cortical ER than was WT ORP5 (fig. S10), most likely reflecting higher levels of PI4P in the PM. Knockdown of ORP5 and ORP8 resulted in prominent PM accumulation of N-PH_{ORP8}, which is normally retained in the cytosol and nucleus but relocates to the PM in response to increased PI4P in this membrane (Figs. 1J and 4E). Likewise, the PS sensor evt2- 2XPH was no longer enriched at the PM in ORP5/ORP8 double-knockdown cells (fig. S11). The requirement of PI4P in the PM for the delivery of PS to this membrane was supported by the lack of enrichment of the PS sensor evt2- 2XPH at the PM in PI4KIII α knockout MEFs (fig. S12).

Because ORP overexpression could result in indirect chronic effects, ORP-mediated PI4P/PS exchange at the ER-PM contacts was further analyzed by means of the acute PM recruitment of ORP constructs by using an FK506/rapamycin-inducible FK506 binding protein (FKBP)–FKBP12- rapamycin-binding (FRB) heterodimerization system (31). A monomeric red fluorescent protein (mRFP) fusion of ORP5 in which its PH domain had been replaced by FKBP12 (mRFP-FKBP- PH-ORP5) was transfected in HeLa cells along with a PM membrane bait [PM-FRB-CFP (32)] and together with PI4P and PS reporters (fig. S13). Upon the addition of rapamycin (fig. S13), this fusion protein was rapidly

recruited to the PM, leading to dissociation of iRFP-P4M (PI4P) but not of GFP-PH^{PLC8} [PI(4,5)P₂] and to PM recruitment of GFP-evt2-2XPH (PS) (Fig. 4F to H). Similar results were observed by replacing the ORP5 portion of the FKBP12 fusion proteins with the corresponding portion of ORP8 (Fig. 4I). In contrast, acute PM recruitment of FKBP12-ORP5 fusion proteins with mutations in the ORD that affect PI4P or PS binding (fig. S2B to E) had no effect on the localization of PI4P and PS reporters (Fig. 4I and fig. S14), and depletion of PI4P in the PM by prior A1 treatment nearly abolished PS increase in this membrane (Fig. 4J).

Physical tethering of the ER to the PM by mRFP-FKBP- PH-ORPs was required for the PI4P/PS exchange to occur. Thus, an mRFP-FKBP- PH-ORP5_{WT} construct in which the C-terminal transmembrane anchor had been deleted, mRFP-FKBP- PH-ORP5_{WT} TM (fig. S13), was recruited to the PM in response to rapamycin (much faster than the ER-anchored construct because this protein is a cytosolic protein) (fig. S15) but failed to dissociate the PI4P marker P4M (Fig. 4K) and to recruit the PS marker evt2-2XPH to the PM (Fig. 4J). Furthermore, the knockdown of Sac1 strongly reduced the dissociation of iRFP-P4M from the PM upon acute recruitment of mRFP-FKBP- PH-ORP5_{WT} (Fig. 4L), implicating PI4P consumption by Sac1 for efficient countertransport, most likely by allowing the ORD to favor PS binding over PI4P binding at the ER membrane interface. These findings support the importance of Osh/ORP-family proteins in the negative regulation of PM PI4P via an action that involve Sac1, as suggested (28), but favor an action of Sac1 “in cis” in ER membrane after delivery of PI4P to this membrane by the ORPs.

Collectively, our results demonstrate that ORP5 and ORP8 function as ER-PM tethers that, via a countertransport mechanism, negatively regulate PI4P in the PM (where the two ORPs are recruited in a PI4P-dependent way) and accounts, at least in part, for the enrichment of PS in this membrane (fig. S16). Thus, a PI4P-dependent countertransport mechanism first proposed to mediate cholesterol delivery from the ER to the Golgi complex by Osh4/OSBP (14) may apply to at least some other ORP/Osh family members [von Filseck et al. (33) provide a similar countertransport by Osh6/Osh7 in yeast], mediate export of another lipid from the ER, and operate at the contact sites of the ER with other membranes. Last, our results suggest a role for plasma membrane PI4P as a homeostatic regulation of PM lipid composition independent from its role as a precursor of PI(4,5)P₂.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Y. Cai, X. Wu, C. Schauder, Y. Saheki, J. Park, and X. Bin for discussion and critical technical suggestions; T. Melia for discussion and advice for lipid experiments; and F. Wilson for outstanding technical support. We thank T. Balla for the kind gift of A1 compound. Generous gifts of plasmids are acknowledged in the materials and methods section of the supplementary materials. This work was supported in part from grants from the NIH (DK082700, R37NS036251, DK45735, and DA018343) to P.D.C., grants from the National University of Singapore via the Life Sciences Institute and a Biomedical Research Council (BMRC)-Science and Engineering Research (SERC) joint grant (BMRC-SERC 112 148 0006) from the Agency for Science, Technology and Research to M.R.W., and grants from the Medical Research Internship program of Okayama University and Japan Student Service Organization to K.M.

REFERENCES AND NOTES

1. Stefan CJ, Manford AG, Emr SD. *Curr. Opin. Cell Biol.* 2013; 25:434–442. [PubMed: 23522446]
2. Holthuis JCM, Menon AK. *Nature.* 2014; 510:48–57. [PubMed: 24899304]
3. Moser von Filseck J, Mesmin B, Bigay J, Antonny B, Drin G. *Biochem. Soc. Trans.* 2014; 42:1465–1470. [PubMed: 25233433]
4. Toulmay A, Prinz WA. *Curr. Opin. Cell Biol.* 2011; 23:458–463. [PubMed: 21555211]
5. Levine T, Loewen C. *Curr. Opin. Cell Biol.* 2006; 18:371–378. [PubMed: 16806880]
6. Elbaz Y, Schuldiner M. *Trends Biochem. Sci.* 2011; 36:616–623. [PubMed: 21958688]
7. Beh CT, McMaster CR, Kozminski KG, Menon AK. *J. Biol. Chem.* 2012; 287:11481–11488. [PubMed: 22334669]
8. Raychaudhuri S, Prinz WA. *Annu. Rev. Cell Dev. Biol.* 2010; 26:157–177. [PubMed: 19575662]
9. de Saint-Jean M, et al. *J. Cell Biol.* 2011; 195:965–978. [PubMed: 22162133]
10. Tong J, Yang H, Yang H, Eom SH, Im YJ. *Structure.* 2013; 21:1203–1213. [PubMed: 23791945]
11. Maeda K, et al. *Nature.* 2013; 501:257–261. [PubMed: 23934110]
12. Olkkonen VM, Li S. *Prog. Lipid Res.* 2013; 52:529–538. [PubMed: 23830809]
13. Im YJ, Raychaudhuri S, Prinz WA, Hurley JH. *Nature.* 2005; 437:154–158. [PubMed: 16136145]
14. Mesmin B, et al. *Cell.* 2013; 155:830–843. [PubMed: 24209621]
15. Leventis PA, Grinstein S. *Annu. Rev. Biophys.* 2010; 39:407–427. [PubMed: 20192774]
16. Du X, et al. *J. Cell Biol.* 2011; 192:121–135. [PubMed: 21220512]
17. Yan D, et al. *J. Biol. Chem.* 2008; 283:332–340. [PubMed: 17991739]
18. Giordano F, et al. *Cell.* 2013; 153:1494–1509. [PubMed: 23791178]
19. Liou J, et al. *Curr. Biol.* 2005; 15:1235–1241. [PubMed: 16005298]
20. Nakatsu F, et al. *J. Cell Biol.* 2012; 199:1003–1016. [PubMed: 23229899]
21. Baird D, Stefan C, Audhya A, Weys S, Emr SD. *J. Cell Biol.* 2008; 183:1061–1074. [PubMed: 19075114]
22. Wu X, et al. *Dev. Cell.* 2014; 28:19–29. [PubMed: 24360784]
23. Balla A, Tuymetova G, Tsiomenko A, Várnai P, Balla T. *Mol. Biol. Cell.* 2005; 16:1282–1295. [PubMed: 15635101]
24. Hammond GRV, Machner MP, Balla T. *J. Cell Biol.* 2014; 205:113–126. [PubMed: 24711504]
25. Bojjireddy N, et al. *J. Biol. Chem.* 2014; 289:6120–6132. [PubMed: 24415756]
26. Vance JE, Steenbergen R. *Prog. Lipid Res.* 2005; 44:207–234. [PubMed: 15979148]
27. Schulz TA, et al. *J. Cell Biol.* 2009; 187:889–903. [PubMed: 20008566]
28. Stefan CJ, et al. *Cell.* 2011; 144:389–401. [PubMed: 21295699]
29. Uchida Y, et al. *Proc. Natl. Acad. Sci. U.S.A.* 2011; 108:15846–15851. [PubMed: 21911378]
30. Yeung T, et al. *Science.* 2008; 319:210–213. [PubMed: 18187657]
31. Muthuswamy SK, Gilman M, Brugge JS. *Mol. Cell. Biol.* 1999; 19:6845–6857. [PubMed: 10490623]
32. Varnai P, Thyagarajan B, Rohacs T, Balla T. *J. Cell Biol.* 2006; 175:377–382. [PubMed: 17088424]
33. Moser von Filseck J, et al. *Science.* 2015; 349:432–436. [PubMed: 26206936]

Fig. 1. PI4P-dependent accumulation of ORP5 and ORP8 at ER-PM contact sites
(A) Confocal images of the middle or of the basal PM focal planes (illustrations at top) of HeLa cells expressing GFP-ORP5, GFP-ORP8L, or GFPORP8S either alone (left) or together with the PI4KIII α complex (right). Scale bars, 10 μ m. **(B)** Ratio of GFP fluorescence visible in the total internal reflection fluorescence (TIRF) (basal PM-associated fluorescence) versus epifluorescence fields (total fluorescence) of cells transfected as in (A) (mean $\pm$ SEM; $P < 0.0001$, t test, $n = 8$ to 20 cells). **(C to E)** Increase of ER-PM contacts produced by GFP-ORP5 expression as revealed with electron microscopy and accompanying morphometric analysis: contact length per unit PM length in (D) (mean $\pm$ SEM; $P < 0.01$, t test, $n = 10$ cells) and number of contacts per PM length in (E) (mean $\pm$ SEM; $P < 0.001$, t test, $n = 10$ cells). Scale bar, 200 nm. **(F)** Confocal live imaging of HeLa

cells transfected with GFP-ORP5 and mCh-ORP8, showing partial recruitment of ORP8 under these conditions. Scale bar, 10 μ m. **(G)** Co-immunoprecipitation of 3XFLAG-ORP8 with GFP-ORP5 in HeLa cells. Asterisk points to a nonspecific band. **(H)** Dissociation from the PM of GFP-ORP5 but not of mRFP-PH_{PLC8} upon acute treatment of cells with the specific PI4KIII α inhibitor A1 (100 nM) induces. **(I)** ORP5 and ORP8 deletion constructs used for the experiments shown in (J). **(J)** Confocal live imaging of HeLa cells expressing the deletion constructs depicted in (I) and with a C-terminal GFP tag revealing that the PI4P-dependent PM recruitment of ORP5 and ORP8 is mediated by their PH domains. The smallest constructs show a partial accumulation in nuclei, as reported previously for other PH domains. Scale bars, 10 μ m.

Fig. 2. PI4KIII α KO cells shows defects in PS metabolism

(A) Confocal live imaging of control (top) or PI4KIII α KO MEFs (middle) coexpressing GFP-ORP5 and RFP-Sec61 β showing ER-PM contacts at base of the cell and their absence in knockout cells. mCh-PI4KIII α expression in the knockout cells rescues the phenotype (bottom). Regions enclosed by rectangles are shown at higher magnification in the insets. Scale bars, main fields, 10 μ m; insets, 2 μ m. (B) Immunoblot of the proteins indicated in lysates from control MEFs, PI4KIII knockout MEFs, and knockout MEFs expressing GFP-PI4KIII α . (C) Lipidomics analysis of PI4KIII α knockout and control MEFs (mean $\pm$ SEM; **** P < 0.0001, *** P < 0.001, t test, n = 3 cells).

Fig. 3. Detection of PS and PIP in the ORD of ORP8

(A) Coomassie blue-stained SDS-polyacrylamide gel electrophoresis gel of purified 3XFLAG-ORD_{ORP8} used for the mass spectrometry studies. (B) Mass spectrometry analysis of 3XFLAG-ORD_{ORP8} showing the occurrence of apo and holo forms (with PS and PIP bound) in native conditions (bottom) and, for comparison, of the apo protein alone in denatured conditions (top). (C and D) Relative abundance of PS (C) and PIP (D) species (different acyl chain lengths) recovered after extraction from 3XFLAG-ORD_{ORP8}. (E) Sucrose-loaded heavy PC/PI4P liposomes (90:10 mol/mol, 2 mM lipids, 400 nm diameter) and light PC/PS liposomes (90:10 mol/mol, 2 mM lipids, 100 nm diameter) were incubated with no protein (-) or with 5 μM either WTORP_{ORP8} or H514A, H515A mutant ORP_{ORP8} for 15 min at 25°C. After centrifugation, supernatant (left) and pellet (right) fractions were collected, and the percentages of PI4P and PS recovered in the two fractions were assessed by means of high-performance liquid chromatography-based lipid analysis (mean ± SEM; *****P* < 0.0001, ****P* < 0.001, *t* test, *n* = 3 cells). Pellet fractions were normalized by PS recovery of (-) sample.

Fig. 4. ORP5 and ORP8 mediate PS/PI4P exchange at the ER-PM contact sites

(A to D) Confocal live imaging of HeLa cells expressing iRFP-P4M [(A) and (B)] or GFP-evt2-2XPH [(C) and (D)] either alone (–) or together with WT or mutant ORP5, as indicated. The main fields show the fluorescence of iRFP-P4M and GFP-evt2-2XPH, respectively. The insets of (A) show at high magnification the GFP-ORP5 fluorescence in the regions indicated in the main fields. The ratio of iRFP (A) or GFP (C) fluorescence visible in the TIRF versus epifluorescence fields is shown in (B) and (D) (mean ± SEM; $P < 0.0001$, t test, $n = 14$ to 25 cells). Scale bars, 10 μm ; insets, 2 μm . (E) Effect of the double knockdown of ORP5 and ORP8 on the subcellular localization of the N-PH domain of ORP8. Scale bars, 10 μm . (F) Confocal live microscopy showing that recruitment of PH-ORP5 to the PM with 1 μM rapamycin induces dissociation of iRFP-P4M. Scale bar, 10 μm for the main fields; 2 μm for the time sequence. (G to L) Quantification of fluorescent signals in the TIRF fields upon rapamycin-induced PM recruitment of FKBP12 fused to the PH-ORP proteins (mean ± SEM). (G) Loss of iRFP-P4M and increase of GFP-evt2-2XPH upon recruitment of PH-ORP5_{WT} ($n = 6$ cells). (H) Loss of iRFP-P4M, but not of GFP-PH-PLC δ , upon recruitment of PH-ORP5_{WT} ($n = 8$ cells). (I) Loss of iRFP-P4M upon PH-ORP5_{WT} or PH-ORP8_{WT} recruitment, but not upon recruitment of PH-ORP5_{H478/479A} or PH-ORP5_{L389D} ($n = 6$ to 10 cells). (J) GFP-evt2-2XPH fluorescence upon recruitment of PH-ORP5_{WT} TM (blue, $n = 18$ cells) or PH-ORP5_{WT} pretreated with 100 nM A1 (10 min) (green, $n = 10$ cells). (K) Loss of iRFP-P4M only if the PH-ORP_{WT} construct is tethered to the ER by its transmembrane region ($n = 4$ cells). (L) The knockdown of Sac1 (inset, immunoblot) impairs the decrease of iRFP-P4M fluorescence upon recruitment of PH-ORP5_{WT} ($n = 6$ to 9 cells).