OSBP-related protein 4L promotes phospholipase Cβ3 translocation from the nucleus to the plasma membrane in Jurkat T-cells

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§ The abbreviations used are: PLC, phosphoinositide phospholipase C; ORP4L, OSBP-related protein 4L; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; OSBP, oxysterol-binding protein; ORD, OSBP-related ligand-binding domain (ORD); VAP, VAMP-associated protein; T-ALL, T-cell acute lymphoblastic leukemia; NLS, nuclear localization signal; NES, nuclear export signal; GST, glutathione-S-transferase; LMB, leptomycin B; CD3, cluster of differentiation 3; RAN, Ras-related nuclear protein; FFAT, two phenylalanines in an acidic tract (FFAT) motif; LMB, leptomycin B; CD3, cluster of differentiation 3; RAN, Ras-related nuclear protein; FFAT, two phenylalanines in an acidic tract (FFAT) motif; EGF, epidermal growth factor; ER, endoplasmic reticulum; HPR, horseradish peroxidase.

Phosphoinositide phospholipases C (PLCs) are a family of eukaryotic intracellular enzymes with important roles in signal transduction. In addition to their location at the plasma membrane, PLCs also exist within the cell nucleus where they are stored. We previously demonstrated that OSBP-related protein 4L (ORP4L) anchors cluster of differentiation 3ε (CD3ε) to the heterotrimeric G protein subunit (Goq11) to control PLCβ3 relocation and activation. However, the underlying mechanism by which ORP4L facilitates PLCβ3 translocation remains unknown. Here, using confocal immunofluorescence microscopy and communoprecipitation assays, we report that ORP4L stimulates PLCβ3 translocation from the nucleus to the plasma membrane in Jurkat T-cells in two steps. First, we found that ORP4L is required for the activation of Ras-related nuclear protein (RAN), a GTP-binding nuclear protein that binds to exportin 1 and eventually promotes the nuclear export of PLCβ3. Second, we also observed that ORP4L interacts with vesicle-associated membrane protein–associated protein A (VAPA) through its two phenylalanines in an acidic tract (FFAT) motif. This complex enabled PLCβ3 movement to the plasma membrane, indicating that PLCβ3 translocation occurs in a VAPA-dependent manner. This study reveals detailed mechanistic insight into the role of ORP4L in PLCβ3 redistribution from storage within the nucleus to the plasma membrane via RAN activation and interaction with VAPA in Jurkat T-cells.

This work was supported by grants from the Basic Research Program of Guangdong province, China (Grant 2017A030308002 to D. Y.) and National Natural Science Foundation of China (NSFC) (Grants 81770438, 91439122, and 30971104 to D. Y.); Academy of Finland Grant 285223 (to V. M. O.); and the Sigrid Juselius Foundation, the Magnus Ehrnrooth Foundation, and the Finnish Foundation for Cardiovascular Research (to V. M. O.). The authors declare that they have no conflicts of interest with the contents of this article. This article contains Figs. S1 and S2.
cation of PLCβ3 from the nucleus to the plasma membrane; and regulates its activation to promote IP₃ production. In this manner, ORP4L serves to regulate IP₃-induced Ca²⁺ signaling, mitochondrial respiration, and ATP generation and thereby maintains the survival of acute T-lymphoblastic leukemia (T-ALL) cells (24). However, the detailed pathway through which ORP4L mediates the translocation of intranuclear PLCβ3 remains to be identified.

Ras-related nuclear protein (RAN), also known as the GTP-binding nuclear protein RAN, is involved in the transport of proteins across the nuclear envelope by interacting with karyopherins and changing their ability to bind or release cargos. Cargo proteins containing a nuclear localization signal (NLS) are transported into the nucleus by binding importins. Inside the nucleus, RAN-GTP binds to importin and releases the import cargo. Cargo that needs to shift from the nucleus into the cytoplasm binds to exportin in a ternary complex with RAN-GTP. Upon hydrolysis of RAN-GTP to RAN-GDP outside the nucleus, the complex dissociates, and the export cargo is released (25, 26). In this study, we reveal mechanistic insight into the role of ORP4L in PLCβ3 redistribution via RAN activation and VAPA involvement and offer a model for the shift of PLCβ3 from storage within the nucleus to its functional site.

Results

PLCβ3 exists in the nucleus of unstimulated cells

The localization of PLCβ3 in Jurkat T-cells was investigated by confocal immunofluorescence microscopy. In unstimulated Jurkat T-cells, PLCβ3 exhibited an intranuclear localization (Fig. 1A). The fractionation of Jurkat T-cells analyzed by Western blotting further confirmed that a majority of PLCβ3 protein localized in the nucleus (Fig. 1B). Consistent with the observations in Jurkat T-cells, a similar intranuclear localization of PLCβ3 was apparent in K562, HeLa, and HepG2 cells (Fig. 1C). The Human Protein Atlas website (available from: https://www.proteinatlas.org/ENSG00000149782-PLCβ3/cell#Human) also provided the PLCβ3 intranuclear localization images in MCF-7, A-431, and U-2 OS cells (Fig. S1). Conversely, our recent study suggested that PLCβ3 catalyzes IP₃ production in T-ALL cells as opposed to PLCγ1 in normal T-cells (24). When we compared the PLCβ3 protein level, we found significantly up-regulated expression of this protein in primary T-ALL cells and Jurkat T-cells as compared with normal T-cells (Fig. 1D). In contrast to intranuclear localization in T-ALL cells, confocal immunofluorescence showed that PLCβ3 is distributed at the peripheral plasma membrane in normal T-cells (Fig. 1E). These results indicated a pathway of T-ALL cells regulating PLCβ3 localization, which was different from normal T-cells.

It has been reported that the interaction between cargo molecules and the transport receptors is frequently mediated by short linear motifs on the cargo called NLSs or nuclear export signals (NESs) (27, 28). Therefore, we dissected the sequence of PLCβ3 with two online platforms, NLStradamus (http://www.moseslab.csb.utoronto.ca/NLStradamus/) and NetNES (http://www.cbs.dtu.dk/services/NetNES/), to discover its NLS and NES. By sequence analysis with NLStradamus (29), we found that PLCβ3 contains two potential NLS sequences within amino acids 466–485 and 967–984 (Fig. 1F). Furthermore, the NetNES 1.1 Server (30) predicts that amino acids 310–315 within PLCβ3 constitute a leucine-rich nuclear export signal (Fig. 1G). These data indicated that the intranuclear localization of PLCβ3 in unstimulated cells is under the control of nuclear import/export machineries, which finally determine its subcellular distribution.

ORP4L regulates PLCβ3 translocation and activation by binding PLCβ3

We previously identified that ORP4L in fact interacts physically with PLCβ3 (24). These previous results suggested that PLCβ3 may be sequestered in the nucleus under unstimulated conditions and upon anti-CD3 stimulation is translocated to the plasma membrane in the presence of ORP4L, thereby gaining access to its substrate,PIP₂ (Fig. S2, A and B).

A coimmunoprecipitation assay showed that ORP4L interacts specifically with PLCβ3 but not with other isotypes of PLCβ3 in Jurkat T-cells (Fig. S2C). PLCβ3 knockdown (Fig. 2A) significantly reduced IP₃ production and Ca²⁺ release from the ER upon anti-CD3 stimulation (Fig. 2B), indicating its dominant role in Jurkat T-cells. ORP4L overexpression promoted IP₃ production and Ca²⁺ release, effects reduced in PLCβ3 knockdown cells (Fig. 2, C and D). ORP4L binds PLCβ3 via its amino acid region 445–513 (31). To investigate the role of this physical interaction, we generated a truncated ORP4L protein lacking the PLCβ3-binding region (ORP4L Δ445–513). ORP4L overexpression enabled PLCβ3 translocation from the nucleus to the plasma membrane, but ORP4L Δ445–513 failed to do this (Fig. 2E). Phosphorylation at Ser537 contributes to the basal activity of PLCβ3 (32). Consequently, ORP4L but not ORP4L Δ445–513 increased PLCβ3 activity (Fig. 2, E and F; phosphorylation of PLCβ3), IP₃ production, and Ca²⁺ release (Fig. 2G). Confocal imaging showed that p-PLCβ3 was present only at the plasma membrane, and not in the nucleus, when cells were stimulated with anti-CD3 (Fig. 2E), indicating a sequestering of PLCβ3 in the nucleus in its inactive state. These results suggested that PLCβ3 translocation and activation are dependent on its direct interaction with ORP4L.

ORP4L regulates PLCβ3 translocation and activation by binding PLCβ3

The small GTPase RAN plays a central role in the coordination and trafficking of nuclear proteins (33, 34). To investigate mechanisms of PLCβ3 export, we performed RAN activity assays. As expected, anti-CD3 stimulation increased the active form of RAN (RAN-GTP) in a time-dependent manner (Fig. 3A), whereas RAN activity declined in ORP4L knockdown cells (Fig. 3B), indicating that ORP4L is required for RAN activation in Jurkat T-cells upon anti-CD3 stimulation. Anti-CD3 stimulation led to phosphorylation of PLCβ3 in a time-dependent manner (Fig. 3C). Leptomycin B (LMB), a specific inhibitor of exportin 1 (34, 35), was added simultaneously with anti-CD3 and blocked the nuclear export of PLCβ3 (Fig. 3D) as well as the phosphorylation of PLCβ3 at the plasma membrane (Fig. 3, D

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ORP4L regulates PLCβ3 translocation

A

PLCβ3  Hoechst 33342  Merge

C

PLCβ3  Hoechst 33342  Merge

B

PM  NU

PLCβ3

H2A

P-cad

D

T-cells  T-ALL  Jurkat

PLCβ3

Actin

C

G532

HepG2

NetNES 1.1: Predicted NES signals in Sequence

| Threshold | NN | HMM | NES Score |
|-----------|----|-----|----------|

17432  J. Biol. Chem. (2018) 293(45) 17430 – 17441
was able to increase the IP$_3$ and Ca$^{2+}$ release (Fig. 4C) upon ORP4L overexpression. These results suggest that ORP4L enables transduction of CD3 signaling to RAN and PLC$\beta_3$ activation in Jurkat T-cells dependent on exportin 1.

**ORP4L interacts with VAPA and functions in PLC$\beta_3$ translocation**

The next question is how PLC$\beta_3$ is transported to the cell periphery. Because yeast ORPs (Osh proteins) and VAPs assemble at ER–plasma membrane contact sites to control the metabolism of plasma membrane phosphoinositides (36) and VAPs interact with microtubules, which play crucial roles in the maintenance and organization of cellular organelles as well as in membrane trafficking (19), we hypothesized that the VAPA may be involved in the translocation of PLC$\beta_3$.

ORP4L, via bimolecular fluorescence complementation, was shown to form a complex with its ER receptor, VAPA, in mammalian cells (14), but there is no direct evidence for the endogenous ORP4L and VAPA interaction. We next sought to investigate whether endogenous ORP4L interacts with VAPA in Jurkat T-cells. Collectively, the confocal immunofluorescence revealed colocalization between endogenous ORP4L and VAPA (Fig. 5A); moreover, the coimmunoprecipitation experiments showed that ORP4L interacted with the integral membrane protein VAPA (Fig. 5B). The GST pulldown experiments further confirmed that WT ORP4L directly interacts with VAPA, whereas the ORP4L lacking the FFAT motif did not (Fig. 5C). These data suggest that ORP4L directly interacts with VAPA via its FFAT motif.

To further dissect the functional role of ORP4L in PLC$\beta_3$ translocation and activation, we transfected Jurkat T-cells with full-length ORP4L or truncated ORP4L lacking the FFAT motif (ORP4L ΔFFAT). Confocal immunofluorescence microscopy images showed that p-PLC$\beta_3$ clearly occurred in the cell periphery in ORP4L-overexpressing cells, but this could not be observed in cells overexpressing ORP4L devoid of the FFAT motif (Fig. 5D). Accordingly, the results of Western blot analysis confirmed the enhanced PLC$\beta_3$ phosphorylation in cells overexpressing WT ORP4L but not in cells expressing ORP4L that lacks the FFAT motif (Fig. 5, E and F).

Next, we measured the IP$_3$ production and Ca$^{2+}$ release from ER upon anti-CD3 stimulation in Jurkat T-cells. The results showed that ORP4L overexpression promotes IP$_3$ production and Ca$^{2+}$ release (Fig. 5, G and H). To our surprise, in contrast to its effect on PLC$\beta_3$ activation, the ORP4L ΔFFAT construct was able to increase the IP$_3$ and Ca$^{2+}$ response (Fig. 5, G and H), although to a lower extent compared with full-length ORP4L. PI$_{3}$F$_{2}$, located in the plasma membrane acts as substrate for PLC catalysis (38). Recently, an important role of the ORD of ORP4L as a transporter for PI$_{3}$F$_{2}$ has been explored (39). We hypothesized that the PLC$\beta_3$ reaction may require the ORD of ORP4L to transport the substrate PI$_{3}$F$_{2}$. Although ORP4L ΔFFAT fails to facilitate PLC$\beta_3$ translocation, the ORD remaining enhances the PI$_{3}$F$_{2}$ transport for plasma membrane (PM) PLC$\beta_3$ catalysis. To this end, we constructed truncated ORP4L lacking FFAT and ORD (ORP4L ΔFFAT&ORD). Compared with full-length ORP4L, ORP4L ΔFFAT&ORD could not increase PLC$\beta_3$ activation or evoke the IP$_3$ and Ca$^{2+}$ response (Fig. 5, G and H), indicating the requirement of the ORD for PLC$\beta_3$ catalysis.

To further investigate the potential dependence of PLC$\beta_3$ translocation on VAPA, RNAi was used to inhibit VAPA expression. The translocation of PLC$\beta_3$ to a peripheral location upon anti-CD3 treatment was prevented by knockdown of VAPA (Fig. 6A), indicating a role of the ORP4L–VAPA complex in controlling PLC$\beta_3$ localization and activity. Moreover, PLC$\beta_3$ could not be shifted to its phosphorylated form in VAPA knockdown Jurkat T-cells, even when ORP4L was overexpressed (Fig. 6, B and C). These results suggested that the nuclear pool of PLC$\beta_3$ in unstimulated Jurkat T-cells is mobilized to the plasma membrane in a manner dependent on the ORP4L–VAPA complex.

**Discussion**

We recently reported that ORP4L organizes a signaling complex involving the translocation and activation of nuclear, ER, mitochondrial, and plasma membrane molecular components (24). However, the detailed role of ORP4L in the shift of PLC$\beta_3$ from the nucleus to plasma membrane remained unclear. The present study offers evidence that the underlying mechanism of PLC$\beta_3$ translocation promoted by ORP4L is related to RAN activation and assistance by the ER receptor for ORP4L, VAPA (Fig. 6D).

PLCs perform a catalytic function at the plasma membrane where their substrate, PI$_{3}$F$_{2}$, is localized. However, PLCs also exist at other subcellular locations such as the cytoplasm and the nucleus (40–43). This separation of an enzyme from its substrates provides a regulatory mechanism, but understanding how the key molecules and translocation events control the function of PLCs requires new insights. In this study, we found that under unstimulated conditions PLC$\beta_3$ mainly exists within the Jurkat T-cell nucleus but translocates to the plasma membrane upon anti-CD3 stimulation. Colocalization of ORP4L and PLC$\beta_3$ was observed in Jurkat T-cells stimulated with anti-CD3, and the two proteins were found to interact physically, which prompted us to consider a role for ORP4L in the PLC$\beta_3$ plasma membrane translocation. Indeed, depletion of ORP4L significantly inhibited the shift of PLC$\beta_3$ to the plasma membrane, consistent with an important role of ORP4L in controlling the distribution and activity of PLC$\beta_3$ in T-ALL cells.

**Figure 1. Localization of PLC$\beta_3$ in unstimulated cells.** A, Immunofluorescence staining with anti-PLC$\beta_3$ showing nuclear localization of the protein in Jurkat T-cells. Scale bars, 10 μm. B, Western blot showing PLC$\beta_3$ in PM and nuclear (NU) fractions of Jurkat T-cells. P-cadherin (P-cad) and histone H2A were used as loading controls for the plasma membrane and nuclear fractions, respectively. C, Immunofluorescence staining with anti-PLC$\beta_3$ showing nuclear localization of the protein in K562, HeLa, and HepG2 cells. Scale bars, 10 μm. D, Western blot showing PLC$\beta_3$ expression in normal T-cells, primary T-ALL cells, and Jurkat T-cells. E, Immunofluorescence staining with anti-PLC$\beta_3$ showing plasma membrane localization of PLC$\beta_3$ in normal T-cells. Scale bars, 10 μm. F, prediction of NLS sequences in PLC$\beta_3$ with NLSyDar (http://www.moseslab.csb.utoronto.ca/NLSyDar/). G, prediction of a NES sequence in PLC$\beta_3$ with NetNES 1.1 Server (http://www.cbs.dtu.dk/services/NetNES/). HMM, hidden Markov model; NN, NetNES 1.1. (Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third-party-hosted site.)
ORP4L regulates PLCβ3 translocation

We have previously provided evidence that ORP4L can specifically interact with CD3ε, Gαq/11, and PLCβ3, and these interactions were modestly enhanced upon anti-CD3 stimulation, suggesting a dynamic nature of the complex formation that is under the control of CD3 signaling (24). Therefore, we hypothesize that formation of the CD3ε–Gαq/11 complex sca-
Folded by ORP4L enhances \( \alpha_{i_{11}} \) activity for activating RAN and PLC\( \beta_3 \) upon anti-CD3 stimulation. The increase in active RAN (RAN-GTP) upon anti-CD3 stimulation and the reduction of the shift of PLC\( \beta_3 \) by leptomycin B, an inhibitor of exportin 1, suggest that PLC\( \beta_3 \) trafficking from the nucleus is mediated by the exportin system.

ORP4L plays an important role in controlling the distribution and activity of PLC\( \beta_3 \) in Jurkat T-cells and primary T-ALL cells (24). The specific nature of this inhibition was further corroborated by data showing that depletion of VAPA, the ER membrane receptor of ORP4L, had a similar effect as depletion of ORP4L. We envision that the deletion of VAPA results in the dysfunction of ORP4L in regulation of PLC\( \beta_3 \).

Moreover, our recent findings showing that ORP4L binds PIP\(_2\) are consistent with a model in which an ORP4L–VAPA complex may target PLC\( \beta_3 \) to distinct PIP\(_2\)-containing microdomains at the plasma membrane (44, 45). The observed affinity of ORP4L for PIP\(_2\), the major plasma membrane phosphoinositide (46), together with an interaction with PLC\( \beta_3 \) suggests an association of ORP4L with components of the plasma membrane. In contrast, interaction with VAPA, an integral membrane protein mainly found in the ER (19), targets ORP4L to ER membranes. This duality in membrane targeting may indicate that ORP4L has the capacity to simultaneously associate with the ER and the plasma membrane. In fact, ORP4L was found to colocalize with PLC\( \beta_3 \) at punctate peripheral sites, which could represent ER–plasma membrane contacts (47). Such regions are commonly referred to as “puncta” (48), and it is within the puncta that key signaling occurs during T-cell activation (49). Such contact sites are also known to play crucial roles in \( Ca^{2+} \) regulation during store-operated \( Ca^{2+} \) entry and muscle excitation-contraction coupling (37). Our findings thus raise the possibility that ORP4L may operate to coordinate the activity of PLC\( \beta_3 \) at ER–plasma membrane contacts.

Figure 3. Anti-CD3 stimulation activates RAN for PLC\( \beta_3 \) exportation from the nucleus in Jurkat T-cells. A, the active form of RAN (RAN-GTP) in Jurkat T-cells upon anti-CD3 stimulation. Cells were stimulated for the indicated times with 10 \( \mu \)g ml\(^{-1} \) anti-CD3. B, the active form of RAN (RAN-GTP) in Jurkat T-cells with ORP4L knockdown upon anti-CD3 stimulation. Cells were stimulated for 3 min with 10 \( \mu \)g ml\(^{-1} \) anti-CD3 for 3 min. The relative GTP-RAN protein content was quantified from Western blots and normalized with the \( \beta \)-actin signal. C and D, Jurkat T-cells were stimulated for the indicated times with 10 \( \mu \)g ml\(^{-1} \) anti-CD3 (C) or preincubated for 2 h with 50 ng ml\(^{-1} \) LMB (D) followed by 5-min stimulation with 10 \( \mu \)g ml\(^{-1} \) anti-CD3, and PLC\( \beta_3 \) phosphorylation at Ser\(^{537} \) was analyzed by Western blotting. E, confocal microscopy analysis of PLC\( \beta_3 \) and p-PLC\( \beta_3 \) in Jurkat T-cells after 5-min anti-CD3 stimulation (10 \( \mu \)g ml\(^{-1} \)) in the presence or absence of LMB. Scale bars, 10 \( \mu \)m. The data represent mean ± S.D. from an experiment performed in triplicate. **, \( p < 0.01; \)***, \( p < 0.001 \), Student’s t test. All error bars represent S.D. N7, nontargeting.

Figure 2. ORP4L binding is required for PLC\( \beta_3 \) translocation and activation in Jurkat T-cells. A, Western blot showing PLC\( \beta_3 \) knockdown in Jurkat T-cells. B, IP\(_3\) production and \( Ca^{2+} \) release upon anti-CD3 stimulation (10 \( \mu \)g ml\(^{-1} \)) in Jurkat T-cells with PLC\( \beta_3 \) knockdown and ORP4L overexpression in Jurkat T-cells. D, IP\(_3\) production and \( Ca^{2+} \) release upon anti-CD3 stimulation (10 \( \mu \)g ml\(^{-1} \)) in Jurkat T-cells upon the indicated genetic manipulations. E, confocal microscopy analysis of the location of p-PLC\( \beta_3 \) phosphorylated at Ser\(^{537} \) in control, ORP4L-, or ORP4L \( \Delta \)445–513-overexpressing cells. The relative p-PLC\( \beta_3 \) protein content was quantified from Western blots and normalized with the \( \beta \)-actin signal. G, IP\(_3\) production and \( Ca^{2+} \) response upon anti-CD3 stimulation (10 \( \mu \)g ml\(^{-1} \)) in Jurkat T-cells overexpressing the indicated constructs. The data represent mean ± S.D. from an experiment performed in triplicate. **, \( p < 0.01; \)***, \( p < 0.001 \), Student’s t test. All error bars represent S.D. N7, nontargeting.
In conclusion, our results suggest that ORP4L facilitates PLCβ3 translocation in T-ALL cells. RAN activation under control of ORP4L regulates the export of inactive PLCβ3 from the nucleus, and interaction of VAPA with ORP4L mediates PLCβ3 transport to the cell periphery. Our study offers novel mechanistic insight into ORP4L functions in T-ALL cells. It also provides a model for a shift of PLCβ3 from subcellular storage to its functional site via RAN activation and VAPA function.

**Experimental procedures**

**Reagents and antibodies**

Alexa Fluor 488 goat anti-mouse IgG (catalogue number A-11001), Alexa Fluor 546 goat anti-rabbit IgG (catalogue number A-11035), and Alexa Fluor 647 donkey anti-goat IgG (catalogue number A-21447) were purchased from Invitrogen. LEAF purified human anti-CD3 (clone HIT3a, catalogue number 300314) and anti-H2A (catalogue number 613301) were purchased from BioLegend (San Diego, CA). Hoechst 33342 and anti-ORP4L (catalogue number HAP021514) were purchased from Sigma-Aldrich. Anti-PLCβ3 (catalogue number Nosc-133231), anti-pan-cadherin (catalogue number sc-1499), and anti-VAPA (catalogue number sc-48698) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-PLCβ3 (catalogue number 2481) was purchased from Cell Signaling Technology (Beverly, MA). Anti-actin (catalogue number 60008-1), HRP-conjugated AffiniPure goat anti-mouse IgG (H+L) (catalogue number SA00001-1), HRP-conjugated AffiniPure goat anti-rabbit IgG (H+L) (catalogue number SA00001-2), and HRP-conjugated AffiniPure donkey anti-foat IgG (H+L) (catalogue number SA00001-3) were purchased from Proteintech Group (Chicago, IL).

**Human leukocyte specimens and cell lines**

Fresh leukocytes were isolated from peripheral blood of healthy human donors and T-ALL patients after obtaining written informed consent. Naïve CD3⁺ T-cells were isolated using an Enhanced Human T Cell Recovery Column kit (Cedarlane, Burlington, Ontario, Canada) according to the manu-
manufacturer's instruction. Jurkat T-cells, K562, HeLa, and HepG2 cells were purchased from American Type Culture Collection. Jurkat T-cells and K562 cells were maintained in RPMI 1640 medium containing 10% FBS, 100 units ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin. HeLa and HepG2 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS, 100 units ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin. All cells were cultured at 37 °C in a humidified incubator with 5% CO₂.

Plasmid constructs

Human full-length ORP4L cDNAs were amplified by PCR amplification (5'-ATTCTAGAATGGGAAAGCGG-3').

Figure 5. ORP4L interacts with VAPA to promote the transport of PLCβ3. A, confocal microscopy analysis of the colocalization between endogenous ORP4L (red) and VAPA (green) in Jurkat T-cells. Scale bars, 10 μm. B, Western blots showing coimmunoprecipitation of endogenous ORP4L and VAPA from Jurkat T-cells. C, GST pulldown experiments with bacterially expressed GST-ORP4L, GST-ORP4L ΔFFAT, and endogenous VAPA from Jurkat T-cells. D, confocal microscopy analysis of the localization of p-PLCβ3 phosphorylated at Ser537 in control, ORP4L-, ORP4L-ΔFFAT-, or ORP4L-ΔFFAT&ORD-overexpressing cells. Scale bars, 10 μm. E, Western blot analysis of p-PLCβ3 phosphorylation at Ser537 in control, ORP4L-, ORP4L-ΔFFAT-, or ORP4L-ΔFFAT&ORD-overexpressing cells. F, the relative p-PLCβ3 protein content was quantified from Western blots (E) and normalized with the β-actin signal. G and H, IP3 production (G) and Ca²⁺ response (H) upon anti-CD3 stimulation (10 μg ml⁻¹) in Jurkat T-cells overexpressing the indicated constructs. The data represent mean ± S.D. from an experiment performed in triplicate. **, p < 0.01; ***, p < 0.001, Student’s t test. All error bars represent S.D. H-chain, heavy chain.
CGGT-3'; 5'-ATTCTAGAAGTGCGCTCAGAAGATGTTCGGGGCACATATGCA-3') from HeLa cell cDNA and subcloned into the pcDNA4HisMaxC (Invitrogen) vector. The FFAT motif deletion was generated by PCR from ORP4L cDNA with primers (Forward 1, 5'-ATTAGATCTATGGGGAAAGCGGCGGCT-3'; Forward 450, 5'-TGATGAAGGATGTGGAGTCTTCCATGGTATCTTCCTCACTGTCC-3'; Reverse 916, 5'-ATTGTCGACGAAGATGTTGGGGCACA-TATGCCA-3'; Reverse 465, 5'-GGACAGTGAGGAAGATGAAATACATGGAAGACTCCACATCCTTCATCA-3'). All constructs were verified by sequencing.

**Isolation of nuclear and plasma membrane fractions**

Nuclear fractions were isolated using a nuclear/cytosol fractionation kit (Biovision) according to the manufacturer's instructions. Briefly, aliquots of 2 x 10⁶ cells were collected by centrifugation followed by adding cytosol extraction buffer-A and homogenization. After cytosol extraction buffer-B was...
added, the sample was centrifuged, and the supernatant fraction was obtained. The pellet was resuspended in nuclear extraction buffer, vortexed, and centrifuged, and the supernatant (nuclear extract) was prepared. For plasma membrane isolation, cells were collected and resuspended in 0.2 mM EDTA in 1 mM NaHCO₃ in an approximate ratio of 1 ml/10⁸ cells and incubated on ice for 30 min to swell the cells. Cells were homogenized using a Dounce homogenizer. The homogenates were centrifuged for 10 min at 175 × g at 4 °C to remove unbroken cells and nuclei, and the supernatant was centrifuged at 25,000 × g for 30 min at 4 °C to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded, and the pellets were resuspended in 0.2 mM potassium phosphate buffer, pH 7.2. The resuspended membranes then were loaded onto the two-phase system with a polymer mixture containing 6.6% Dextran T500 (GE Healthcare), 6.6% (w/v) poly(ethylene glycol) 3350 (Fisher Scientific), and 0.2 M potassium phosphate, pH 7.2. The phases were separated by centrifugation at 1150 × g for 5 min at 4 °C. The upper phase containing primarily plasma membranes was collected.

**Gene transfer**

For ORP4L knockdown, high-titer lentivirus carrying shRNA (TCAGAGTCAAGCTCAGGTGTA) prepared by Shanghai GenePharma (Shanghai, China) was used. 1 × 10⁶ cells were resuspended in 100 μl of medium with lentivirus (multiplicity of infection, 100) and 5 μg ml⁻¹ Polybrene in a 24-well culture plate. Infections were carried out for 6 h at 37 °C in 5% CO₂. At the end of infection, 400 μl of medium was added. Efficacy of knockdown of ORP4L was verified by Western blotting after 3-day infection. A 4D-Nucleofector™ System (Lonza, Basel, Switzerland) was used for transient transfection of ORP4L, ORP4L ΔFFAT, and ORP4L ΔFFAT&ORD constructs and VAPA siRNA (catalogue number sc-61768, Santa Cruz Biotechnology) in Jurkat T-cells.

**Coimmunoprecipitation and GST pulldown assay**

For the coimmunoprecipitation assay, 1 × 10⁷ Jurkat T-cells were washed twice with ice-cold PBS and incubated on ice for 30 min with 1 ml of lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.5 mM MgCl₂, 10% glycerol, and 0.5% Triton X-100, pH 8.0) supplemented with protease inhibitor mixture (Roche Applied Science). Cell lysates were centrifuged for 15 min at 15,000 × g. The supernatant was preabsorbed with 50 μl of Protein G–agarose (Invitrogen) for 1 h at 4 °C. The recovered supernatant was incubated overnight with VAPA or control antibody at 4 °C. The beads were washed four times with lysis buffer and boiled in SDS-PAGE loading buffer. Samples were resolved on 10% SDS-polyacrylamide gels and subjected to Western blot analysis with antibodies.

For GST pulldown assay, pGEX-4T-1 (Addgene, Cambridge, MA), full-length ORP4L constructs (pGEX-4T-1-ORP4L), or the truncated ORP4L constructs lacking the FFAT motif (pGEX-4T-1-ORP4L ΔFFAT) were transformed into E. coli Rosetta™ (DE3) (Novagen) and cultured at 37 °C to an OD₆₀₀ of 0.5–1.0 followed by induction with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 16–18 h at 18 °C. The cells were collected, and crude bacterial lysates were prepared by sonication in lysis buffer 1 (50 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, pH 8.0) in the presence of the protease inhibitor mixture (Roche Applied Science). Bacterial lysates were centrifuged for 20 min at 12,000 × g, and the supernatants were used for fusion protein purification with GST-Bind beads (Novagen) according to the manufacturer’s protocol. Jurkat T-cells were washed twice with cold PBS, lysed in lysis buffer, and shaken for 30 min on ice, and the lysate was clarified by a 10-min centrifugation at 12,000 rpm in a microcentrifuge. For pulldown, 10 μg of GST/ORP4L/GST-ORP4L ΔFFAT and 30 μl of GST-Bind beads were incubated for 30 min on ice followed by washing three times with PBS. Jurkat T-cells lysates were then added and incubated at 4 °C overnight. Then the beads were washed three times with lysis buffer, resuspended into 2× SDS-PAGE loading buffer at 98 °C for 5 min, and resolved on 10% SDS-polyacrylamide gels for Western blotting.

**Measurement of RAN activity**

RAN activity was measured using a RAN activation assay kit according to the manufacturer’s instructions (NewEast Biosciences). Briefly, anti-active RAN mouse mAb was incubated with cell lysates containing RAN-GTP. The bound active RAN was pulled down by Protein A/G–agarose, eluted, and detected by immunoblot analysis using polyclonal rabbit anti-Ran antibody.

**Measurement of IP₃ production**

IP₃ was measured using an ELISA kit for IP₃ (CEC037Ge, Cloud-Clone Corp.) according to the manufacturer’s instructions. Briefly, 0.5 × 10⁶ Jurkat T-cells transfected with the indicated construct were lysed with lysis buffer and incubated with Detection Reagent A for 1 h at 37 °C. After aspirating and washing three times, Detection Reagent B was added, incubated for 30 min at 37 °C, then aspirated, and washed five times. Substrate Solution was added and incubated for 7 min at 37 °C. Finally, the reactions were blocked with Stop Solution and read at 450 nm immediately.

**Ca²⁺ imaging**

Cells (0.5 × 10⁶ cells ml⁻¹) transfected with the indicated construct were plated onto glass-bottomed dishes and incubated with 1 μM Fluo-4-AM for 30 min at 37 °C in ECB buffer (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 25 mM Heps, pH 7.5, 1 mg ml⁻¹ BSA, and 5 mM glucose). The buffer was replaced, and incubation continued for 20 min at 37 °C to permit dye de-esterification. Culture dishes were mounted on the stage of an inverted confocal microscope (Olympus FV3000 laser-scanning confocal microscope system) equipped with a 40× objective. Calcium measurements were performed in fresh ECB buffer at 37 °C with 5% CO₂. The Ca²⁺ images of
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cells excited with low-intensity 488-nm laser excitation were acquired at 2-s intervals alternately under time-lapse mode. Image data were subsequently analyzed using ImageJ (National Institutes of Health) and are presented as a ratio of $F/F_0$ in the final results where $F_0$ represents baseline fluorescence intensity in each cell.

Immunofluorescence microscopy

Cells were seeded onto coverslips, stimulated with anti-CD3 for the indicated times, and then fixed with 4% paraformaldehyde for 30 min at room temperature followed by permeabilization with 0.1% Triton X-100 for 5 min and blocking with 10% FBS for 30 min at room temperature. Cells were then incubated with primary antibodies in 5% FBS at 4 °C overnight. After washing three times (10 min each) with PBS, cells were incubated with fluorescent secondary antibody conjugates at 37 °C for 30 min followed by staining with Hoechst 33342 at room temperature for 10 min. Finally, the specimens were analyzed using a Zeiss LSM 510 Meta laser-scanning confocal microscope system or Olympus FV3000 laser-scanning confocal microscope system.

Western blot analysis

Cellular total protein samples were mixed with loading sample buffer, boiled for 10 min, and subjected to SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Millipore, Life Science). After blocking and incubations of the membranes with primary antibodies and HRP-secondary antibody conjugates (Bio-Rad), the blots were developed by enhanced chemiluminescence (Millipore, Life Science). Proteins were quantified by densitometry using ImageJ (National Institutes of Health), and the data were normalized using the β-actin signal.

Statistical analyses

All data are expressed as mean ± S.D. of at least three independent experiments. Differences between groups were analyzed by unpaired two-tailed Student’s t test. $p$ values of <0.05 were considered statistically significant.

Author contributions—G. P., X. C., C. Lai, and W. Z. data curation; G. P. software; G. P. validation; G. P., B. L., C. Li, and D. L. visualization; G. P. and W. Z. methodology; X. C., C. Li, and D. L. investigation; V. M. O. and W. Z. conceptualization; V. M. O., W. Z., and D. Y. project administration; D. Y. funding acquisition.

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