An optogenetic system to control membrane phospholipid asymmetry through flippase activation in budding yeast

Tomomi Suzuki\(^{1,2,}\), Tetsuo Mioka\(^3\), Kazuma Tanaka\(^3\) & Akira Nagatani\(^{1,}\)

Lipid asymmetry in biological membranes is essential for various cell functions, such as cell polarity, cytokinesis, and apoptosis. P4-ATPases (flippases) are involved in the generation of such asymmetry. In \textit{Saccharomyces cerevisiae}, the protein kinases Fpk1p/Fpk2p activate the P4-ATPases Dnf1p/Dnf2p by phosphorylation. Previously, we have shown that a blue-light-dependent protein kinase, phototropin from \textit{Chlamydomonas reinhardtii} (CrPHOT), complements defects in an \textit{fpk1}\(^{-}\)\textit{fpk2}\(^{-}\) mutant. Herein, we investigated whether CrPHOT optically regulates P4-ATPase activity. First, we demonstrated that the translocation of NBD-labelled phospholipids to the cytoplasmic leaflet via P4-ATPases was promoted by blue-light irradiation in \textit{fpk1}\(^{-}\)\textit{fpk2}\(^{-}\} cells with CrPHOT. In addition, blue light completely suppressed the defects in membrane functions (such as endocytic recycling, actin depolarization, and apical-isotropic growth switching) caused by \textit{fpk1}\(^{-}\)\textit{fpk2}\(^{-}\) mutations. All responses required the kinase activity of CrPHOT. Hence, these results indicate the utility of CrPHOT as a powerful and first tool for optogenetic manipulation of P4-ATPase activity.

In eukaryotic cells, lipid bilayers exhibit asymmetric phospholipid distributions. This is particularly evident in the plasma membrane: the cytoplasmic leaflet has an abundance of phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and its derivatives, whereas the exoplasmic leaflets are rich in phosphatidylcholine (PC), sphingomyelin (SM) and glycosphingolipids. Lipid asymmetries across membranes play a crucial role in many cell functions, such as vesicular transport, cytokinesis, cell signalling, apoptosis, cell migration, and the immune response. The asymmetry of phospholipids in biological membranes is established and maintained by lipid transporters. A family of such transporters is P4-ATPase, which transports specific lipids from the exoplasmic leaflet to the cytoplasmic leaflet using the energy of ATP hydrolysis. Most P4-ATPases interact with a noncatalytic subunit, Cdc50 family protein, for proper subcellular localization and ATPase activity.

The P4-ATPase subfamily is widely conserved in eukaryotes. To date, many studies in mammals, \textit{Caenorhabditis elegans}, \textit{Saccharomyces cerevisiae}, \textit{Arabidopsis thaliana}, and others have been reported. As an example, mammalian studies have reported that knockdown of a P4-ATPase ATP8A1 disrupts the asymmetric distribution of PS in recycling endosomes (REs) and suppresses vesicle transport from endosomes. The involvement of P4-ATPases in vesicular transport has been reported in various organisms and is considered to be a general function of P4-ATPases. Probes that specifically bind to PS (e.g. LactC2-GFP and GFP-evt2-2XPH) enable direct observation of the PS distribution in the cytosolic leaflet. Analysis using a biotinylated PE probe in yeast cells showed that PE is specifically exposed on the surface of polarized ends such as the bud site and bud neck. Mutation of P4-ATPase genes disrupts these PE polarized distributions in yeast cells. As described above, genetic analysis using mutants and overexpression reveals many physiological functions of P4-ATPases in various organisms. Cell biological analysis using specific lipid probes reveals the location of

---

\(^{1}\)Department of Botany, Graduate School of Science, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan. \(^{2}\)PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan. \(^{3}\)Division of Molecular Interaction, Institute for Genetic Medicine, Hokkaido University Graduate School of Life Science, Kita-ku, Sapporo 060-0815, Japan. \(^{5}\)email: tomo@physiol.bot.kyoto-u.ac.jp; nagatani@physiol.bot.kyoto-u.ac.jp
intracellular phospholipids. In contrast, many issues regarding the spatiotemporal regulation of P4-ATPases and translocated phospholipids remain. To elucidate them, the development of a new tool is required.

The budding yeast *S. cerevisiae*, has five P4-ATPases, Drs2p, Dnf1p, Dnf2p, Dnl3p, and Neo1p. Neo1p is essential, while the others are redundantly essential for viability[32]. Dnf1p and Dnf2p have 69% amino acid sequence identity and are localized in the plasma membrane and inner membranes (trans-Golgi network (TGN), early endosomes and transport vesicles);[22,23,24,25] Dnf1p/Dnf2p transport PE, PC, their lyso-forms, and monosaccharide glycosphingolipids like glucosylceramide (GlcCer) and galactosylceramide (GalCer). GlcCer is primarily transported by Dnf2p[23,26,27,28]. Both Dnf1p and Dnf2p require interaction with Lem3p, a member of the Cdc50p family, for subcellular localization and function; hence, *lem3Δ* and *dnf1Δ* mutants are phenocopies[29]. In both *dnf1Δ* dnf2Δ and *lem3Δ* mutants at a late mitotic phase, translocation of PE to the inner leaflet, actin depolarization at the bud tip and switching from apical to isotropic growth are significantly delayed compared to those in wild-type cells[23,30,31,32]. These results indicate that Dnf1p/Dnf2p-Lem3p are involved in the maintenance of cell polarity.

Dnf1p/Dnf2p-Lem3p are also involved in endocytic recycling of the vesicle-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) Snc1p. Mutation of another P4-ATPase, Drs2p, or its partner subunit Cdc50p causes an internal accumulation of Snc1p, and a combination of *dnf1Δ* dnf2Δ (or *lem3Δ*) and *drs2Δ* (or *cdc50Δ*) mutations results in severe vesicle-transport abnormalities[22,29,32,33]. Hence, Dnf1p/Dnf2p-Lem3p and Drs2p-Cdc50p are redundant in the regulation of vesicle transport in this pathway. Furthermore, Dnf1p/Dnf2p-dependent cellular processes, such as vesicle transport, were regulated in these cells by BL. Our results suggest the potential of *Cr* PHOT as an optogenetic tool to regulate membrane functions.

**Results**

**Optical control of yeast cell growth by Chlamydomonas PHOT.** To establish a system to control P4-ATPase (flipase) activity by light (Fig. 1a), we attempted to prove that GrPHOT regulates P4-ATPases at the molecular level. On the basis of the highest sequence homology among the kinase domains of Fpk1p/Fpk2p and GrPHOT[34], we have shown that GrPHOT complements the synthetic lethality of a *PGAL1-CDC50 fpk1Δ* fpk2Δ yeast mutant in a BL-dependent manner[35]. In this study, we introduced GrPHOT and its derivatives (Fig. 1b) into the mutant and examined its growth activity again. Consequently, we reconfirmed that GrPHOT suppresses the growth defect of the *PGAL1-CDC50 fpk1Δ* fpk2Δ yeast mutant in glucose-containing medium under BL but not under red light (RL) or in darkness (Fig. 1c, GrPHOT). Furthermore, a K-fragment with constitutive kinase activity suppressed the growth defect regardless of the light condition, and KDm with a kinase-dead mutation, ΔP4ATPases in a light-dependent manner. Therefore, we newly constructed LOV1/2m, which has Cys to Ala mutations in two LOV domains from *Chlamydomonas reinhardtii* (GrPHOT) into an *fpk1Δ* fpk2Δ mutant with a conditional Cdc50p mutant (*PGAL1-CDC50 fpk1Δ* fpk2Δ). As a result, we have shown that GrPHOT complements the growth defect of the *fpk1Δ* fpk2Δ mutant in a BL-dependent manner. This result indicates the possibility of CrPHOT controls P4-ATPases in a light-dependent manner. Therefore, we examined which system could be used as a new optogenetic technology.

In this study, we reintroduced GrPHOT into the *fpk1Δ* fpk2Δ mutant or the *PGAL1-CDC50 fpk1Δ* fpk2Δ mutant and biochemically assessed the lipid translocation activity to show that it was indeed regulated by BL irradiation. Furthermore, Dnf1p/Dnf2p-dependent cellular processes, such as vesicle transport, were regulated in these cells by BL. Our results suggest the potential of CrPHOT as an optogenetic tool to regulate membrane functions.

CrPHOT and Dnf1p/Dnf2p P4-ATPases are localized in similar subcellular compartments. Fpk1p/Fpk2p, which regulate P4-ATPase activity, are mainly distributed throughout the cytoplasm but are partially localized in the early endosome/TGN and plasma membrane[35,36,37]. To clarify the control of P4-ATPases by CrPHOT, we compared the intracellular localization of fluorescent protein-tagged CrPHOT with that of Fpk1p. Before analysis, we confirmed that the tagged proteins were functional by a growth assay of the *PGAL1-CDC50 fpk1Δ* fpk2Δ mutant (Supplementary Fig. S1). An *fpk1Δ* fpk2Δ mutant expressing GFP-Fpk1p or GFP-CrPHOT cultured in darkness was briefly stained with a lipophilic dye, FM4-64[32,40], which was employed as a marker for the plasma membrane and endosomal/TGN compartments (Fig. 2a). GFP-CrPHOT was occasionally observed at the plasma membrane but primarily localized to intracellular punctate structures, which mostly merged with the fluorescence of FM4-64 (64.4% of the GFP-CrPHOT speckles were merged, n = 163; Fig. 2a).
Figure 1. Light control of yeast cell growth by CrPHOT. (a) A schematic of BL-induced activation of flippases through CrPHOT in yeast. (b) Schematic illustration of Fpk1p and CrPHOT and its derivatives. K-fragment, kinase domain fragment; KDm, kinase-dead mutant with D549N; LOV1/2 m and LOV2m, mutants with a decreased extent of light activation by substitution of conserved Cys in LOV1 and LOV2 domain or LOV2 domain only, respectively, to Ala. Flavin mononucleotide (FMN) is a chromophore of phototropin. (c) Growth of the yeast conditional mutant PGAL1-CDC50 fpk1Δ fpk2Δ carrying pRS416-CrPHOT or its derivatives. Yeast cells were serially diluted and spotted onto plates containing galactose (SGA-Ura) or glucose (SDA-Ura), which were incubated in darkness (Dark) or under 10 μmol m⁻² s⁻¹ RL (Red) or BL (Blue) irradiation at 28 °C for 3 days. The experiments were performed more than three times with different biological samples. A representative image is shown in (c). (d) The protein level of CrPHOT and its derivatives is not affected by light conditions. HA-fused Fpk1p and CrPHOT and its derivatives were extracted from the yeast transformants (same strains shown in c) cultured in darkness (d) or under 10 μmol m⁻² s⁻¹ BL irradiation (b) at 28 °C for 16 h, which were then resolved by SDS-PAGE and analysed by immunoblotting with anti-HA antibodies. Asterisk indicates non-specific bands.
The localization was similar to that of GFP-Fpk1p (66.0% of the GFP-Fpk1p speckles were merged, n = 152; 67.5% in previous analysis); Fig. 2a). The speckles of GFP-CrPHOT, as with GFP-Fpk1p, were colocalized with another TGN marker, Sec7p-mRFP (Supplementary Fig. S2). These results suggest that GFP-CrPHOT is primarily localized to endosomal/TGN compartments in yeast cells.

We then examined the effect of BL on the subcellular localization of GFP-CrPHOT. This was because BL irradiation changes phototropin intracellular localization in plants. The localization analysis by FM4-64 staining in yeast cells was performed under BL irradiation. The results showed that the localization of GFP-CrPHOT under BL was the same as that in the dark and that most of its speckles merged with FM4-64 (62.5%, n = 120; Fig. 2a). To investigate the effect of kinase activity on CrPHOT localization, we observed the localization of GFP-KDm. The localization pattern of GFP-KDm was the same as that of GFP-CrPHOT regardless of the light condition (68.0% in the dark and 57.9% under BL, n = 166 and n = 164, respectively; Fig. 2a). These results suggest that the localization of CrPHOT in yeast cells is not dependent on either BL or its own kinase activity.

Under normal culture conditions, Dnf1p and Dnf2p are primarily localized to early endosomal/TGN compartments and partially to the plasma membrane of the bud and bud neck. Fpk1p is known to colocalize with Dnf1p/Dnf2p at endosomal/TGN compartments and the plasma membrane. Dnf1p and Dnf2p are primarily localized to early endosomal/TGN compartments and partially to the plasma membrane of the bud and bud neck. Fpk1p is known to colocalize with Dnf1p/Dnf2p at endosomal/TGN compartments and the plasma membrane (Fig. 2b). The influence of BL or the introduction of mRFP-KDm on the localization of Dnf1p-GFP was hardly observed (Fig. 2b). The same results were found for the localization of Dnf2p-GFP (Supplementary Information).
Fig. S2). These results suggest that CrPHOT, as is the case with Fpk1p, constitutively colocalizes with Dnf1p/Dnf2p in endosomal/TGN compartments.

Optical control of phospholipid translocation by CrPHOT in a kinase activity-dependent manner. At the plasma membrane, Dnf1p/Dnf2p are involved in flipping phospholipids (mainly PC and PE) and glycosphingolipids13,21,23,26,28,29,30. Fpk1p/Fpk2p regulates phospholipid uptake through Dnf1p/Dnf2p across the plasma membrane32,34,39,43. Since CrPHOT was able to complement Fpk1p/Fpk2p function in yeast cell growth35 (Fig. 1b), BL irradiation likely activates CrPHOT to control phospholipid uptake in the same way as Fpk1p/Fpk2p.

We thus examined whether CrPHOT controls the uptake of nitrobenzoxadiazole (NBD)-labelled phospholipids (NBD-PE and NBD-PC) in the fpk1Δ fpk2Δ mutant. NBD-phospholipids taken up by P4-ATPases are transported mainly to the endoplasmic reticulum (ER) in a short time, and strong fluorescence at the ER membrane is observed30,44. When NBD-PE was added, fluorescence was observed at the ER membrane in wild-type cells but not in the fpk1Δ fpk2Δ mutant (Fig. 3a, WT + vector and fpk1Δ fpk2Δ + vector). The internalized NBD-PE was quantified by flow cytometry of the cells (Fig. 3b). When the fpk1Δ fpk2Δ mutant harboured empty vector, the amount of NBD-PE was significantly decreased as described previously32 (53 ± 8% in the dark and 53 ± 8% under BL relative to wild-type levels, Fig. 3b). When the fpk1Δ fpk2Δ mutant harboured CrPHOT plasmids, intracellular fluorescence was hardly observed and showed a low value in the dark (Fig. 3a; 73 ± 5%, Fig. 3b) but was observed to the same extent as that in the wild type under BL (Fig. 3a; 99 ± 2%, Fig. 3b). In contrast, no promotion of uptake by CrPHOT under BL was observed in the fpk1Δ fpk2Δ lem3Δ background strain (Fig. 3a; 31 ± 9% in the dark and 32 ± 7% under BL for NBD-PE, Fig. 3b). This indicates that NBD-PE uptake promoted by CrPHOT depends on Dnf1p/Dnf2p-Lem3p. The same results were obtained when NBD-PC was added (Fig. 3b, Supplementary Fig. S3). These results suggest that P4-ATPase-mediated phospholipid uptake can be photo-controlled via CrPHOT. In addition, when the fpk1Δ fpk2Δ mutant harboured KDM plasmids, intracellular fluorescence was hardly observed even under BL (Fig. 3a; 50 ± 8% for NBD-PE and 64 ± 13% for NBD-PC under BL, Fig. 3b; Supplementary Fig. S3), suggesting that control by CrPHOT is dependent on its own kinase activity. These results were consistent with the altered sensitivity of the fpk1Δ fpk2Δ mutant to duramycin in a BL-dependent manner (Supplementary Fig. S4)35. Duramycin is a peptide toxin that specifically binds to PE in biological membranes45,46. The fpk1Δ fpk2Δ mutant is sensitive to duramycin because PE is not enriched in the inner leaflet and is exposed on the outer leaflet of the plasma membrane32,34,39,43. CrPHOT suppressed the sensitivity of the fpk1Δ fpk2Δ mutant to duramycin in a BL-dependent manner, and the K-fragment suppressed it regardless of light conditions. In contrast, the fpk1Δ fpk2Δ mutant harbouring KDM showed increased sensitivity to duramycin even under BL (Supplementary Fig. S4)37. Under BL, the resistance activity by LOV1/2 m remained moderate, although it decreased the suppression compared to that of CrPHOT (Supplementary Fig. S4). These results reaffirm the suggestion of controlling phospholipid flipping by light as described above.

We next investigated the time-course of phospholipid uptake in response to BL. Cells grown in the dark were further incubated for a total of 3 h. During incubation, cells were irradiated with different lengths of BL (0, 0.5, 1, 3 h) towards the end of the period. NBD-PE was added 1 h before the end of incubation time (Fig. 3c). As a result of quantification by flow cytometry, the internalization of NBD-PE reached the same level as that of the wild-type cells grown in the dark, but it was distributed throughout the cells under BL as FPK1 was (Fig. 4c; 23.0% in the dark and 79.6% under BL, Fig. 4b). These results suggest that CrPHOT is able to control both actin depolarization and switching of cell growth in a BL-dependent manner.

Light regulates both actin depolarization and switching of apical-isotropic growth. In small-budded cells (at G2/early mitotic phase), cortical actin patches (small assemblage of actin filaments) are polarized at the tip of the bud, and daughter cells exhibit apical growth35. In large-budded cells (at a late mitotic phase), the actin patches are randomly distributed, and the cells switch to isotropic growth47. Dnf1p/Dnf2p-Lem3p and Fpk1p/Fpk2p are involved in actin depolarization and switching from apical to isotropic growth. Therefore, defects in these genes result in prolonged polarization of actin patches at the tip and extended elongation of the bud even at the late mitotic phase31,32. We thus investigated the photo-regulation of Myo2p-Pho80-GFP expression in the fpk1Δ fpk2Δ mutant. Cells cultured in each light condition were stained with phalloidin-TRITC (tetrathydrodiamine-2-phenylindole) to visualize actin and with DAPI (4’,6-diamidino-2-phenylindole) to confirm the cell cycle stage. As a result of quantification by flow cytometry, the internalization of NBD-PE reached the same level as that of the wild-type cells in 1 h BL irradiation (112 ± 7% relative to wild-type levels, Fig. 3c), although the effect of 0.5 h BL was not clear. These data demonstrate that this CrPHOT system results in a superior, light-induced control of flipping activity.
cells expressing the K-fragment regardless of light irradiation (77.2% in the dark and 86.7% under BL), while prolonged polarization was observed even under BL in Kdm- (Fig. 4c; 19.3% in the dark and 25.0% under BL, Fig. 4d) or LOV1/2 m-expressing cells (Fig. 4c; 24.7% in the dark and 46.6% under BL; Fig. 4d). These results suggest that light can regulate cell polarity switching in a CrPHOT kinase activity-dependent manner.

**Light regulates endocytic recycling to the TGN in the CrPHOT system.** Dnf1p/Dnf2p-Lem3p and Fpk1p/Fpk2p, redundantly with Drs2p-Cdc50p, regulate endocytic recycling. The lem3Δ or fpk1Δ fpk2Δ mutant
of Dnf1p are required for the optimal function of Dnf1p32,35. The control of P4-ATPase activity by Fpk1p/Fpk2p
Snc1p and Tlg1p accumulate in abnormal structures in the cell (Fig. 5a, vector)32,33.

Figure 3. CrPHOT promotes NBD-phospholipid flipping by flipases in a BL-dependent manner. (a) NBD-PE internalization by CrPHOT under BL. Wild type (WT) harbouring vector plasmid, fpk1Δ fpk2Δ mutant harbouring vector, CrPHOT or Kdm plasmids, and KKT274 (fpk1Δ fpk2Δ lem3Δ) harbouring vector or
CrPHOT plasmids were grown in SC medium in darkness or under 10 μmol m−2 s−1 BL at 30 °C and treated with NBD-PE. A representative cell image obtained by microscopic observation is shown. Scale bar = 5 μm. (b) Quantification of internalized NBD-labelled phospholipids. Cells grown in SDA-U medium in the dark at 30 °C were labelled with NBD-PE or -PC for 60 min in the dark or under 10 μmol m−2 s−1 BL, and then washed with SD containing 2.5% BSA before flow cytometry. (c) Time course of NBD-labelled PE internalization by BL. Cells grown in the dark were incubated for a total of 3 h in the indicated light condition (dark, BL 0.5 h, BL 1.0 h, or BL 3.0 h), in which the last 1 h was incubated with the NBD-PE. The internalized NBD-phospholipids were quantitated by flow cytometry. Data are presented as the average percentage ± SD relative to wild-type (WT) harbours vector, Cr

Discussion
In this study, we succeeded in photo-controlling phospholipid (such as PC and PE) flipping and biological mem-
brane functions (actin depolarization and endocytic recycling) in yeast. To date, some optogenetic techniques have been established to transiently control the amounts of phosphatidylinositol 4,5-bisphosphate (PI (4,5) P2) and its metabolites in cells using PI-metabolizing enzymes21–23. The potential benefits of the proposed technique include control of the intracellular distribution of non-Pi phospholipids.

With Cdc50p-depletion exhibits severe defects in the retrieval pathway from early endosomes to the TGN22,22,39. We thus investigated whether CrPHOT can regulate this pathway in a light-dependent manner. An exocytic vesicle-SNARE Snc1p is recycled from the plasma membrane via early endosomes to the TGN by this pathway48. mRFP-Snc1p is primarily localized at the plasma membrane of daughter cells during bud formation in partially Cdc50p-depleted fpk2Δ cells (Fig. 5a, FPK1)32. A target-SNARE, Tlg1p, is recycled between the TGN and early endosomes, and thus, Tlg1p-GFP was observed in punctate fluorescence, indicating endosome/TGN localization in this cell (Fig. 5a, FPK1)36. When the retrieval pathway from early endosomes to the TGN is inhibited, Snc1p and Tlg1p accumulate in abnormal structures in the cell (Fig. 5a, vector).32,33.

Plasmids encoding CrPHOT or its derivatives were introduced into the Cdc50p-depleted fpk1Δ fpk2Δ mutant expressing mRFP-Snc1p and GFP-Tlg1p and cultured in the dark or under BL. After fixing the cells with formaldehyde, the localization of those marker proteins was observed using a microscope. In CrPHOT-expressing cells, mRFP-Snc1p and GFP-Tlg1p were observed in the abnormal aggregates in the dark; cells in which mRFP-Snc1p was normally localized in the plasma membrane were hardly observed (Fig. 5a; 13.3%, Fig. 5b, CrPHOT) as in the case of vector (15.0% in the dark, 20.9% under BL). Under BL irradiation, GFP-Tlg1p was localized in the normal punctate structures, and mRFP-Snc1p was localized in the cell periphery (Fig. 5a; 70.7%, Fig. 5b, CrPHOT), as in the case of the cells expressing Fpk1p (Fig. 5a; 74.5% in the dark, 74.2% under BL, Fig. 5b, FPK1). LOV1/2 m exhibited abnormal aggregation of both marker proteins and failed to restore them to normal localization even under BL irradiation (Fig. 5a; 15.5% in the dark, 29.2% under BL, Fig. 5b, LOV1/2 m). These results suggest that the endocytic recycling pathway can be photo-controlled by CrPHOT. We then investigated the necessity for kinase activity in the photo-control of endocytic recycling. As a result, both mRFP-Snc1p and GFP-Tlg1p showed normal localization in cells harbouring the K-fragment regardless of light irradiation (Fig. 5a; 67.1% in the dark and 75.0% under BL, Fig. 5b), but failed for Kdm even under BL (Fig. 5a; 13.9% in the dark and 15.5% under BL, Fig. 5b). These results suggest that the control of endocytic recycling by light requires CrPHOT kinase activity.

How does CrPHOT regulate P4-ATPases? All P4-ATPase-related responses analysed in this study were dependent on the kinase activity of CrPHOT (Figs. 1, 3, 4, 5, Supplementary Fig. S3 and S4). Yeast Fpk1p phosphorylates Dnf1p/Dnf2p-P4-ATPases in vivo and in vitro35,36,37. The kinase activity of Fpk1p and phosphorylation of Dnf1p are required for the optimal function of Dnf1p35,37. The control of P4-ATPase activity by Fpk1p/Fpk2p and control of P4-ATPase activity by CrPHOT under BL irradiation were remarkably consistent; therefore, we conclude that CrPHOT also regulates Dnf1p/Dnf2p by phosphorylation. Some phosphorylation sites of Dnf1p by Fpk1p have been identified, and the combination of mutations at those sites within Dnf1p reduces PE flipping in yeast cells35. The molecular mechanism underlying P4-ATPase regulation by kinase-related phosphorylation is still unknown, but this system that switches the activity of P4-ATPases by light will be quite useful for elucidating it in the future.
Figure 4. Optical control of actin depolarization associated with apical-isotropic growth switching. (a, b) Optical control of F-actin distribution by CrPHOT. Yeast fpk1Δ fpk2Δ cells carrying pKT1639 (pRS416-FPK1) or pRS416-CrPHOT were cultured in darkness (Dark) or under 10 μmol m⁻² s⁻¹ BL irradiation (Blue) at 18 °C, followed by staining with phalloidin-TRITC and DAPI to visualize actin and nuclei, respectively. (a) A representative cell image by microscopic observation. Scale bar = 5 μm. (b) Large-budded cells with divided nuclei were classified as showing actin polarized to the bud tip (grey) or its distribution in whole daughter cells (black). n = 155–253. (c, d) Optical control of Myo2p-GFP localization in a kinase-dependent manner. KKT353 (fpk1Δ fpk2Δ MYO2-GFP) cells carrying pKT1639 (pRS416-FPK1) or pRS416-CrPHOT or its derivatives were cultured in YPDA medium in darkness (Dark) or under BL irradiation (Blue) at 18 °C, followed by staining with DAPI to visualize nuclei. (c) A representative cell image obtained by microscopic observation. Arrowheads indicate Myo2p-GFP polarized to the bud tip. Scale bar = 5 μm. (d) Large-budded cells with divided nuclei were classified as showing Myo2p-GFP distributed in whole daughter cells (black), polarized to the bud tip (grey) or delocalized (white). n = 96–180.
Next, it is necessary to consider the effect of CrPHOT on factors other than Dnf1p/Dnf2p, e.g., other P4-ATPases. In microscopic observation, localization of CrPHOT to TGN/endosomes was mainly observed (Fig. 2, Supplementary Fig. S2). Drs2p and Dnf3p are P4-ATPases mainly localized to the TGN/endosomes. Furthermore, their function in the endocytic recycling pathway is partially redundant with that of Dnf1p/Dnf2p\(^\text{22,23,24,33}\). Drs2p and Dnf3p are also phosphorylated by Fpk1p in vitro\(^\text{32,36}\), and Dnf3p is isolated as a phosphorylation substrate for Fpk1p/Fpk2p in vivo\(^\text{34,35}\). However, the necessity of Fpk1p/Fpk2p activity to the functions of Drs2p and Dnf3p is currently unknown. Further analysis is needed to determine whether CrPHOT regulates the functions of other P4-ATPases, such as Drs2p and Dnf3p.

Another is the effects of Fpk1p/Fpk2p on factors other than P4-ATPases. The protein kinases Ypk1p and Akl1p are known as phosphorylation substrates for Fpk1\(^\text{34,35,36,43}\). Target of rapamycin complex 2 (TORC2) serves as a sensor and regulator for plasma membrane status and is involved in actin-cytoskeleton regulation, sphingolipid synthesis, and endocytosis in \textit{S. cerevisiae}\(^\text{52,53}\). Under plasma membrane stresses, TORC2 phosphorylates and negatively regulates Fpk1p through activation of Ypk1p\(^\text{34}\). Fpk1p activated under normal conditions suppresses the upstream inhibitor Ypk1p by phosphorylation and promotes the endocytic pathway from the plasma membrane through inhibition of Akl1p by phosphorylation, independent of Dnf1p/Dnf2p activation\(^\text{35,36}\).

To investigate the involvement of CrPHOT in this endocytic pathway, CrPHOT or FPK1 was introduced into the \textit{PGAL1-CDC50 fpk1Δ fpk2Δ} strain, and endocytosis from the plasma membrane was observed by FM4-64 staining. Unfortunately, the \textit{PGAL1-CDC50 fpk1Δ fpk2Δ} cells failed to show any FM4-64 uptake delay even when the corresponding empty vector was introduced, despite experiments at various temperatures (4–24 °C) and depression conditions (data not shown). This observation indicated that the endocytic pathway functioned properly even in the absence of Dnf1p/Dnf2p-Lem3p and Drs2p-Cdc50p in our experimental conditions. Therefore, the involvement of CrPHOT in endocytosis could not be investigated. We would need to investigate whether CrPHOT regulates this pathway, including the phosphorylation of Akl1p and Ypk1p.

RSK3 (belonging to the p90-S6K subfamily) and Ca\(^{2+}\)-dependent protein kinase C (PKC) are known as kinases that would be involved in phosphorylation of P4-ATPases in mammals. RSK3 has been identified as a functional counterpart of Fpk1p/Fpk2p in yeast screening\(^\text{43}\), but P4-ATPase regulation and phosphorylation in mammalian cells are not understood. PKC controls the endocytosis of the P4-ATPase ATP11C (relates to B-cell maturation,
erythrocyte shape, anaemia and hyperbilirubinemia), and phosphorylation of the ATP11C C-terminal region is required for endocytosis\(^{34}\). Hence, P4-ATPases are likely regulated by kinases in many eukaryotes, and CrPHOT may also function in mammalian cells. Although an analysis of photo-reversibility and improvement in controllability are required, this study is able to propose the basis of a P4-ATPase light control system.

### Methods

#### Media and growth conditions.

Yeast strains were cultured in YPDA-rich medium (1% yeast extract [BD Biosciences, San Jose, CA], 2% bacto-peptone [BD], 2% glucose [Nacalai Tesque, Kyoto, Japan], and 0.01% adenine [FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan]). Strains carrying plasmids were selected in synthetic medium (SD) containing the required nutritional supplements\(^{35}\). When appropriate, 0.5% casamino acids [BD] were added to SD medium without uracil [FUJIFILM Wako (SD-Ura)]. For induction of the GAL1 promoter, 3% galactose [FUJIFILM Wako] and 0.2% sucrose [Nacalai Tesque, Kyoto, Japan] were used as carbon sources instead of glucose (YPG and SGA-Ura). Blue (peak at 470 nm) and red (peak at 660 nm) light-emitting diode panels [SL-150X150 series; CCS, Tokyo, Japan] were used as light sources. In all analyses, BL and RL were used at an intensity of 10 μmol m\(^{-2}\) s\(^{-1}\) unless otherwise noted. When grown on the agar medium, the yeast cells were irradiated with light vertically from a height of about 10 cm above the plate. When cultured in a liquid medium, the entire test tube was irradiated with light from a distance of about 10 cm from the side of the tube. *Escherichia coli* strains were cultured in LB medium [Nacalai] containing appropriate antibiotics as needed. The lithium acetate method was used to introduce plasmids into yeast cells\(^{36,37}\).

#### Strains and plasmids.

The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast strains carrying monomeric red fluorescent protein (mRFP)-tagged SNC1 were constructed by integrating linearized pRS306-mRFP-SNC1 into the *URA3* locus, followed by a marker change from *URA3* to *TRP1*. Strain carrying *SEC7* mRFP was constructed by PCR-based procedures as described\(^{38,39}\). *E. coli* strain DH5α was used for the construction and amplification of plasmids.

The plasmids used in this study are listed in Table 2. pRS416-GFP-CrPHOT, pRS416-mRFP-CrPHOT, pRS416-GFP-KDm and pRS416-mRFP-KDm were constructed as follows. GFP- or mRFP-tagged *CrPHOT* or *CrPHOT*(DS49N)\(^{40}\) was constructed by megaprimer PCR-based procedures\(^{40}\) and cloned into the BamHI/SalI site of pRS416\(^{41}\). pRS416-LOV1/2 m was generated using a QuikChange site-directed mutagenesis kit [Agilent Technologies, Santa Clara, CA] with pRS416-LOV2m\(^{17}\). The genes inserted into pRS416 were constitutively expressed in a form targeted with two N-terminal tandem repeats of the influenza virus haemagglutinin epitope (2HA) under control of the *TPI1* promoter. All regions constructed by PCR-based procedures were verified by DNA sequencing.

#### Yeast growth assay.

The yeast cells were grown at 28 °C in liquid SGA-Ura medium to an A\(_{600}\) of 0.6–0.8 and diluted with sterile water to an A\(_{600}\) of 0.1. Ten-fold serial dilutions (10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\)) were made in sterile water. A 10-μl aliquot of each of the diluted cell suspensions was then spotted on a plate with SGA-Ura or SDA-Ura medium. The plates were then placed at 28 °C under different light conditions for 3 days. For growth sensitivity to duramycin, the yeast cells grown in liquid SDA-Ura were diluted as above, and 2 μl of each of the diluted cell suspensions were examined on YPDA plates containing 20 μM duramycin [Sigma-Aldrich, St. Louis, MO] at 28 °C.

#### Immunoblot analysis.

Yeast cells were grown to logarithmic phase in YPGA medium at 28 °C under different light conditions. Protein extraction from yeast cells was performed as described\(^{40}\). The extracted proteins (40 μg) were separated in a 4–15% SDS-polyacrylamide gel [Bio-Rad Laboratories, Hercules, CA] and blotted onto polyvinylidene difluoride (PVDF) membrane [Bio-Rad], and immunoblotting was performed using mouse anti-HA monoclonal antibody [MBL, Nagoya, Japan] and horseradish peroxidase (HRP)-conjugated anti-mouse IgG [Promega, Madison, WI]. The signals were detected using an LAS-3000 imaging system [FUJIFILM, Tokyo, Japan].

### Table 1.

| Yeast strains used in this study. | KKT strains are isogenic derivatives of BY4743. YKT strains are isogenic derivatives of YEF473. |
|----------------------------------|---------------------------------------------------------------------------------------------------|

---

### Table 2.

| Strain | Relevant genotype | Derivation/source |
|--------|-------------------|-------------------|
| BY4743 | MATa/α LYS2/α ara3Δ0/ara3Δ0 his3Δ1/α his3Δ1 leu2Δ0/α leu2Δ0 met15Δ0/Δ MET15 | Brachmann et al., 1998\(^{43}\) |
| YEF473 | MATa/α lys2-810/α lys2-810 ara3-52/ara3-52 his3Δ-200/Δ his3Δ-200 trp1Δ-63/trp1Δ-63 leu2Δ-1/Δ leu2Δ-1 | Bi and Pringle, 1990\(^{44}\) |
| KKT330 | MATa/α LYS2/α his3Δ1 leu2Δ0/Δ MET15 HIS3MX6-Pcα7, 3HA-CDC50 fpk1Δ:HphMX4 fpk2Δ:KanMX6 (designated here as Pcα7, CDC50 fpk1Δ fpk2Δ) | Nakano et al., 2008\(^{32}\) |
| KKT268 | MATa/α LYS2/α his3Δ1 leu2Δ0/Δ MET15 fpk1Δ:HphMX4 fpk2Δ:KanMX6 (designated here as fpk1Δ fpk2Δ) | Nakano et al., 2008\(^{32}\) |
| KKT353 | MATa/α LYS2/α his3Δ1 leu2Δ0/Δ MET15 MYO2-GFP; HIS3MX6 fpk1Δ:HphMX4 fpk2Δ:KanMX6 | Nakano et al., 2008\(^{32}\) |
| YKT905 | MATa/α ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 lys2-801 SEC7-mRFP::TRP1 | Sakane et al., 2006\(^{65}\) |
| KKT492 | MATa/α LYS2/α his3Δ1 leu2Δ0/Δ MET15 HIS3MX6-Pcα7, 3HA-CDC50 fpk1Δ:HphMX4 fpk2Δ:KanMX6 ura3::TRP1::mRFP-SNC1 | This study |
| KKT332 | MATa/α lys2Δ0/Δ his3Δ1 leu2Δ0 met15Δ0 DNF1-GFP::HIS3MX6 fpk1Δ:HphMX4 fpk2Δ:KanMX6 | Nakano et al., 2008\(^{32}\) |
| KKT336 | MATa/α LYS2/α his3Δ1 leu2Δ0/Δ MET15 DNF2-GFP; HIS3MX6 fpk1Δ:HphMX4 fpk2Δ:KanMX6 | Nakano et al., 2008\(^{32}\) |
| KKT274 | MATa/α LYS2/α his3Δ1 leu2Δ0/Δ MET15 leu3Δ3:KanMX6 fpk1Δ:HphMX4 fpk2Δ:His3MX6 | This study |

---

Scientific Reports, Vol:.(1234567890), pp.1-10, https://doi.org/10.1038/s41598-020-69459-0

---

---

---
**Microscopic observations.** Microscopic experiments were performed as previously described. Yeast cells were grown to early-midlogarithmic phase in YPDA liquid medium at 18 °C in darkness or under BL irradiation. All experiments requiring dark conditions were performed in a dark room with the aid of green safe light. Most GFP- or mRFP-tagged proteins were observed in living cells, which were observed using a Fluoview FV1000 confocal microscope [Olympus Corporation, Tokyo, Japan]. The lipophilic styryl dye FM4-64 [Thermo Fisher Scientific, Waltham, MA, USA] was used to visualize endosomal structures. Cells grown in the logarithmic phase were fixed by the addition of formaldehyde (3.7% final concentration) [FUJIFILM Wako] into the medium and incubated at 18 °C for 10 min and then 30 °C for 10 min. After fixation, cells were washed twice with phosphate-buffered saline (PBS) and immediately observed using a confocal microscope. To observe Myo2p-GFP in cells with divided nuclei, cells fixed as above were stained with DAPI (0.5 μg ml⁻¹ final concentration) and then incubated at 25 °C for 20 min. Labelled cells were immediately observed using a confocal microscope. Localization of mRFP-Snc1p and GFP-Tlg1p or Sec7p-mRFP was observed in fixed cells. Cells grown in the logarithmic phase were harvested by centrifugation, washed twice with ice-cold SC medium, resuspended in 100 μl of water at room temperature for 10 min. To visualize F-actin, cells were fixed by the addition of formaldehyde (5.0% final concentration) into the medium and incubated at 18 °C for 10 min and then 25 °C for 30 min. After fixation, the cells were labelled with phalloidin-TRITC (0.1925 μM final concentration) [Sigma-Aldrich] in PBS at room temperature for 30 min, washed three times with PBS, and then stained with DAPI as described above. Cells stained with DAPI were washed three times with water and immediately observed using a BX51 biological microscope [Olympus] with the appropriate fluorescence filter sets [Olympus]. Images were acquired with an ORCA-fusion digital CMOS camera [C14440-20UP; Hamamatsu Photonics, Hamamatsu, Japan].

**Internalization of fluorescence-labelled phospholipids into yeast cells.** Large unilamellar vesicles containing NBD-phospholipids were prepared as described. 1-palmitoyl-2-(6-NBD-aminocaproyl)-PE (NBD-PE), 1-palmitoyl-2-(6-NBD-aminocaproyl)-PC (NBD-PC), and dioleoylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Fluorescently labelled phospholipid internalization experiments were performed as described. Briefly, cells were grown to early logarithmic phase in SDA-U medium at 30 °C in the dark. After dilution to 0.35 A600 ml⁻¹, cells were incubated for 60 min at 30 °C with liposomes containing 40% NBD-phospholipid and 60% DOPC at a final concentration of 20 μM in the dark or under BL. Cells were then suspended in cold SD containing 20 mM sodium azide and 2.5% bovine serum albumin (BSA), incubated for 20 min, and washed with PBS. Flow cytometry of fluorescently labelled cells was performed on a FACSCanto II cytometer [BD]. For investigation of time response, overnight cultures were diluted to 0.2 A600 ml⁻¹ and incubated for a total of 3 h in the indicated condition (dark, BL 0.5 h, BL 1.0 h, or BL 3.0 h), in which

| Plasmid Characteristics | Derivation/source |
|-------------------------|------------------|
| CYplac111               | LEU2 CEN4        |
| YPlac181                | LEU2 2 μm        |
| YPlac195                | URA3 2 μm        |
| pKO10                   | Ura3::HA URA3 2 μm |
| pRS416                  | URA3 CEN6        |
| pRS315                  | LEU2 CEN6        |
| pKT1634 [pRS416-GFP-FPK1]| P<sub>TIR</sub>-GFP-FPK1 URA3 CEN6 |
| pKT1638 [pRS416-mRFP-FPK1]| P<sub>TIR</sub>-mRFP-FPK1 URA3 CEN6 |
| pKT1639 [pRS416-HA-FPK1]| P<sub>TIR</sub>-HA-FPK1 URA3 CEN6 |
| pKT1651 [pRS315-GFP-TLG1]| P<sub>TIR</sub>-GFP-TLG1 LEU2 CEN6 |
| pKT2177 [pRS306-mRFP-SNC1]| P<sub>TIR</sub>-mRFP-SNC1 URA3 |
| pRS416-CrPHOT           | P<sub>TIR</sub>-HA-CrPHOT URA3 CEN6 |
|                         | (identical to Kinase-fragment in Aihara et al., 2012<sup>37</sup>) |
| pRS416-K-fragment       | P<sub>TIR</sub>-HA-GPHOTAN URA3 CEN6 |
|                         | (identical to Kinase-dead [D549N] in Aihara et al., 2012<sup>27</sup>) |
| pRS416-KDm              | P<sub>TIR</sub>-HA-GPHOT[D549N] URA3 CEN6 |
|                         | (identical to Kinase-dead [D549N] in Aihara et al., 2012<sup>27</sup>) |
| pRS416-LOV2m            | P<sub>TIR</sub>-HA-GPHOT(C250A) URA3 CEN6 |
|                         | (identical to (C250A) in Aihara et al., 2012<sup>27</sup>) |
| pRS416-LOV1/2 m         | P<sub>TIR</sub>-HA-GPHOT(C57A, C250A) URA3 CEN6 |
| pRS416-GFP-CrPHOT       | P<sub>TIR</sub>-HA-GFP-CrPHOT URA3 CEN6 |
| pRS416-mRFP-CrPHOT      | P<sub>TIR</sub>-HA-mRFP-CrPHOT URA3 CEN6 |
| pRS416-GFP-KDm          | P<sub>TIR</sub>-HA-GFP-CrPHOTΔN URA3 CEN6 |
| pRS416-mRFP-KDm         | P<sub>TIR</sub>-HA-mRFP-CrPHOTΔN URA3 CEN6 |

Table 2. Plasmids used in this study.
Received: 27 January 2020; Accepted: 8 July 2020
Published online: 27 July 2020

References

1. Zachowski, A. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem. J. 294, 1–14 (1993).
2. Pomorski, T., Hrafnsdóttir, S., Devaux, P. F. & van Meer, G. Lipid distribution and transport across cellular membranes. Semin. Cell Dev. Biol. 12, 139–148 (2001).
3. Muthusamy, B.-P., Natarajan, P., Zhou, X. & Graham, T. R. Linking phospholipid flippases to vesicle-mediated protein transport. Biochim. Biophys. Acta 1791, 612–619 (2009).
4. Emoto, K. et al. Redistribution of phosphatidylethanolamine at the cleavage furrow of dividing cells during cytokinesis. Proc. Natl. Acad. Sci. USA 93, 12867–12872 (1996).
5. Luo, J. et al. Phosphatidylethanolamine Is Required for Normal Cell Morphology and Cytokinesis in the Fission Yeast S. cerevisiae. PLoS One 3, e3313 (2008).
6. Groves, J. T. & Kuriyan, J. Molecular mechanisms in signal transduction at the membrane. Nat. Struct. Mol. Biol. 17, 659–665 (2010).
7. Kay, J. G. & Fairn, G. D. Distribution, dynamics and functional roles of phosphatidylserine within the cell. Cell. Mol. Life Sci. 71, 17, 126 (2019).
8. Kato, U. et al. Role for phospholipid flippase complex of ATP8A1 and CDC50A proteins in cell migration. J. Biol. Chem. 288, 4922–4934 (2013).
9. O’Donnell, V. B., Rossjohn, J. & Wakelam, M. J. O. Phospholipid signaling in innate immune cells. J. Clin. Investig. 128, 2670–2679 (2018).
10. Montigny, C., Lyons, J., Champell, P., Nissen, P. & Lenoir, G. On the molecular mechanism of flippase- and scramblase-mediated phospholipid transport. Biochim. Biophys. Acta 1861, 767–783 (2016).
11. Andersen, J. P. et al. P4-ATPases as phospholipid flippases—structure, function, and enigmas. Front. Physiol. 7, 275 (2016).
12. Palmgren, M., Österberg, J. T., Nintemann, S. J., Poulsen, L. R. & López-Marqués, R. L. Evolution and a revised nomenclature of P4-ATPases, a eukaryotic family of lipid flippases. Biochim. Biophys. Acta 1861, 1135–1151 (2019).
13. Panatella, R., Henrich, H. & Holthuis, J. C. M. Inner workings and biological impact of phospholipid flippases. J. Cell Sci. 128, 2021–2032 (2015).
14. Lopez-Márquez, R. L., Theorin, L., Palmgren, M. G. & Pomorski, T. G. P4-ATPases: lipid flippases in cell membranes. Pfug. Arch. 466, 1227–1240 (2014).
15. Nintemann, S. J., Palmgren, M. & López-Marqués, R. L. Catch you on the flip side: a critical review of flippase mutant phenotypes. Trends Plant Sci. 24, 468–478 (2019).
16. Lee, S. et al. Transport through recycling endosomes requires EHD 1 recruitment by a phosphatidylserine translocase. EMBO J. 34, 669–688 (2015).
17. Bost, J. T., Xu, P. & Graham, T. R. Phospholipid flippases in membrane remodeling and transport carrier biogenesis. Curr. Opin. Cell Biol. 29, 8–15 (2019).
18. Yeung, T. et al. Membrane phosphatidylserine regulates surface charge and protein localization. Science 319, 210–213 (2008).
19. Uchida, Y. et al. Intracellular phosphatidylserine is essential for retrograde membrane traffic through endosomes. Proc. Natl. Acad. Sci. USA 108, 15846–15851 (2011).
20. Aoki, Y., Uenaka, T., Aoki, J., Umeda, M. & Inoue, K. A novel peptide probe for studying the transvillayer movement of phosphatidylethanolamine. J. Biochem. (Tokyo) 116, 291–297 (1994).
21. Iwamoto, K. et al. Local exposure of phosphatidylethanolamine on the yeast plasma membrane is implicated in cell polarity: polarized PE exposure on plasma membrane. Genes Cells 9, 891–903 (2004).
22. Hua, Z., Fatheddin, P. & Graham, T. R. An essential subfamily of Drs2p-related P-Type ATPases is required for protein trafficking between golgi complex and endosomal/vacuolar system. Mol. Biol. Cell 13, 3162–3177 (2002).
23. Pomorski, T. et al. Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. Mol. Biol. Cell 14, 1240–1254 (2003).
24. Liu, K., Hua, Z., Nepute, J. A. & Graham, T. R. Yeast P4-ATPases Drs2p and Dnf1p are essential cargoes of the NPFXD/Sla1p endocytic pathway. Mol. Biol. Cell 18, 487–500 (2007).
25. Sartorel, E., Barrey, E., Lau, R. K. & Thorner, J. Plasma membrane aminoglycerolipid flippase function is required for signaling competence in the yeast mating pheromone response pathway. Mol. Biol. Cell 26, 134–150 (2015).
26. Baldridge, R. D., Xu, P. & Graham, T. R. Type IV P-type ATPases distinguish mono- versus diacyl phosphatidylserine using a cytosolic exit gate in the membrane domain. J. Biol. Chem. 288, 19516–19527 (2013).
27. Riekhof, W. R. et al. Lyso-phosphatidylcholine Metabolism in Saccharomyces cerevisiae: the role of p-type ATPases in transport and a broad specificity acyltransferase in acylation. J. Biol. Chem. 282, 36683–36681 (2007).
28. Roland, B. P. et al. Yeast and human P4-ATPases transport glycosphingolipids using conserved structural motifs. J. Biol. Chem. 294, 1794–1806 (2019).
29. Saito, K. et al. Cdc25p, a protein required for polarized growth, associates with the Drs2p P-type atpase implicated in phospholipid translocation in Saccharomyces cerevisiae. Mol. Biol. Cell 15, 3418–3432 (2004).
30. Kato, U. et al. A novel membrane protein, Ros3p, is required for phospholipid translocation across the plasma membrane in Saccharomyces cerevisiae. J. Biol. Chem. 277, 37853–37862 (2002).
31. Saito, K. et al. Transblayer Phospholipid Flipping Regulates Cdc42p Signaling during Polarized Cell Growth via Rga GTPase-Activating Proteins. Dev. Cell 13, 743–751 (2007).
32. Nakano, K., Yamamoto, T., Kishimoto, T., Noji, T. & Tanaka, K. Protein kinases Fpk1p and Fpk2p are novel regulators of phospholipid asymmetry. Mol. Biol. Cell 19, 1783–1797 (2008).
33. Furuta, N., Fujimura-Kamada, K., Saito, K., Yamamoto, T. & Tanaka, K. Endocytic Recycling in Yeast Is Regulated by Putative Phospholipid Translocases and the Ypt31p/32p–Rcy1p Pathway. Mol. Biol. Cell 18, 295–312 (2007).
34. Roelants, F. M., Baltz, A. G., Trott, A. E., Fereses, S. & Thorner, J. A protein kinase network regulates the function of aminophospholipid flippases. Proc. Natl. Acad. Sci. USA 107, 34–39 (2010).
35. Roelants, F. M. et al. TOR complex 2-regulated protein kinase Fpk1 stimulates endocytosis via inhibition of Ark1/Pkr1-related protein kinase Akl1 in Saccharomyces cerevisiae. Mol. Cell. Biol. 37, e00627 (2017).
37. Aihara, Y. 38. Matsuoka, D. & Tokutomi, S. Blue light-regulated molecular switch of Ser/Thr kinase in phototropin. Reprints and permissions information Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-69459-0.

The authors declare no competing interests.

Author contributions T.S. conceived and conducted most of the experiments and data analysis and wrote the paper. T.M. and K.T. advised on most of experiments, and designed and conducted the lipid incorporation analysis. A.N. supervised the project and edited the manuscript. All authors read and approved the final manuscript.

Competing interests The authors declare no competing interests.

Additional information Supplementary information is available at https://doi.org/10.1038/s41598-020-69459-0.

Correspondence and requests for materials should be addressed to T.S. or A.N.

Reprints and permissions information is available at www.nature.com/reprints.
Publisher’s note  Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access  This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020