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Regulation of a Golgi Flippase by Phosphoinositides and an ArfGEF

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SUMMARY

The essential role for phosphatidylinositol 4-phosphate (PtdIns[4]P) in vesicle-mediated protein transport from the *trans*-Golgi network (TGN) was first described in budding yeast1–3. However, the identity of downstream effectors of PtdIns[4]P in this system has been elusive. Here, we show that Drs2p, a type IV P-type ATPase required for phospholipid translocase (flippase) activity and transport vesicle budding from the TGN4–8, is an effector of PtdIns[4]P. Drs2p-dependent flip of a fluorescent phosphatidylserine analogue across purified TGN membranes requires synthesis of PtdIns[4]P by the PtdIns 4-kinase Pik1p. PtdIns[4]P binds to a regulatory domain in the C-terminal tail of Drs2p that has homology to a split PH domain and is required for Drs2p activity. In addition, basic residues required for phosphoinositide binding overlap a binding site for the ArfGEF Gea2p that was previously mapped9. ArfGEF binding to this C-terminal domain also stimulates flippase activity in TGN membrane preparations. These interactions imply the presence of a novel coincidence detection system used to activate phospholipid translocation at sites of vesicle formation.

Phosphoinositides exert control over numerous aspects of cell physiology, including signal transduction cascades, cytoskeletal dynamics, cell motility and protein trafficking10–12. In the latter role, phosphoinositides provide unique signposts that help establish organelle identity through recruitment of effectors13. For example, PtdIns[4]P is predominantly found in TGN membranes where in mammalian cells it recruits AP-1, GGA and epsinR adaptors to facilitate budding of clathrin-coated vesicles, as well as a glucosylceramide transfer protein

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests

AUTHOR CONTRIBUTIONS

P.N. performed most of the project planning, experimental work, data analysis and manuscript preparation. Experimental work was also contributed by K.L. (Fig 1b), D.V.P (Fig. 4c) V.A.S. (Fig 1a), and C.L.J. (Fig, 5c). TRG contributed to project planning, data analysis and manuscript preparation.

(FAPP) required for budding of exocytic carriers14–18. In yeast, GGA clathrin adaptors and oxysterol binding proteins require Pik1p activity for TGN association19–21, but other effectors must be present to explain the spectrum of protein transport defects observed in *pik1^{ts}* cells1–3, 22. Drs2p activity is required for the formation of one exocytic vesicle class as well as AP-1/clathrin-coated vesicles4, 7. The discovery of a strong genetic interaction between *drs2* and *pik1^{ts}* suggested a potential biochemical relationship between PtdIns[4]P and flippase activity at the TGN23.

To determine if Drs2p acts upstream of Pik1p to facilitate PtdIns[4]P synthesis, we compared phosphoinositide levels in wild-type (WT) and *drs2*Δ cells labeled with myo-[^{3}H]inositol. No significant difference was observed between *drs2*Δ and WT cells in the relative amount of any of the four major phosphoinositide species produced in *Saccharomyces cerevisiae*, including PtdIns[4]P. In addition, membrane localization of Osh1-PH-GFP, which specifically binds PtdIns[4]P in the TGN21, was also unaffected by loss of Drs2p (Fig. 1). Thus, Drs2p is not required for the synthesis of PtdIns[4]P at the TGN, nor does Drs2p influence the synthesis of any other phosphoinositide.

Next we tested whether PtdIns[4]P influences Drs2p-dependent phospholipid flippase activity. TGN membranes were purified from WT cells and cells carrying temperature-sensitive (ts) alleles of *DRS2* (*drs2^{ts}*) or *PIK1* (*pik1^{ts}*), which encode proteins that are functional at 27°C but lose activity at the nonpermissive temperature of 37°C. These strains were grown at 27°C to maintain normal structure and function of the Golgi before purifying the TGN. The WT and *pik1^{ts}* membranes were first incubated with γ^{32}P ATP at 27°C and 37°C to determine if the TGN membranes retained PtdIns 4-kinase (*pik1^{ts}*) activity and if the ts kinase could be inactivated by temperature shift *in vitro*. Labeled lipids were extracted and separated by thin-layer chromatography. Robust synthesis of PtdIns[4]P was detected with both WT and *pik1^{ts}* TGN membranes at 27°C. Relative to WT membranes, PtdIns[4]P production was significantly reduced, although not eliminated, in *pik1^{ts}* membranes incubated for two hours at the nonpermissive temperature of 37°C (Fig. 2a–b). A relatively minor amount of PtdIns[3]P synthesis was detected with these membrane preparations, but synthesis of this lipid was not significantly altered by *pik1^{ts}* inactivation.

The WT, *pik1^{ts}* and *drs2^{ts}* TGN membranes were then assayed for Drs2p-dependent flippase activity at both the permissive and nonpermissive temperature. NBD-phosphatidylserine (NBD-PS) was incorporated into the TGN membrane on ice. The presence of the NBD fluor and short acyl chain (six-carbon) in the *sn-2* position makes this lipid sufficiently water soluble to allow selective incorporation into, and subsequent back-extraction from, the TGN cytosolic leaflet. Before incubation at higher temperature (Fig. 2c–e, 0 hrs), 100% of the NBD-PS probe could be back-extracted onto fatty acid free bovine serum albumin (BSA), indicating that the probe resided in the cytosolic leaflet. However, during incubation at 27°C or 37°C without ATP, spontaneous flip-flop allowed a portion of the NBD-PS probe to slowly diffuse into the inner lumenal leaflet, where it was resistant to back-extraction by BSA. At two hours, ATP was added to half of each membrane sample to stimulate the Drs2p-dependent flippase activity as well as synthesis of PtdIns[4]P. For WT, *pik1^{ts}* and *drs2^{ts}* membranes incubated at 27°C, addition of ATP stimulated NBD-PS translocation to the cytosolic leaflet relative to the same membranes without ATP (Fig. 2c, e and g). WT

membranes retained flippase activity at 37°C (Fig. 2d); however, inactivation of PtdIns 4-kinase by incubation of *pik1^{ts}* membranes at 37°C dramatically reduced the flippase activity in these membranes (Fig. 2f). As previously shown6, TGN membranes from a *drs2^{ts}* strain also showed a loss of NBD-PS flippase activity at the nonpermissive temperature of 37°C (Fig. 2h). To facilitate comparisons, the difference in the percentage of NBD-PS in the cytosolic leaflet of wild-type TGN membranes after 4 hours at 37°C with or without ATP was defined as 100% NBD-PS flippase activity (Fig. 2d, asterisk) and used to normalize flippase assay results for other conditions tested (Fig. 3).

The modest flippase activity remaining in *pik1^{ts}* membranes at 37°C could be stimulated to near WT levels by incorporation of exogenous PtdIns[4]P (at 3.3 mol% of the total endogenous phospholipid) into the TGN membrane (Fig. 3a). Thus, the Pik1p requirement was bypassed by addition of its lipid product. Addition of PtdIns[3]P also stimulated NBD-PS flippase activity, although not as potently as PtdIns[4]P, and PtdIns[5]P was least effective (Fig. 3a). TGN membranes were also purified from strains carrying temperature-conditional alleles of sole yeast PtdIns 3-kinase (*vps34^{ts}*). NBD-PS flippase activity in the TGN membranes from the *vps34^{ts}* strain was not significantly different from WT (Fig. 3a). Addition of wortmannin, a PtdIns 3-kinase inhibitor, modestly reduced flippase activity in wild-type membranes (Fig. 3b). The most effective tool for eliminating phosphoinositide synthesis in the TGN membranes was the Sac1 phosphoinositide phosphatase domain from Inp53p. Addition of sufficient GST-Sac1 to destroy both PtdIns[3]P and PtdIns[4]P in the TGN membrane abrogated flippase activity in wild-type membranes while GST alone had no effect (Fig. 3b). These data indicate that the Drs2p-dependent flippase activity requires phosphoinositides and is primarily stimulated by PtdIns[4]P produced by Pik1p.

A mechanism for regulating Drs2p-dependent flippase activity by phosphoinositides was suggested by the following observations. We had previously found that the cytosolic C-terminal tail (C-tail) of Drs2p is required for the function of Drs2p in protein trafficking from the Golgi *in vivo*9, 24. The C-tail sequences responsible for this essential function were mapped to residues 1259 – 1282, which impinges on a stretch of amino acids that are highly conserved in mammalian Drs2p homologs (1274 – 1296) and an ArfGEF binding site (1250–1270)9. More recently, we noticed a short region of homology between residues 1268–1289 in the Drs2p C-tail and part of the split PH domain (also called the GLUE domain) of Vps36p (Fig. 4a). The R261 residue of Vps36p, directly associates with PtdIns[3]P and is within a loop N-terminal to an alpha-helical segment of the split PH domain25. Within this region of homology, Drs2p has a patch of basic resides followed by a predicted alpha-helical segment. We hypothesized that this basic patch of residues (RMKKQR) within the functionally essential C-tail region conferred regulation of Drs2p by PtdIns[4]P.

To test if the basic patch can interact with phosphoinositides, we purified the membrane proximal two thirds of the Drs2p C-tail (residues 1219 – 1305) containing either the WT basic patch (RMKKQR), a deletion of the basic patch (ΔRMKKQR), or basic residue to alanine mutations (AMAAQA). This part of the Drs2p C-tail contains two other predicted alpha-helices (boxes in the C-tail region of Fig. 4a) and based on the circular dichroism spectrum, the purified His6-tagged C-tail has significant alpha-helical content

(Supplementary information, Fig. S1). The wild-type (RMKKQR) C-tail bound specifically to phosphoinositides immobilized on nitrocellulose with preference for PtdIns[4]P and PtdIns[3,4,5]P_3 (Fig. 4b and Supplementary information, Fig. S2a). In contrast, binding of mono- and di-phosphorylated phosphoinositides, and particularly PtdIns[4]P, was substantially reduced by the AMAAQA and ΔRMKKQR mutations. Binding of PtdIns[3,4,5]P_3 was unaffected by the AMAAQA mutations (Fig. 4b and Supplementary information, Fig. S2a), but this phosphoinositide is not produced in yeast and so the basis for this interaction was not further pursued. Among the other phospholipids tested, only the negatively charged PS and phosphatidic acid (PA) interacted with the WT C-tail above background levels (Fig. 4b and Supplementary information, Fig. S2a). In binding assays with liposomes, the WT C-tail preferentially interacted with phosphatidylcholine (PC)/PE membranes that contain 3 mol% PtdIns[4]P relative to control PC/PE or PC/PE/PI liposomes. C-tail interaction was also observed with liposomes containing PtdIns[5]P, PtdIns[3]P and PS (Supplementary information, Fig. S2b).

If the C-tail interaction with phosphoinositides is required for Drs2p activity, then mutation of the basic residues in the context of full length Drs2p should perturb the function of this protein *in vivo*. Plasmids carrying the *drs2* alleles shown in Fig. 4c were tested for their ability to complement the cold-sensitive growth defect of *drs2*Δ. The *drs2*Δ strain carrying an empty plasmid control (Fig. 4c, *drs2*Δ) showed normal growth at 30°C and the strong growth defect at 20°C typical for cells deficient for Drs2p activity. The wild-type (WT) *DRS2* gene fully complemented the cold-sensitive growth defect. In contrast, *drs2* alleles carrying the basic patch to alanine C-tail mutations (*drs2-AMAAQA*) or a deletion of the basic patch (*drs2-*Δ*RMKKQR*) failed to fully complement the *drs2*Δ mutant. Deletion of the predicted alpha-helical segment homologous to Vps36p (*drs2-*Δ*GFAFSQAEE*), or the Gea2p binding region (*drs2-*Δ*1250-1263*), perturbed complementation, although not as drastically as mutation of the basic patch. A western blot showed no difference in the stability of the mutant Drs2 proteins relative to WT Drs2p (Supplementary information, Fig. S3a).

To further assess the influence of the basic patch on Drs2p activity, we purified TGN membranes from the wild-type, *drs2-AMAAQA* and *drs2-*Δ*RMKKQR* strains to assay for NBD-PS flippase activity. An equivalent amount of Drs2p was recovered in each TGN sample, indicating that the basic patch is not required for TGN localization of Drs2p (Supplementary information, Fig. S3a). However, NBD-PS flippase activity was strongly abrogated by mutation of the C-tail basic patch (Fig. 4d). The *drs2-*Δ*RMKKQR* mutant displayed less activity than *drs2-AMAAQA*, consistent with the more severe growth defect of cells expressing *drs2-*Δ*RMKKQR* relative to *drs2-AMAAQA* (Fig. 4c and d).

The RMKKQR basic patch overlaps a binding site for the Sec7 (exchange factor) domain of the ArfGEF Gea2p that we had previously mapped (Fig.4a)9. Therefore, we tested the influence of the ΔRMKKQR and AMAAQA mutations on ArfGEF interaction. The basic patch deletion partially disrupted the Gea2p interaction although the alanine mutations had no effect (Fig. 5a). This observation suggested that the more severe effect of the ΔRMKKQR mutation on flippase activity and *in vivo* function was caused by loss of both phosphoinositide and ArfGEF interaction.

To test the influence of ArfGEF interaction with Drs2p on flippase activity, we purified TGN membranes from a yeast strain carrying a *gea2* mutation (*gea2-V698G*) that was specifically selected in a reverse two-hybrid assay for loss of interaction with Drs2p9. The *gea2-V698G* TGN membranes were assayed at 37°C with or without addition of the wild-type Gea2p-Sec7 domain (Fig. 5b). Relative to wild-type membranes, flippase activity with *gea2* membranes was reduced 50%. Addition of recombinant Gea2p-Sec7 domain to the mutant membranes significantly stimulated flippase activity.

The Drs2p C-tail interacts with both Gea1p and Gea2p9, a closely related essential pair of ArfGEFs, although it was primarily Gea2p associated with the TGN membrane preparations (Supplemental information, Fig. S3b). Therefore, TGN membranes were purified from a *pik1^{ts} gea2*Δ double mutant and assayed for flippase activity. Relative to *pik1^{ts}* membranes at 27°C, flippase activity in the *pik1^{ts} gea2*Δ membranes was reduced at 27°C and nearly eliminated at 37°C. Addition of PtdIns[4]P stimulated the flippase activity while addition of 200 ng of Gea2p-Sec7 domain provided marginal stimulation. However, addition of both molecules provided a synergistic activation of Drs2p activity (Fig. 5c). The ability of Gea2p to stimulate flippase activity is unlikely to be mediated by activation of Arf as no GTP was added to the reaction, addition of an inhibitor of the Arf exchange factor activity of Gea2p, brefeldin A, had no effect (Supplemental information, Fig. S3c), and we cannot detect an interaction between Arf and Drs2p7.

In further support of the in vivo significance of these interactions, we found that the *drs2-*Δ*1250-1263* allele, defective in binding Gea2p, is synthetically lethal with *pik1^{ts}* (Fig. 5d). For this plasmid shuffle assay, a *pik1^{ts} drs2*Δ strain carrying wild-type *DRS2* on a *URA3*-based plasmid was transformed with *LEU2*-based plasmids carrying the indicated *drs2* allele. Failure to grow on 5 fluoro-orotic acid (5-FOA), which is toxic to cells retaining the *URA3-DRS2* plasmid, indicates that *drs2-*Δ*1250-1263* cannot support viability of *pik1^{ts}* cells. In contrast, viable *pik1^{ts} drs2-AMAAQA* double mutants could be produced, and the *drs2-*Δ*RMKKQR* allele yielded only a few viable colonies. Thus, a strain with reduced PtdIns[4]P levels can tolerate a mutation in the Drs2p PtdIns[4]P binding site, but cannot tolerate loss of interaction between Drs2p and ArfGEF.

Our data indicate that the activity of Drs2p in the TGN is strongly stimulated by binding of PtdIns[4]P and ArfGEF to a regulatory domain in the Drs2p C-tail. The observed synergistic activation of Drs2p by PtdIns[4]P and ArfGEF imply a novel coincidence detection system for constraining Drs2p activity to membrane sites enriched for both activators. Liposome-binding experiments suggest that PtdIns[4]P and Gea2p compete for interaction with the Drs2p C-tail (Supplemental information, Fig. S4). In this case, synergistic activation could be achieved if binding of one activator enhances the probability of interaction with the second, or if the interactions occur sequentially at the surface of the TGN membrane (Supplemental information, Fig. S6). Conversely, it is also possible that in the context of full-length Drs2p in the TGN membrane, a tripartite complex of PtdIns[4]P, the Drs2p C-tail and Gea2p is stabilized to provide synergistic activation (Supplemental information, Fig. S6). The structural basis by which Drs2p C-tail interaction with phosphoinositides and ArfGEF stimulate flippase activity is unknown. For the plasma membrane Ca^{2+} ATPase, the C-tail is an autoinhibitory domain and binding of calmodulin to the C-tail relieves

autoinhibition and stimulates this pump26. It is possible that the Drs2p C-tail is also an autoinhibitory domain, although we have not been able to detect an inhibitory activity of the C-tail when added in trans to the TGN NBD-PS flippase assay (Supplemental information, Fig. S5). As an integral membrane protein required for a phospholipid flippase activity, Drs2p is a novel effector of phosphoinositides involved in protein trafficking. This work also describes first function for an ArfGEF beyond the activation of Arf, and provides further evidence that Drs2p activity is coupled to the vesicle budding machinery of the TGN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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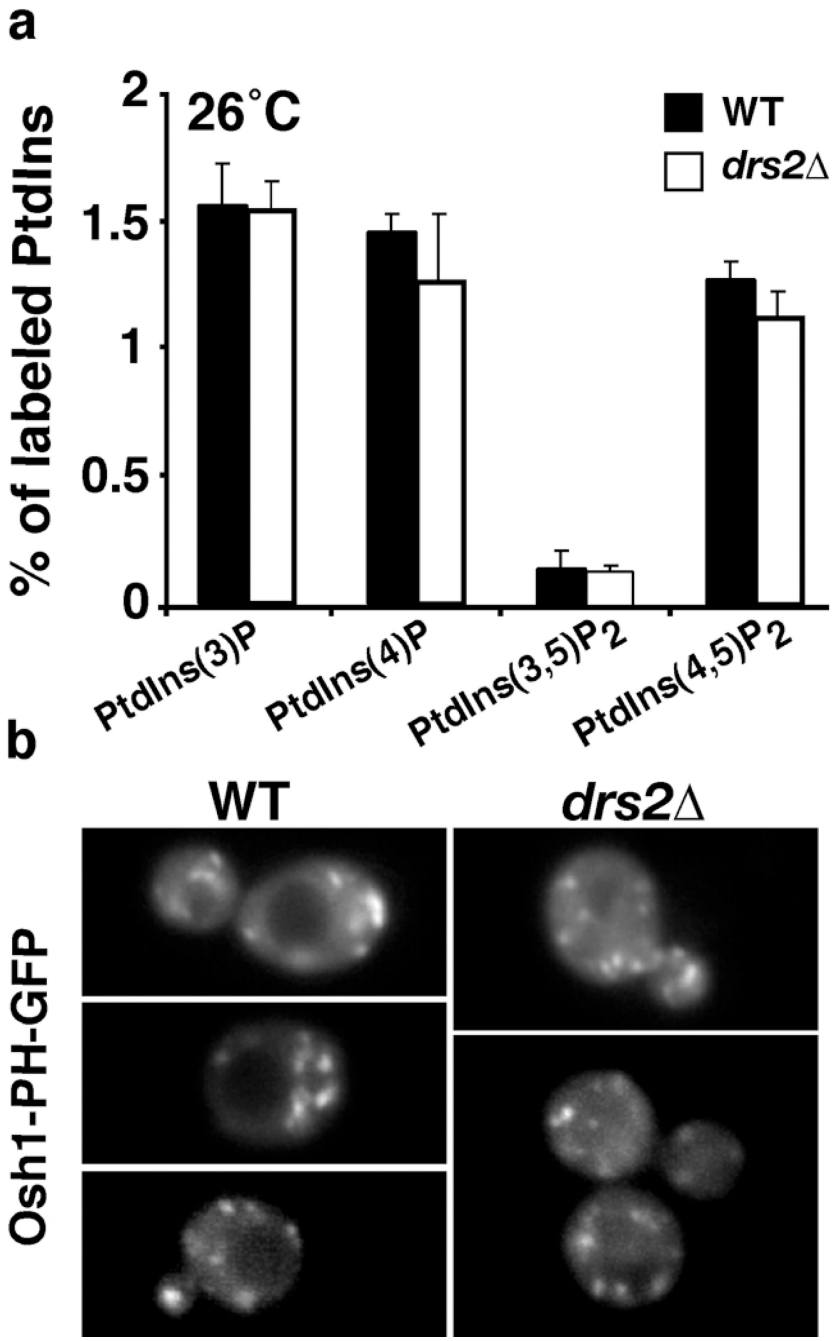

Figure 1.

Loss of Drs2p does not perturb phosphorinositide metabolism. **(a)** Wild-type (WT) and *drs2*Δ cells were labeled with myo-[2-^{3}H] inositol and phosphoinositides were quantified as described in Rudge et. al27. **(b)** Fluorescence microscopy of WT and *drs2*Δ yeast cells expressing Osh1 PH-GFP. This GFP fusion protein specifically binds PtdIns[4]P at the TGN. Scale bar, 5μm. The graph in **a** depicts average ± SD (n=3).

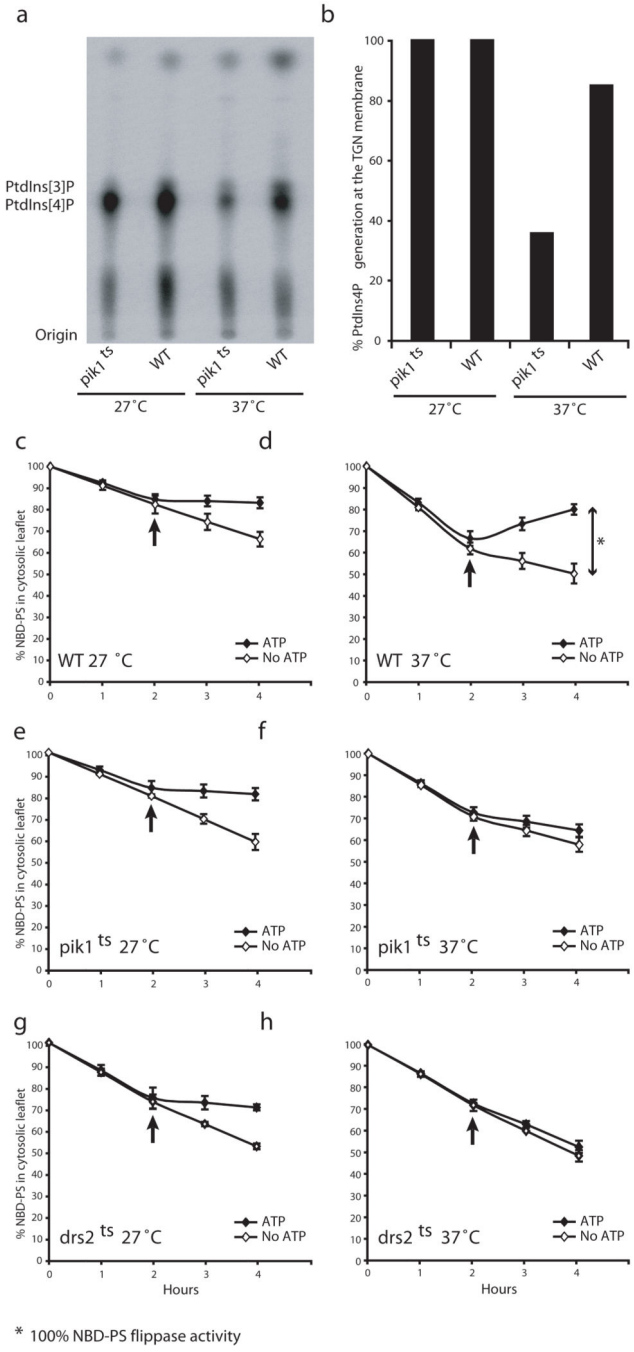

Figure 2.

NBD-PS flippase activity in TGN membranes requires Drs2p and PtdIns 4-kinase activity catalyzed by Pik1p. **(a)** Thin layer chromatography (TLC) of PtdIns kinase assay reaction products performed with TGN membranes isolated from *WT* and *pik1*^{ts} cells. **(b)** Quantification of PtdIns[4]P produced on TGN membranes by Phosphor-Imager (Molecular Dynamics). Data are normalized to the 27°C samples. **(c to h)** TGN membranes from WT, *pik1*^{ts} and *drs2*^{ts} strains were assayed for NBD-PS flippase activity at 27°C and 37°C. The arrow indicates time of ATP addition to half of each sample. The difference in cytosolic

leaflet NBD-PS after 4 hrs at 37°C with or without ATP was considered 100% NBD-PS flippase activity (asterisk). The methods for purifying TGN membranes and assaying NBD-PS flippase activity are previously described6, 28, 29. The graphs in **c** to **h** depict average ± SD (n=3).

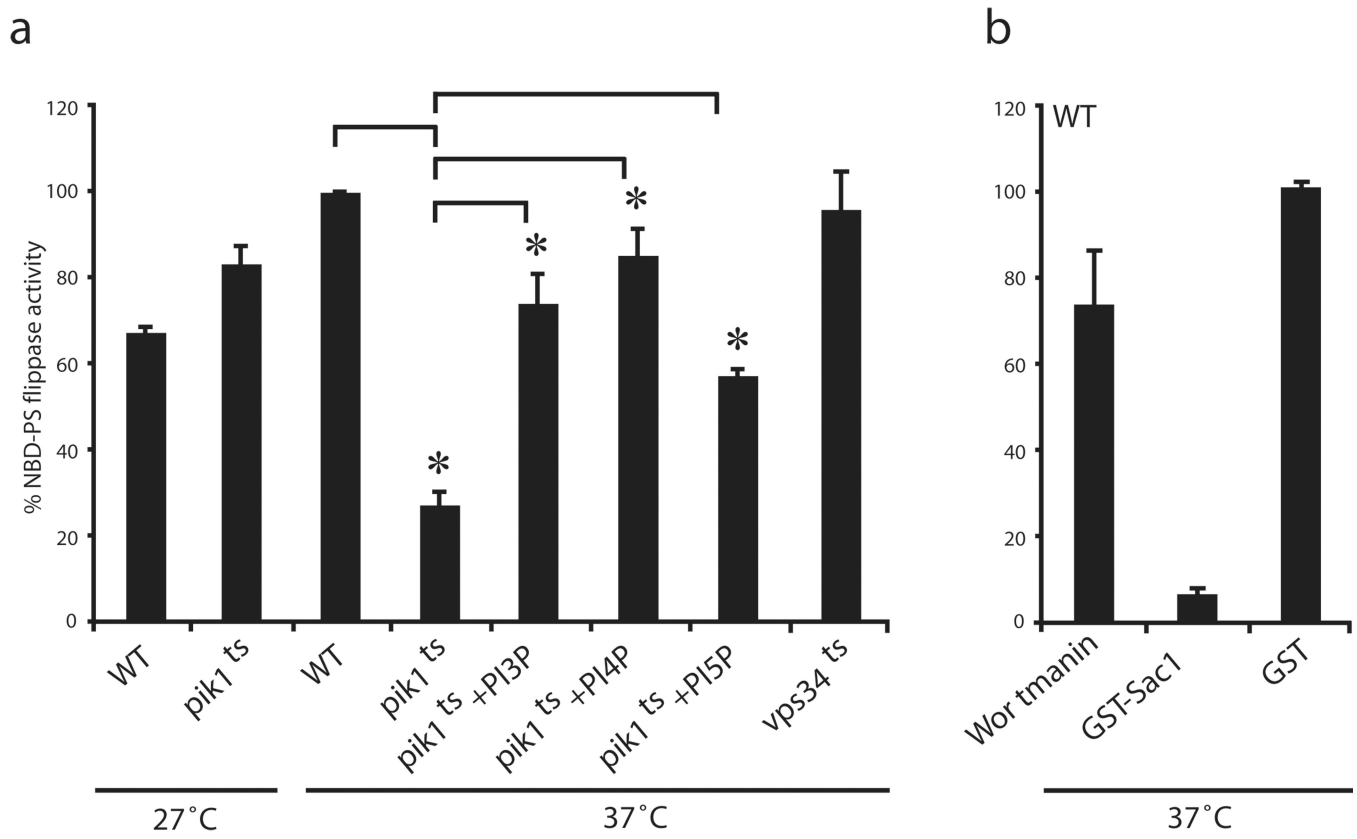

Figure 3.

Stimulation of NBD-PS flippase activity by phosphoinositides. **(a)** NBD-PS flippase activity in TGN membranes from the indicated yeast strains were normalized to 100% activity of WT membranes at 37°C. PtdIns3P, 4P and 5P (D-myo-phosphatidylinositol phosphate (PtdInsP) D (+)-sn-1,2-di-O-butanoylglyceryl, 3-O-phospho linked) were added to *pik1*^{ts} TGN membranes at 3.3 mol% relative to endogenous phospholipids (**P*< 0.05; *t* test). **(b)** NBD-PS flippase activity in WT TGN membranes incubated with either 10 μM wortmannin, 1 μg GST-Sac1p (GST-Inp53-Sac1 phosphoinositide phosphatase) or 1 μg GST (**P*< 0.001; *t* test). The graphs in **a** and **b** depict average ± SD (n=3).

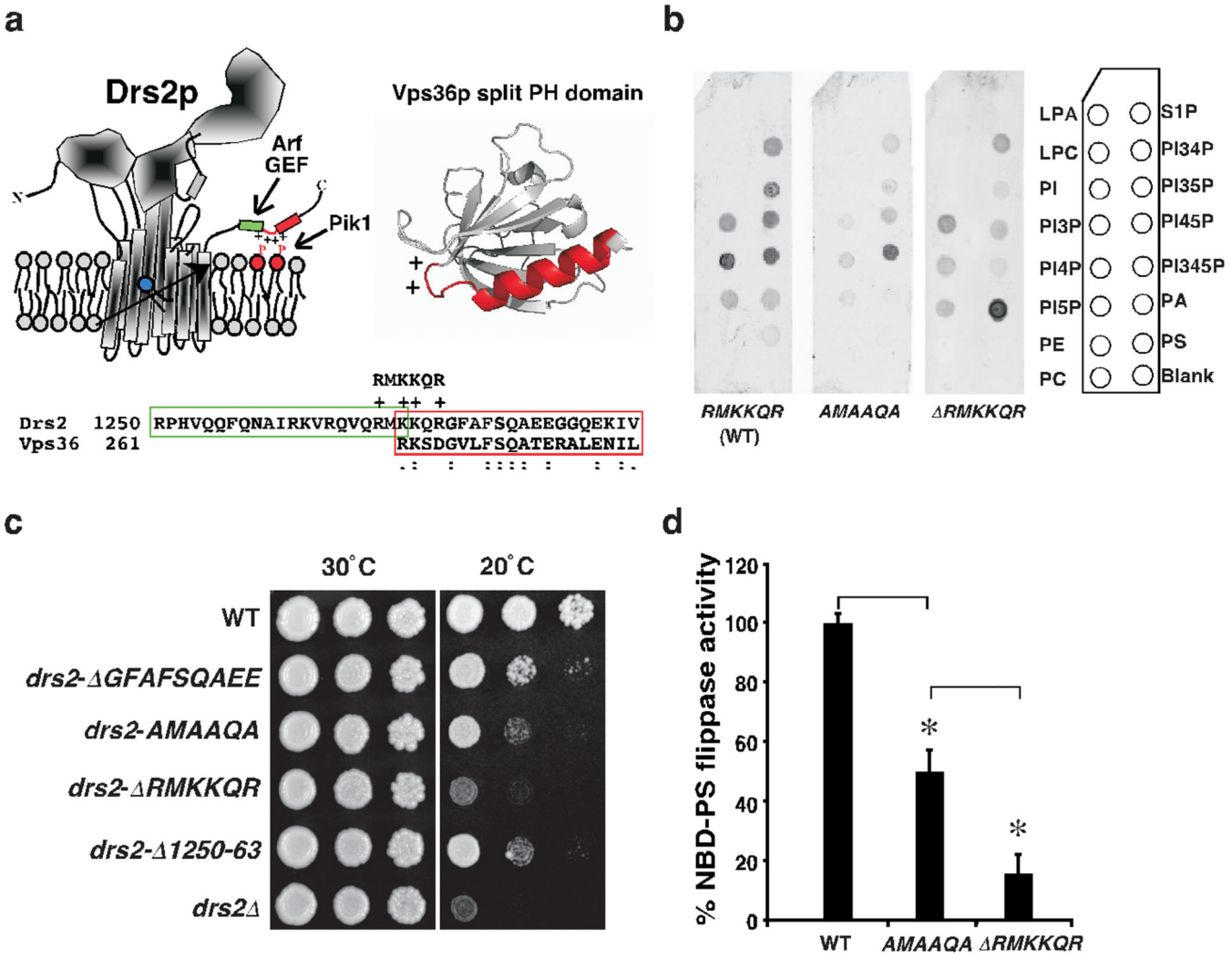

Figure 4.

Functional requirement for a phosphoinositide binding motif in the Drs2p C-terminal tail. **(a)** A potential phosphoinositide-binding motif (basic patch of amino acids at 1268-73), based on homology with part of the split PH domain of Vps36p (red), overlaps an ArfGEF (Gea2p-Sec7 domain) interaction domain in the Drs2p C-terminal tail (1250–1270, green). **(b)** Binding of His-tagged Drs2p C-tail (residues 1219 – 1305) containing either the wild-type basic patch (RMKKQR), basic residue -> alanine mutations (AMAAQA) or deletion of basic patch (ΔRMKKQR) to phospholipids (PIP strips, Echelon Biosciences). LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SIP, sphingosine 1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. **(c)** Complementation of *drs2*Δ cells with plasmids carrying either wild-type *DRS2* (WT), *drs2-ΔGFAFSQAEE*, *drs2-AMAAQA*, *drs2-ΔRMKKQR*, *drs2-Δ1250-63* or an empty plasmid (*drs2*Δ). Cells were serially diluted and grown at 30°C and 20°C. **(d)** NBD-PS flippase activity in TGN membranes purified from WT, *drs2-AMAAQA* and *drs2-ΔRMKKQR* cells (*$P < 0.01$; *t* test). The graph in **d** depicts average ± SD (n=3).

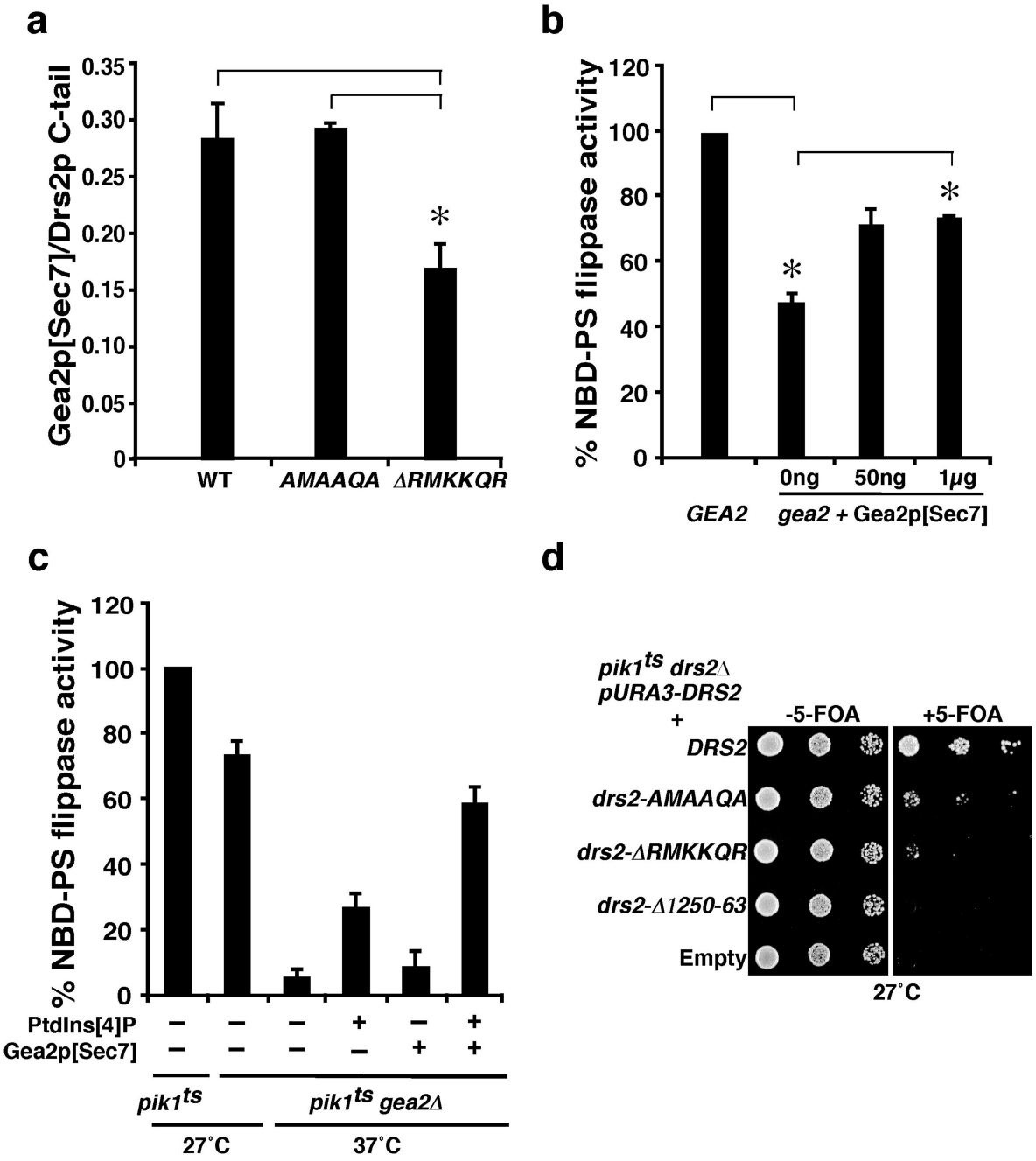

Figure 5.

Synergistic activation of NBD-PS flippase activity by phosphoinositide and ArfGEF **(a)** Quantification of His-tagged Drs2p-C-tail protein interaction with the Gea2p-Sec7 domain. The ratio of Gea2p bound to the Drs2p-C-tail was determined as described under Methods (**P*< 0.05; *t* test). **(b)** ArfGEF dependent stimulation of NBD-PS flippase activity in TGN membranes purified from wild-type (*GEA2*) or *gea2-V698G (gea2)* strains incubated with or without recombinant Gea2p (Sec7 domain) at 37°C (**P*<0.05; *t* test). **(c)** PtdIns[4]P and ArfGEF stimulation of NBD-PS flippase activity in TGN membranes purified from a

pik1^{ts}*gea2*Δ strain incubated with either 3 mol% PtdIns[4]P, 200 ng Gea2p[Sec7] or both activators at 37°C. **(d)** Test for complementation of *pik1*^{ts} *drs2*Δ synthetic lethality with *LEU2* plasmids carrying either wild-type *DRS2*, *drs2-AMAAQA*, *drs2-ΔRMKKQR*, *drs2-Δ1250-63* or an empty plasmid. Cells were serially diluted and grown at 27°C with or without 5-fluororotic acid (5FOA) to counter select the *URA3-DRS2* plasmid. The graphs in **a** to **c** depict average ± SD (n=3).