TORC1 Determines Fab1 Lipid Kinase Function at Signaling Endosomes and Vacuoles

Graphical Abstract

Highlights

- The Fab1 lipid kinase is a substrate of the Target of Rapamycin Complex 1 (TORC1)

- Fab1 and TORC1 localize to signaling endosomes and the vacuole

- Fab1 phosphorylation shifts TORC1 to signaling endosomes and changes its activity

- Fab1 and TORC1 function in a regulatory feedback loop, which controls signaling

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In Brief

Chen, Malia, Hatakeyama, et al. demonstrate that the yeast Target of Rapamycin Complex 1 (TORC1) phosphorylates the lipid kinase Fab1, a homolog of metazoan PIKfyve. Fab1 phosphorylation shifts both complexes from the vacuole to signaling endosomes and changes TORC1 activity, suggesting a regulatory feedback loop in cellular signaling from these organelles.
SUMMARY

Organelles of the endomembrane system maintain their identity and integrity during growth or stress conditions by homeostatic mechanisms that regulate membrane flux and biogenesis. At lysosomes and endosomes, the Fab1 lipid kinase complex and the nutrient-regulated target of rapamycin complex 1 (TORC1) control the integrity of the endolysosomal homeostasis and cellular metabolism. Both complexes are functionally connected as Fab1-dependent generation of PI(3,5)P2 supports TORC1 activity. Here, we identify Fab1 as a target of TORC1 on signaling endosomes, which are distinct from multivesicular bodies, and provide mechanistic insight into their crosstalk. Accordingly, TORC1 can phosphorylate Fab1 proximal to its PI3P-interacting FYVE domain, which causes Fab1 to shift to signaling endosomes, where it generates PI(3,5)P2. This, in turn, regulates (1) vacuole morphology, (2) recruitment of TORC1 and the TORC1-regulatory Rag GTPase-containing EGO complex to signaling endosomes, and (3) TORC1 activity. Thus, our study unravels a regulatory feedback loop between TORC1 and the Fab1 complex that controls signaling at endolysosomes.

INTRODUCTION

Eukaryotic organelles differ in the lipid and protein composition of their surfaces and thus maintain a unique identity. Along the endomembrane system, proteins are transported by vesicles that are generated at the donor compartment and fuse with an acceptor organelle. Despite this active transport between organelles, each organelle maintains its identity. Apart from the fusion and fission machineries involved in each transport step, organelle-specific phosphatidylinositol phosphates (PIPs) are important markers that contribute to the identity of distinct organelles. PIPs are phosphorylated derivatives of phosphatidylinositol (PI), which can be modified at three positions by specific lipid kinases and phosphatases to generate seven distinct isoforms. Organelle-specific PIPs are proposed to recruit specific proteins to their surface, which then have decisive roles in cell signaling, membrane trafficking, and lipid metabolism. Proteins may recognize not only PIPs but additionally also membrane-associated proteins, which increases the specificity of their localization—a mechanism termed coincidence detection. Consequently, PIP production and turnover is tightly controlled to maintain organelle identity.

Within the endosomal system, proteins and lipids are transported either from the plasma membrane or Golgi to the early endosome. These endosomes then undergo maturation and sorting processes, whereby a number of membrane proteins are sorted into intraluminal vesicles by the endosomal sorting complexes required for transport (ESCRT) machinery, whereas others are recycled with the support of retromer complexes. ESCRTs function in consecutively acting complexes on endosomes. Early acting complexes (0–II) recognize ubiquitinated membrane proteins, whereas ESCRT-III and the interacting ATPase Vps4 corral the proteins and promote formation of intraluminal vesicles. Mature late endosomes, also called multivesicular bodies (MVBs), eventually fuse with the lysosome. Two PIPs, phosphatidylinositol-3-phosphate (PI3P) and phosphatidylinositol-3,5-bisphosphate (PI(3,5)P2), are essential for endosomal and lysosomal biogenesis. The Vps34 kinase complex II...
produces PI(3,5)P₂ at endosomes, which is also found at vacuoles in yeast (or lysosomes in metazoan cells).²³ Subunits of both the retromer and ESCRT complexes have PX and FYVE domains, which bind specifically to PI(3,5)P₂ and thus endosomes.²⁵ PI(3,5)P₂ can be further phosphorylated to the low abundant PI(3,5)P₃, which is strongly upregulated during osmotic stress.¹⁷ In yeast, osmotic stress results in fragmentation of the vacuole to maintain organelle integrity.²⁵ Vacular fragmentation is also required for the inheritance of vacuoles to daughter cells, and also here PI(3,5)P₂ levels play an important regulatory role.²² Moreover, nutrient supply as an activator of TORC1 also affects vacuole fragmentation,¹⁰ though the TORC1 substrate(s) that is relevant for this process remains unknown.

The PI(3,5)K-farnese Fab1, called PIKfyve in metazoans, is part of a multisubunit complex, which includes the PI(3,5)P₂ phosphatase Fig4, the scaffold protein Vac14, and Atg18.¹¹,¹²,¹³ Fab1, Fig4, and Vac14 form the core of the complex.¹¹,¹²,¹³,¹⁴ Full Fab1 activity in yeast also requires the vacuolar membrane protein Vac7¹⁷ and is controlled by Atg18.¹⁶,¹⁷ Consequently, mutants lacking either Fab1, Vac7, or Vac14 exhibit very large vacuoles and defects in protein sorting at endosomes and vacuoles.¹⁸–²¹ Likewise, inactivation of metazoan PIKfyve affects PI(3,5)P₂ production and lysosomal biogenesis and homeostasis.¹²,²²–²⁴ In humans, such mutations result in devastating neurodegenerative diseases, demonstrating that PI(3,5)P₂ levels need to be tightly controlled.²⁵–²⁸

The identification of PI(3,5)P₂ effectors starts to shed light on the role of this lipid in lysosomal biogenesis and homeostasis. In yeast, the most prominent interactor is Atg18,¹⁶ which can directly induce membrane fragmentation.²⁹ Additional PI(3,5)P₂-interactors include lysosomal ion channels, the vacuolar ATPase, and a subunit of the lysosomal target of rapamycin complex 1 (TORC1).³⁰–³³ TORC1 was initially identified in yeast, and is conserved across eukaryotic species.³⁴ It consists of the protein kinases Tor1 or Tor2 (mTOR in humans), Kog1/Raptor, Lst8/mLST8, and the yeast-specific subunit Tco89.³⁴ TORC1 is a cellular pacemaker that promotes anabolic processes and growth. Nutrients, particularly amino acids, are required for TORC1 activity.³⁵ Amino acids are sensed by a conserved lysosomal complex, the Rag GTPase-containing EGO complex (Ragulator-Rag complex in metazoans) and its regulators, such as the SEACIT and SEACAT complexes (GATOR1 and 2 in metazoans).³⁶–³⁸ The FYVE domain-containing yeast protein Pib2 represents another amino acid-sensing branch that regulates TORC1, although its conservation in higher eukaryotes has not been demonstrated.³⁹–⁴³ Recent data suggest that yeast TORC1 also responds to glucose as it oligomerizes upon starvation in structures known as TORC1 bodies or TORC1 dots.¹³,¹⁴ Tor1 or Tor2 (mTOR in humans), Kog1/Raptor, Lst8/mLST8, and the yeast-specific subunit Tco89. TORC1 is a cellular pacemaker that promotes anabolic processes and growth. Nutrients, particularly amino acids, are required for TORC1 activity. Amino acids are sensed by a conserved lysosomal complex, the Rag GTPase-containing EGO complex (Ragulator-Rag complex in metazoans) and its regulators, such as the SEACIT and SEACAT complexes (GATOR1 and 2 in metazoans). The FYVE domain-containing yeast protein Pib2 represents another amino acid-sensing branch that regulates TORC1, although its conservation in higher eukaryotes has not been demonstrated. Recent data suggest that yeast TORC1 also responds to glucose as it oligomerizes upon starvation in structures known as TORC1 bodies or TORC1 dots. Tor1 or Tor2 (mTOR in humans), Kog1/Raptor, Lst8/mLST8, and the yeast-specific subunit Tco89. TORC1 is a cellular pacemaker that promotes anabolic processes and growth. Nutrients, particularly amino acids, are required for TORC1 activity. Amino acids are sensed by a conserved lysosomal complex, the Rag GTPase-containing EGO complex (Ragulator-Rag complex in metazoans) and its regulators, such as the SEACIT and SEACAT complexes (GATOR1 and 2 in metazoans). The FYVE domain-containing yeast protein Pib2 represents another amino acid-sensing branch that regulates TORC1, although its conservation in higher eukaryotes has not been demonstrated. Recent data suggest that yeast TORC1 also responds to glucose as it oligomerizes upon starvation in structures known as TORC1 bodies or TORC1 dots.

The EGO complex and TORC1 localize to a special endosomal population in yeast that we coined “signaling endosomes.”⁴⁶,⁴⁷ Such endosomes are distinct from previously described metazoan endosomes involved in signaling via, for instance, the epithelial growth factor.⁴⁸ Ivy1, an effector of the vacuolar Rab GTPase Ypt7⁴⁹ and inhibitor of the Fab1 complex⁵⁰ colocalizes with Fab1 and the EGO complex to dots proximal to the vacuole.⁵¹

We now present evidence that signaling endosomes have a key function in endosome and vacuole membrane homeostasis. Ivy1, Fab1, the EGO complex, and TORC1 all localize to signaling endosomes in addition to their vacuolar localization. We uncover that Fab1 is a TORC1 substrate and that phosphorylation of Fab1 shifts not only the primary localization of the Fab1 complex from vacuoles to signaling endosomes but also of TORC1. This in turn results in a local PI(3,5)P₂ accumulation at signaling endosomes and altered TORC1 activity. Our data highlight a novel feedback loop between a lipid kinase and the metabolically regulated TORC1 at endomembranes.

**RESULTS**

**TORC1 and Fab1 Localize to Signaling Endosomes and Interact with Each Other**

Both Ivy1, as a Fab1 inhibitor, and TORC1 localize to the vacuole and distinct dots.⁴⁹–⁵¹ As Ivy1 localization was unaffected by ESCRT-III deletions,⁴⁹ we previously discarded the possibility that Ivy1 resides also on endosomes. However, it was recently shown that a second population of endosomes exists, which contains an endosomal pool of TORC1 and the EGO complex.⁵² Ivy1 also strongly colocalizes with EGO components in dots proximal to the vacuole.⁴⁹ we wondered if all proteins, including TORC1 and Fab1, function together on signaling endosomes.

To ask if Ivy1, Fab1, and TORC1 are present on these endosomes, we analyzed the localization of GFP-tagged Ivy1 relative to mCherry-tagged Kog1 (a subunit of TORC1), Vps4 (the disassembling ATPase of ESCRT-III proteins at MVBs), and Vps8 (a subunit of endosomal CORVET, which binds the Rab5-like Vps21 protein). Indeed, we observed that a fraction of Ivy1 colocalized with Kog1 and Vps8 positive dots but not with dots positive for Vps4 (Figures 1A and 1B). This suggests that a significant portion of Ivy1 is present on signaling endosome, but not MVBs, together with the endosomal TORC1.

We then analyzed if Fab1 is also present on these signaling endosomes with Ivy1 and TORC1. Indeed, a significant amount of mNeonGreen-tagged Fab1 colocalized with mCherry-tagged Kog1, Ivy1, and Vps8 in dots proximal to the vacuole (Figures 1C–1E). Fab1 was, however, also present on Vps4-positive MVBs, and thus has a broader distribution (Figures 1C–1E). To determine if TORC1 and Fab1 contact each other on signaling endosomes, we added the C-terminal half of the Venus fluorescence protein to Fab1 (VC) and the N-terminal half to Kog1 and Vps8 positive dots and thus has a broader distribution (Figures 1C–1E).

Overall, our data reveal that Ivy1, Fab1, and TORC1 localize both to vacuoles and to signaling endosomes (Figure 1E), where Fab1 and TORC1 are in close proximity.

**Fab1 Is Phosphorylated by TORC1**

As Fab1 and TORC1 are in close proximity on signaling endosomes, we asked whether Fab1 is a direct target of TORC1 and thus conducted two lines of independent assays. As a first approach, we compared the phosphoproteome of yeast strains
in the absence and presence of rapamycin, a TORC1 inhibitor, and identified serine residue 208 (Ser^{208}) in Fab1 as a potential target of TORC1 in vivo, whereas other sites in Fab1 (Ser^{125} and Ser^{155}) were unaffected. The same assay also identified the known bona fide TORC1 target residue Thr^{723} in Sch9 (Figure 2A). Ser^{208} is located next to the PI3P interacting FYVE domain in Fab1 (residues 230–320; Figures 2A and 2B). In a second approach, we subjected Fab1 to in vitro phosphorylation assay in bacteria and identified serine residue 208 (Ser^{208}) in Fab1 as a potential TORC1 target residue. Thr^{206} and Ser^{210} (Figure 2A). We confirmed via mass spectrometry that Fab1 1–600 construct was phosphorylated by TORC1 at Ser^{208}, Ser^{204}, and Ser^{210}. Recombinant variants of Fab1 1–600 were only marginally phosphorylated by TORC1 (Figure 2D). These TORC1 phosphorylation sites are conserved between yeast and human, and Ser^{210} is also phosphorylated in vivo by TORC1 in human cells. Fab1 1–600 was phosphorylated by TORC1 at Ser^{208}, Ser^{204}, and Ser^{210} (Figure 2A). Recombinant variants of Fab1 1–600 were only marginally phosphorylated by TORC1 (Figure 2D). Based on these results, we conclude that TORC1 phosphorylates Fab1 at mainly Ser^{208}, Ser^{204}, and Ser^{210}.

**Fab1 Phosphorylation Affects Vacuole Morphology and EGO Localization**

To determine whether Fab1 phosphorylation by TORC1 affected endosome and vacuole biogenesis, we generated (using CRISPR/Cas9 gene editing) fab1^{64A} and fab1^{60D} mutant strains. Here, the entire cluster of Ser/Thr residues between Ser^{202} and Ser^{210} was replaced by either Ala or phosphomimetic aspartates (Figure 2B). We initially analyzed strains expressing Fab1-
A TORC1 phosphorylates multiple residues within the N-terminal part of Fab1. The table shows the rapamycin sensitivity of phosphorylation of Ser125, Ser155, Ser204, Ser208, and Ser210 detected on spectrometric analysis, and the identification of the phosphopeptide containing phosphorylated Ser203, Ser204, Ser206, Ser208, and Ser210 (5A) were subjected to in vitro phosphorylation by TORC1 (purified from yeast) in the absence (+) or presence (−) of the TORC1 inhibitor WM. Representative SYPRO Ruby staining and autoradiography (32P) blots are shown. B TORC1 phosphorylates multiple residues within the N-terminal part of Fab1. The table shows the rapamycin sensitivity of phosphorylation of Ser125, Ser155, Ser204, Ser208, and Ser210 detected on spectrometric analysis, and the identification of the phosphopeptide containing phosphorylated Ser125, Ser155, Ser204, Ser208, and Ser210 detected on purified Fab11–600 following in vitro phosphorylation by TORC1. Wortmannin (WM) was used to inhibit TORC1 in vitro (see STAR Methods). PEP, posterior error probability; the smaller the number, the better is the hit. >100 implies that the phosphorylation levels of the respective amino acid residues were at least two orders of magnitude higher in samples treated with TORC1 when compared to the ones treated with TORC1 in the presence of WM. C Model of the Fab1 sequence including the potential TORC1 target phosphosite. The Fab1 domains are indicated as FYVE domain (230–320), chapman domain (1181–1500), and kinase domain (1900–2266). The region between residues 201 and 210 is highlighted with the identified Ser204, Ser208, and Ser210 shown in blue, the corresponding mutant alleles are indicated in red. D TORC1 kinase predominantly phosphorylates the N-terminal part (encompassing amino acids 1–600) of Fab1. Purified recombinant His6-Fab1 fragments (His6-Fab11–600, His6-Fab1580–1180, His6-Fab11160–1760, and His6-Fab11760–2266) were subjected to in vitro phosphorylation by TORC1 (purified from yeast) in the absence (−) or presence (+) of the TORC1 inhibitor WM (see STAR Methods). Representative SYPRO Ruby staining and autoradiography (32P) blots are shown. E Mutation of identified phosphosites strongly impairs the in vitro Fab1 phosphorylation by TORC1. Purified recombinant His6-Fab11–600 containing, mNeonGreen variants to trace their localization. While the wild-type Fab1 and Fab16A protein localized mainly to round, wild-type-like vacuoles and to some dots, Fab16D was enriched in dot-like structures around fragmented vacuoles (Figures 3A and 3B), which we identified as endosomes, as they colocalize with the endosomal Vps8 protein (Figure 6A). Interestingly, mCherry-tagged Ivy1 and the TORC1 subunit Kog1 showed a similar subcellular distribution pattern as Fab1, Fab16A, or Fab16D in each of the strains expressing the respective Fab1 allele (Figures 3C–3E). Moreover, the EGO complex subunit Ego1 also colocalized with Kog1 in wild type, fab16A, and fab16D mutant cells (Figure 3D). Fab16D, however, did not colocalize with the MVB-marker Vps4 (Figures 3F and 3G; see inset), indicating that a fraction of Fab16D accumulates on signaling endosomes. Finally, Fab16D-expressing cells exhibited a significantly higher number of Ivy1, Ego1, and Kog1 dots next to vacuoles than wild-type cells or, even more, than Fab16A-expressing cells (Figure 3H). These data indicate that Fab1 phosphorylation results in a significant shift of Fab1, TORC1, and the EGO complex from their vacuole localization to signaling endosomes. If, however, Fab1 cannot be phosphorylated, TORC1 and the EGO complex seem to become more confined to the vacuole, though some dot-like structures remain.

We next asked if the phosphomimetic fab16D mutant, which showed an aberrant vacuole morphology, has defects in vacuole biogenesis or endolysosomal protein trafficking. As the vacuolar fragmentation observed in fab16D cells can be the result of either increased fission or impaired fusion,29 we stained vacuoles with FM4-64 and subjected cells to a brief incubation in water, which promotes vacuole fusion. Under these circumstances, the vacuoles in the fab16D strain looked like the ones in wild type, suggesting that vacuole fragmentation in the fab16D mutant is not due to defects in fusion (Figure S1A). Vacuole fragmentation often indicates defects in vacuole protein sorting and biogenesis, and fab16A cells also have an endosomal protein sorting defect.31 Therefore, we tested the trafficking of selected cargo proteins from the Golgi or plasma membrane to the vacuole. Sorting of carboxypeptidase Y (CPY) to vacuoles as monitored by its abundance in cells was functional in wild-type and fab1 mutants but defective if vacuole biogenesis was impaired by deleting the HOPS subunit Vps39 (Figure S1B). Likewise, trafficking of the plasma membrane localized dicarboxylic acid transporter Dip5 or carboxypeptidase S (Cps1) via MVBs into the vacuole lumen was unaffected in fab1 mutants but impaired if the ESCRT protein Vps4 was lacking (Figures S1C and S1D). Similarly, a synthetic AP-3 cargo55 was sorted efficiently to vacuoles in both fab1 mutants but was missorted in a mutant lacking the AP-3 subunit Apl5 (Figure S1E). We thus conclude that phosphorylation of Fab1 results in both vacuole fragmentation and redistribution of Fab1 as well as the EGO complex and TORC1 components to signaling endosomes. However, it does not impair overall endocytosis or MVB biogenesis.
Strikingly, we observed that cells carrying fab1 alleles would also affect TORC1 activity. To test for TORC1 activity, we used growth on rapamycin as a readout. Strikingly, we observed that cells carrying fab1ΔΔ grew as poorly as tor1Δ cells. In contrast, cells with fab16Δ were slightly more resistant to rapamycin than wild-type cells when grown at either 30°C or 37°C (Figure 4A). All cells grew similarly well in the absence of rapamycin. To reveal if tor1Δ and fab16Δ strains differ, we compared their sensitivity on plates containing increasing amounts of rapamycin and observed that tor1Δ cells already showed growth deficiency at 1 ng/mL, whereas fab16Δ strains began to show this phenotype at 4 ng/mL (Figure S2A). This indicates that Fab1 mutants modulate TORC1 activity rather than abolish it. When we transformed these cells with a vector encoding wild-type Fab1, we observed rescue of the growth phenotypes on rapamycin (Figure 4B). This indicates that the mutations did not cause secondary defects within the genome and that the Fab1 mutant alleles are not dominant over the wild-type allele. We conclude that Fab1 phosphorylation also affects TORC1 activity.

To determine if Fab1 phosphorylation specifically affects TORC1 activity on endosomes or vacuoles (Figure 4C), we used established TORC1 activity reporters that specifically localize to endosomes (ET) or to vacuoles (VT)47,52 (see STAR Methods). Fab1 localization relative to FM4-64 stained vacuolar membranes was analyzed by fluorescence microscopy. Scale bar, 5 μm. Scale bar (inset), 1 μm. (B) Quantification of vacuole morphology from (A). At least 200 cells were counted from three independent experiments. Results are means ± SD. Significance was determined with a two-tailed Student’s t test (***p < 0.001; **p < 0.01; *p < 0.05).

(C) Kog1 localization in fab1 mutants. Cells expressing Fab1-mNeonGreen with mCherry-Kog1 were analyzed by fluorescence microscopy. Scale bar, 5 μm. Scale bar (inset), 1 μm. (D) Localization of Ego1 and Kog1 proteins in fab1 mutants. Ego1 was tagged C-terminally with GFP, and Kog1 was tagged N-terminally with mCherry under the control of the ADH1′ promoter. All images were taken by fluorescence microscopy. Scale bar, 5 μm. Scale bar (inset), 1 μm. (E and F) Ivy1 and Vps4 localization in fab1 mutants. Ivy1-mCherry (E) or Vps4-3HA-mCherry (F) was co-expressed with Fab1-mNeonGreen, and analysis was as in (C).

(G) Quantification of colocalization between Fab1 and Kog1, Vps4, or Ivy1 dots in fab1 mutants. Fab1 dots colocalizing with mCherry signal were counted and plotted as percent of total number of Fab1 dots. At least 80 Fab1 dots were counted in each of the three independent experiments. Results are means ± SD. Significance was determined with a two-tailed Student’s t test (**p < 0.01; *p < 0.05; ns, no significant difference).

(H) Quantification of dot-like structures in (C–E). At least 100 cells were counted from three independent experiments. Results are means ± SD. Significance was determined with a two-tailed Student’s t test (***p < 0.0001).

See also Figure S1.
Fab1 is a lipid kinase, we wondered if Fab1 would then generate PI(3,5)P₂ at signaling endosomes. To determine the local PI(3,5)P₂ pools, we searched for a suitable reporter. The S6Kinase homolog Sch9 is a TORC1 target that localizes to vacuoles in a Fab1- and thus PI(3,5)P₂-dependent manner, and its N-terminal region (residues 1–390) directly interacts with PI(3,5)P₂ in vitro. To identify a minimal PI(3,5)P₂-interacting fragment within Sch9, we localized several truncations in wild-type cells as GFP-fusion proteins. Whereas full-length GFP-Sch9 and an internally truncated GFP-Sch9Δ184–397 protein were found in part at the vacuole, all fragments lacking the first 183 residues were cytosolic (Figure 5A). However, GFP-tagged Sch9 1–397, and to a greater extent Sch9 1–183, localized strongly to perivacuolar dots and faintly to vacuoles (Figure 5B). This dot localization was completely lost in mutants lacking Fab1, Vac7 or Vac14, showing that its localization requires PI(3,5)P₂ synthesis.

**TORC1 Controls Local Synthesis of PI(3,5)P₂ at the Signaling Endosome**

Our data so far imply that TORC1-mediated phosphorylation shifts a significant amount of Fab1 to signaling endosomes. As fabΔ D mutant background, suggesting that phosphorylation of Fab1 causes reduced TORC1 activity. Next, we asked if the expression of the hyperactive Tor₁I₁₉₅₄V allele could suppress this phenotype and observed that it generally conferred some resistance to rapamycin in all strains. Nevertheless, the fab1Δ strain remained significantly more sensitive to rapamycin when compared to the respective fab1Δ and wild-type strains even in the presence of the Tor₁I₁₉₅₄V allele (Figure 4E). This suggests that fab1Δ mutant is dominant over known hyperactive mutant alleles of tor₁. Thus, phosphorylation of Fab1 seems to control TORC1 activity possibly by regulating PI(3,5)P₂ synthesis.
Of note, Sch9 1–183 exclusively decorated vacuoles in atg18Δ cells, suggesting that Atg18 is required to shift PI(3,5)P₂ synthesis from vacuoles to perivacuolar dots, which will be followed up elsewhere.

To confirm the specificity of our probe, we isolated recombinant Sch91–183 from bacteria and incubated the protein with liposomes containing PI3P or PI(3,5)P₂ and separated liposomes from proteins by centrifugation. At 10 mol% PI-lipids (Figure 5D), but even more so at lower concentrations, Sch9 1–183 strongly accumulated in the pellet fraction of liposomes carrying PI(3,5)P₂ (Figure 5E). We therefore used Sch9 1–183 in further experiments as a reporter of local PI(3,5)P₂ levels at vacuoles and endosomes.

To determine if increased PI(3,5)P₂ levels are present at signaling endosomes, we analyzed the localization of mCherry-tagged Sch9 1–183 relative to GFP-Tor1, which is also present on signaling endosomes. Indeed, we found both GFP-Tor1 and mCherry-tagged Sch9 1–183 in the same dot-like structure (Figure 5F).

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(Figure 5F). A pool of Fab1-GFP was likewise found in mCherry-Sch91–183 positive dots in wild-type cells. These Sch91–183-positive dots increased in size in the fab16D mutant but were reduced in the fab16A mutant (Figure 5G), suggesting that phosphorylation of Fab1 determines also local synthesis of PI(3,5)P2 at the signaling endosome.

As TORC1-mediated phosphorylation results in Fab1 accumulation at signaling endosomes, we next asked whether TORC1 localization also affects endosomal PI(3,5)P2 levels. To alter TORC1 localization, we fused TORC1 to the PI3P-specific FYVE domain of EA1, which targets TORC1 to endosomes,47 and still observed mCherry-Sch91–183 in dots (Figure 5H). In turn, we also expressed mCherry-Sch91–183 in a strain expressing Tor1 with three internal GFP-tags, which due to its tagging is found exclusively at the vacuole.44,47,58 Here, mCherry-Sch91–183 was to a large extent lost from membranes similar to its localization in the fab16A mutant (Figure 5I). These data indicate that endosomal TORC1 is required to maintain a pool of active Fab1 kinase at signaling endosomes, although we do not exclude the possibility that TORC1 regulates Fab1 at the vacuole as well.

Given that TORC1 has multiple targets apart from Fab1 at the signaling endosome and vacuole, we asked if inactivation of TORC1 reverses the aberrant vacuole morphology of the fab16D mutant. We therefore monitored the vacuole morphology of wild-type and fab1 mutant cells after addition of rapamycin (Figure S2B). Whereas wild-type and the fab16A mutant cells did not show any change in their vacuole morphology, vacuoles of the fab16D mutant became less fragmented (Figures S2B and S2C). This indicates that the vacuole morphology observed in the Fab1 mutant cells is caused by active TORC1.

In summary, we observe that TORC1-mediated phosphorylation of Fab1 causes re-localization of a pool of Fab1 and subsequent local PI(3,5)P2 synthesis at signaling endosomes.

**Phosphorylation of Fab1 Determines Its FYVE-Dependent Localization**

How could phosphorylation alter the localization of Fab1? Intriguingly, the identified TORC1 phosphorylation sites (S204, 206, 208) are just 20 residues upstream of the FYVE domain of Fab1, a known PI3P interacting domain, which is localized in Fab1 between residues 230 and 320. To test if phosphorylation targets Fab1 via its FYVE domain to PI3P positive endosomes, we deleted the N-terminal 211 residues preceding the FYVE domain and allow its binding to endosomal PI3P. In turn, we also expressed mCherry-Sch91–183 in a strain expressing Tor1 with three internal GFP-tags, which due to its tagging is found exclusively at the vacuole.44,47,58 Here, mCherry-Sch91–183 was to a large extent lost from membranes similar to its localization in the fab16A mutant (Figure 5I). These data indicate that endosomal TORC1 is required to maintain a pool of active Fab1 kinase at signaling endosomes, although we do not exclude the possibility that TORC1 regulates Fab1 at the vacuole as well.

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In summary, we observe that TORC1-mediated phosphorylation of Fab1 causes re-localization of a pool of Fab1 and subsequent local PI(3,5)P2 synthesis at signaling endosomes.

**Phosphorylation of Fab1 Determines Its FYVE-Dependent Localization**

How could phosphorylation alter the localization of Fab1? Intriguingly, the identified TORC1 phosphorylation sites (S204, 206, 208) are just 20 residues upstream of the FYVE domain of Fab1, a known PI3P interacting domain, which is localized in Fab1 between residues 230 and 320. To test if phosphorylation targets Fab1 via its FYVE domain to PI3P positive endosomes, we deleted the N-terminal 211 residues preceding the FYVE domain and allow its binding to endosomal PI3P. In turn, we also expressed mCherry-Sch91–183 in a strain expressing Tor1 with three internal GFP-tags, which due to its tagging is found exclusively at the vacuole.44,47,58 Here, mCherry-Sch91–183 was to a large extent lost from membranes similar to its localization in the fab16A mutant (Figure 5I). These data indicate that endosomal TORC1 is required to maintain a pool of active Fab1 kinase at signaling endosomes, although we do not exclude the possibility that TORC1 regulates Fab1 at the vacuole as well.

In summary, we observe that TORC1-mediated phosphorylation of Fab1 causes re-localization of a pool of Fab1 and subsequent local PI(3,5)P2 synthesis at signaling endosomes.

**DISCUSSION**

This study sheds light on the enigmatic crosstalk between the metabolically regulated TORC1 and the conserved Fab1 lipid kinase complex, which generates the low-abundant lipid PI(3,5)P2.1,7 In the past, a number of studies unraveled the regulation of Fab1 during osmotic stress, which results in a rapid synthesis of PI(3,5)P2 and subsequent vacuole fragmentation.11,13,15,16,19,30,59,60 Here, we uncovered that TORC1 phosphorylates Fab1, which in turn controls TORC1 activity. Phosphorylation of Fab1 results in its preferential shift to signaling endosomes, which also accumulate the EGO complex, TORC1, and Ivy1. These structures have been coined vacuolar microdomains in the past,49,61 yet are clearly endosomes (Figure 1),47 which lack the ESCRT-III-releasing Vps4 ATPase—as shown here—and thus differ from MVBs. Our data are consistent with the idea that the lipid kinase Fab1 and TORC1 are counter-regulated in space and activity.

We identify an N-terminal part of Fab1, which precedes the PI3P interacting FYVE domain, as a regulator of the Fab1 localization to vacuoles or endosomes. If the N-terminal segment is missing or phosphorylated, Fab1 is found primarily at the signaling endosome. We hypothesize that the FYVE domain is somehow blocked by the N-terminal segment, possibly by folding back to the FYVE domain, creating an autoinhibition. TORC1-mediated phosphorylation of Fab1 could then relieve this block and enable the Fab1 FYVE domain to efficiently bind PI3P at the signaling endosome (Figure S4), a model that requires further testing. Importantly, we observed that signaling endosomes are the main organelles presenting PI(3,5)P2 on their surface, and this lipid may be delivered by fusion to vacuoles, though alternative transport pathways are possible.
The analysis of Fab1 and its metazoan homolog PIKfyve has been challenging as their enzymatic product, PI(3,5)P₂, is a critical lipid for endosome and vacuole/lysosome biogenesis and cellular physiology. Fab1 activity is tightly regulated by intramolecular inhibition,⁵⁷ which is relieved in hyperactive alleles ⁵⁸⁻⁶⁰ or by cyclin-dependent kinases (i.e., Pho80/85) ⁶³. Yet, these data could not explain why Fab1 deletions have such an impact on endosomal biogenesis if Fab1 is primarily present on vacuoles. ²¹

We speculate that the lipid kinase and PI3P effector Fab1 and the PI(3,5)P₂ effector TORC1 sense and co-regulate the balance between PI3P and PI(3,5)P₂ at endosomes and at the vacuole. The respective balance of these lipids may locally define the membrane positioning of TORC1 relative to its regulator(s) such as Pib2, which binds PI3P. ⁴⁰,⁴²,⁴³,⁴⁵ In addition, the balance between PI3P and PI(3,5)P₂ is likely important for the activity of other PI3P and PI(3,5)P₂ effectors on the vacuole such as the V-ATPase or Atg18. ¹⁷,²⁹,³⁰,⁶⁷

How could Fab1 and TORC1 sense and regulate the balance between PI3P and PI(3,5)P₂ at endosomes or vacuoles? We propose that the FYVE domain of Fab1 combines two functions: on the one hand, it serves as a sensor for PI3P levels. On the other hand, it functions as a targeting module, which is co-regulated by TORC1-mediated phosphorylation of a stretch of amino acids that is located in its immediate proximity in the N-terminal part of Fab1 (Figure S4). If this stretch is not phosphorylated, it likely auto-inhibits the FYVE domain, which causes Fab1 to primarily localize to the vacuole (Figure S4). On the vacuolar membrane, Fab1 changes the PI3P to PI(3,5)P₂ ratio by consuming PI3P and generating PI(3,5)P₂, which results in enhanced recruitment of TORC1 to the vacuole.

Depending on its metabolic activation state, which is controlled by nutrient cues such as amino acids, TORC1 can then phosphorylate Fab1 proximal to its FYVE domain and thereby release the FYVE domain from autoinhibition (Figure S4). This would then allow the FYVE domain to bind PI3P and consequently favor a redistribution of Fab1 to the signaling endosome, where Fab1 can generate PI(3,5)P₂. In turn, increasing PI(3,5)P₂ will trigger the recruitment of TORC1 to signaling endosomes and hence sequester TORC1 away from its vacuolar targets (Figure S4). As long as TORC1 receives metabolic inputs at signaling endosomes, it will then be able to keep Fab1 phosphorylated in a positive feedback loop. It is likely that phosphorylation of other TORC1 targets is likewise promoted by phosphorylated Fab1 at the signaling endosome and vacuole, ⁴⁶ which may maintain their identity.
Re-localization of Fab1 to the vacuole (Figure S4) may occur as a result of any of several possible events that are not necessarily mutually exclusive: (1) if TORC1 receives diminished metabolic inputs at the signaling endosome, the autoinhibitory stretch of Fab1 may be preferentially dephosphorylated; (2) Fab1 may consume the majority of the PI3P molecules present on signaling endosomes, thus running out of substrate to associate with; (3) Fab1 is actively dephosphorylated by a yet to be identified phosphatase; or (4) signaling endosomes fuse with vacuoles, thus diluting out PI3P and PI(3,5)P₂, separating TORC1 from Fab1, and favoring dephosphorylation of Fab1 as illustrated (Figure S4). In either of these scenarios, Fab1 and TORC1 cooperate in a finely tuned balance. This balance can be affected by superimposed metabolic or osmotic stress control mechanism, which may even differ at the signaling endosome and at the vacuole. In sum, we hypothesize that it is exactly this—the PI3P to PI(3,5)P₂ ratio at each organelle—that determines the intimate crosstalk between Fab1 and TORC1.

There are, of course, several open questions. It is intriguing that the PI(3,5)P₂ phosphatase Fig4 is part of the Fab1 complex and could also be involved in counterbalancing the Fab1-mediated PI(3,5)P₂ synthesis at signaling endosomes or at vacuoles. In addition, the supply routes of PI3P to vacuoles and signaling endosomes likely differ. Vacuoles may receive their PI3P mainly via MVBs, whereas signaling endosomes may perhaps receive PI3P mostly during their biogenesis. Finally, our model may be extended to other vacuolar PI(3,5)P₂ effectors, which could be tuned to recognize fine-grained alterations in the respective phosphoinositide levels.

Our model is congruent and supported by all of our observations and compatible with findings of many others in the field. We also suspect that a similar crosstalk extends to metazoans, where the Fab1 homolog PIKfyve also has been linked to mTORC1 activity. Whether this crosstalk also occurs in metazoans, as shown here for yeast Fab1 and TORC1, is an attractive hypothesis that remains to be evaluated. Intriguingly, the Raptor subunit of mTORC1 also binds PI(3,5)P₂, and PIKfyve depletion strongly affects mTORC1 localization in adipocytes. However, PIKfyve depletion has drastic pleiotropic effects on the endolysosomal system. The analysis of a possible mTORC1 and PIKfyve crosstalk will thus require more targeted mutant studies in the future.

PI(3,5)P₂ levels are crucial for the correct function of lysosomes, and mutations in subunits of the Fab complex result in neurodegenerative diseases in humans. We believe that our novel insights into Fab1-regulation will provide a fresh view on how to understand the origin of these diseases.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.10.026.

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AUTHOR CONTRIBUTIONS

Z.C. and P.C.M. conducted all experiments on Fab1 localization and function; R.H. conducted experiments on development and analysis of the Sch9<sup>1-183</sup> probe; R.N., Z.H., M.-P.P.-G., and J.D. did the phosphorylation assays and analyses; and E.E. and J.W. conceived and performed the initial Sch9 mapping. Z.C. and P.C.M. conducted all experiments on Fab1 localization and function; R.H. conducted experiments on development and analysis of the Sch9<sup>1-183</sup> probe; R.N., Z.H., M.-P.P.-G., and J.D. did the phosphorylation assays and analyses; and E.E. and J.W. conceived and performed the initial Sch9 mapping. T.N. and C.J.S. did the lipid analysis of the mutant alleles. J.G. analyzed microscopy and quantitation data.

STAR+METHODS

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit anti-CPY     | Horvath et al. 68 | N/A |
| Rabbit anti-Pgk1    | Fulvio Reggiori | N/A |
| Goat anti-Sch9      | De Virgilio lab | N/A |
| Rabbit anti-Sch9-pThr737 | De Virgilio lab | N/A |
| Mouse Monoclonal anti-GFP | Roche | 118144600001; RRID: AB_390913 |
| Mouse Monoclonal anti-HIS tag | SIGMA-ALDRICH | SAB1305538 |
| **Bacterial and Virus Strains** |        |            |
| DH5<sup>x</sup> Competent Cells | Invitrogen | Cat#18265017 |
| Rosetta<sup>INDE3</sup> Competent Cells | Novagen | Cat#70954 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Rapamycin           | LC Laboratories | Cat#R-5000 |
| Wortmannin          | LC Laboratories | Cat#W-2990 |
| G418 (Geneticin)    | Carl Roth GmbH | Art. No. 2039.3; CAS: 108321-42-2 |
| Hygromycin B        | Carl Roth GmbH | Art. No. 1287.2; CAS: 31282-04-9 |
| Nourseothricin (clonNAT) | Jena Bioscience | CAS: 96736-11-7 |
| Ampicillin sodium salt | Carl Roth GmbH | Art. No. K029.2; CAS: 69-52-3 |
| Kanamycin           | Carl Roth GmbH | Art. No. T832.3; CAS: 25389-94-0 |
| Cycloheximide       | Applichem | Cat#10020730 |
| 30% Acrylamide/Bis Solution, 37.5:1 | Carl Roth GmbH | Art.Nr. 3029.1 |
| Pefabloc<sup>®</sup> SC-Protease-Inhibitor | Carl Roth GmbH | Art.Nr. A154.1; CAS: 30827-99-7 |
| Pepstatin A         | Carl Roth GmbH | Art.Nr. 2936.2; CAS: 26305-03-3 |
| Leupeptin           | Roche | Cat#1017101 |
| 1,10-Phenanthroline 1-hydrate | PanReac AppliChem ITW Reagents | 13132; CAS: 5144-89-9 |
| Phenylmethyl sulphonyl fluoride | Carl Roth GmbH | Art.Nr. 6367.2 |
| CHAPS               | Carl Roth GmbH | Art.Nr. 149.2 |
| EDTA Disodium Salt 2-hydrate | PanReac AppliChem ITW Reagents | A2937; CAS: 6381-92-6 |
| DTT                 | PanReac AppliChem ITW Reagents | A1101; CAS: 3483-12-3 |
| Tris                | PanReac AppliChem ITW Reagents | A1379; CAS: 77-86-1 |
| Glycerol            | Carl Roth GmbH | Art. No. 7530.4; CAS: 56-81-5 |
| Imidazole           | Carl Roth GmbH | Art. No. X998.4; CAS: 288-32-4 |
| CSM(powder)         | MP Biomedicals | SKU: 114500022 |
| CSM-Uralpowder      | MP Biomedicals | SKU 114511212 |
| 18:1 (Δ9-Cis) PC (DOPO) | Avanti Polar Lipids | SKU: 850375P |
| 18:1 (Δ9-Cis) PE (DOPE) | Avanti Polar Lipids | SKU: 850725P |

(Continued on next page)
REAGENT or RESOURCE  SOURCE  IDENTIFIER
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Pi(3)P diC16  Echelon Bioscience  SKU: P-3016-
Pi(3,5)P2 diC16  Echelon Bioscience  SKU: P-3516-
ATTO-DPPE dye  ATTO-TEC  AD550-151
CellTracker Blue CMAC Dye  Invitrogen  Cat#C2110
FM™ 4-64 Dye  Invitrogen  Cat#T3166
Coomassie  Brilliant blue G-250  PanReac AppliChem  A3480; CAS: 6104-58-1
Glutathione Sepharose  Merck  SKU: GE17-0756-01
Protino  Ni-NTA Agarose  Macherey-Nagel  CAS: 64-17-5
Dynabeads M-270 Epoxy  Thermo Fisher  Cat#14304
[γ-32P]-ATP  Hartmann Analytic  Cat#SRP-501
Sypro Ruby Protein Gel Stain  Merck  Cat#S4942
Arg10  Cambridge Isotope Laboratories  CNLM-539-H
Lys8  Cambridge Isotope Laboratories  CNLM-291-H
10kD MW-cutoff filters  PALL  OD030C34
Titanium dioxide  GL Sciences  5020-75010

Critical Commercial Assays
NucleoSpin Gel and PCR Clean-up  Macherey-Nagel  REF 740609.250
GeneJET Plasmid-Miniprep-Kit  Thermo Fisher Scientific  Cat#K0503

Experimental Models: Cell Lines
S. cerevisiae: Strain background: BY4741  Brachmann et al.  N/A
S. cerevisiae: Strain background: BY4727  Brachmann et al.  N/A

Experimental Models: Organisms/Strains
CUY518: MATa; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0  tor1Δ::KanMX  Heitman et al.  N/A
CUY10936: MATa; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0  FAB1::mNeonGreen::hphNT1  This study  N/A
CUY11107: MATa; his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 FAB1::VC::KanMX  This study  N/A
CUY11172: MATa; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0  FAB1::mNeonGreen::hphNT1 ADH1pr-mCherry::KOG1  This study  N/A
CUY11260: MATa; his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 FAB1::VC::KanMX TRP1::CET1pr-VN::KOG1  This study  N/A
CUY11579: MATa; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0  FAB1::mNeonGreen::hphNT1 VPS8::mKate::ClonNAT  This study  N/A
CUY11710: MATa; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0  IVY1::GFP::hphNT1 VPS8::mkate::ClonNAT  This study  N/A
CUY11845: MATa; his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 FAB1::VC::KanMX TRP1::CET1pr-VN::KOG1 VPS8::mkate::ClonNAT  This study  N/A
CUY11922: MATa; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 fab 3202A/3203A/3204A/7206A/S208A/S210A::mNeonGreen::hphNT1  This study  N/A
CUY11923: MATa; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 fab 3202D/3203D/3204D/7206D/S208D/S210D::mNeonGreen::hphNT1  This study  N/A
CUY11972: MATa; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 FAB1::mNeonGreen-hphNT1 IVY1::3xmCherry::ClonNAT  This study  N/A

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| REAGENT or RESOURCE SOURCE IDENTIFIER | This study | N/A |
|--------------------------------------|------------|-----|
| CUY11973: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 fab1 S202A/S203A/S204A/T206A/S208A/S210A::mNeonGreen::hphNT1 IVY1::3xmCherry-ClonNAT | This study | N/A |
| CUY11974: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 fab1 S202D/S203D/S204D/T206D/S208D/S210D::mNeonGreen::hphNT1 IVY1::3xmCherry-ClonNAT | This study | N/A |
| CUY11976: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 fab1 S202A/S203A/S204A/T206A/S208A/S210A::mNeonGreen::hphNT1 KOG1::CloNAT-ADHpr | This study | N/A |
| CUY11977: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 fab1 S202A/S203A/S204A/T206A/S208A/S210A::mNeonGreen::hphNT1 KOG1::CloNAT-ADHpr | This study | N/A |
| CUY12077: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 fab1 S202D/S203D/S204D/T206D/S208D/S210D | This study | N/A |
| CUY12078: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 fab1 S202D/S203D/S204D/T206D/S208D/S210D | This study | N/A |
| CUY12219: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 EGO1::GFP::hphNT1 CloNAT::ADH1pr-mCherry::KOG1 | This study | N/A |
| CUY12220: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 fab1 S202A/S203A/S204A/T206A/S208A/S210A::EGO1::GFP::hphNT1 CloNAT::ADH1pr-mCherry::KOG1 | This study | N/A |
| CUY12221: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 fab1 S202D/S203D/S204D/T206D/S208D/S210D::EGO1::GFP::hphNT1 CloNAT::ADH1pr-mCherry::KOG1 | This study | N/A |
| CUY12227: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 IVY1::mGFP-hphNT1 VPS4::3xHA-mCherry::TRP1 FAB1::mNeonGreen::hphNT1 | This study | N/A |
| CUY12259: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 URA3::PHO5pr-GFP::FAB1 | This study | N/A |
| CUY12260: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 URA3::PHO5pr-GFP::FAB1-211aa-end | This study | N/A |
| CUY12341: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 URA3::PHO5pr-GFP::FAB1-211aa-end | This study | N/A |
| CUY12342: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 URA3::PHO5pr-GFP::FAB1-211aa-end | This study | N/A |
| CUY12354: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 URA3::PHO5pr-GFP::FAB1-211aa-end VPS8::mKate::hphNT1 | This study | N/A |
| CUY12355: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 URA3::PHO5pr-GFP::FAB1-211aa-end VPS8::mKate::hphNT1 | This study | N/A |
| CUY12356: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 URA3::PHO5pr-GFP::FAB1-211aa-end VPS8::mKate::hphNT1 | This study | N/A |
| CUY12357: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 URA3::PHO5pr-GFP::FAB1-211aa-end VPS8::mKate::hphNT1 | This study | N/A |
| CUY12362: MATa; his3 J1, leu2 J0, met15 J0, ura3 J0 URA3::PHO5pr-GFP::FAB1-151aa-end | This study | N/A |

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| REAGENT or RESOURCE SOURCE IDENTIFIER | SOURCE | IDENTIFIER |
|--------------------------------------|--------|------------|
| CUY12365: MATα; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 URA::PHO5pr-GFP-FAB1-191aa-end | This study | N/A |
| CUY12367: MATα; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 URA::PHO5pr-GFP-FAB1-321aa-end | This study | N/A |
| CUY12445: MATα; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 URA::PHO5pr-GFP-FAB1-51aa-end | This study | N/A |
| CUY12446: MATα; his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 URA::PHO5pr-GFP-FAB1-101aa-end | This study | N/A |
| CUY12650: MATα; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 IVY1::mGFP-hphNT1 KOG1::CloNAT-ADHpr | This study | N/A |
| CUY12653: MATα; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FAB1::mNeonGreen-hphnt1 VPS4::3xHA-mCherry-KanMX | This study | N/A |
| CUY12654: MATα; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FAB1 S202A,S203A,S204A,T206A,S208A,S210A::mNeonGreen-hpHNt1 VPS4::3xHA-mCherry-KanMX | This study | N/A |
| CUY12655: MATα; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FAB1 S202D,S203D,S204D,T206D,S208D,S210D::mNeonGreen-hpHNt1 VPS4::3xHA-mCherry-KanMX | This study | N/A |
| CUY12656: MATα; leu2Δ0, ura3Δ0 SCH91-183::SpHIS5 TOR1::hphNT1 trp1Δ::KanMX | This study | N/A |
| RLK395: MATα; his3Δ1, leu2Δ0, ura3Δ0 LEU2::GFP-TOR1 | This study | N/A |
| YL516: MATα; his3Δ1, leu2Δ0, ura3Δ0 | Binda et al. 71 | N/A |
| RHK395: MATα; his3Δ1, leu2Δ0, ura3Δ0 LEU2::GFP-TOR1 | Horvath et al. 68 | N/A |
| RHK486: MATα; leu2Δ0, ura3Δ0 his3Δ1::GFP-SCH91-183::SpHIS5 | This study | N/A |
| RHK488: MATα; leu2Δ0, ura3Δ0 his3Δ1::GFP-SCH91-183::SpHIS5 fab1Δ::KanMX | This study | N/A |
| RHK490: MATα; leu2Δ0, ura3Δ0 his3Δ1::GFP-SCH91-183::SpHIS5 vac7Δ::KanMX | This study | N/A |
| RHK492: MATα; leu2Δ0, ura3Δ0 his3Δ1::GFP-SCH91-183::SpHIS5 vac14Δ::KanMX | This study | N/A |
| RHK555: MATα; leu2Δ0, ura3Δ0 his3Δ1::GFP-SCH91-183::SpHIS5 atg18Δ::KanMX | This study | N/A |
| RKH78: MATα; his3Δ1, leu2Δ0, ura3Δ0 tor1Δ::hphNT1 trp1Δ::KanMX | This study | N/A |
| MP52-2A: MATα; his3Δ1, leu2Δ0, ura3Δ0 tor1Δ::hphNT1 trp1Δ::GFP | Heitman et al. 70 | N/A |

**Oligonucleotides**

- **Fab1-Forward**: GGACAGGGAT GCGGTCAACGAAGAAGgtttta
gagctagaaatagcaagttaaaataagg
- **Fab1-Reverse**: CTTCTTCGTTGAT CCGGATCTCTGGTGCCAcatcatattt
cattctagtgag
- **HDR-Fab1 5A**: TAAATGCAGGA CAGGAAACTCTGGCAGAGG
cgGATCCTTATCGCAAGA CACTTTATCGCC
- **HDR-Fab1 6D**: TAAATGCAGGA CAGGAACTCTGGCAGAGG
cgGATCCTTATCGCAAGA CACTTTATCGCC
- **HDR-Fab1 6D**

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| REAGENT or RESOURCE SOURCE IDENTIFIER |
|--------------------------------------|
| GST-p40-PX                           |
| Takatori et al.²²                    |
| pRS315: CEN/ARS, LEU2                 |
| Sikorski and Hieter²³                |
| pRS413: CEN/ARS, HIS3                 |
| Sikorski and Hieter²³                |
| pRS414: CEN/ARS, TRP1                 |
| Sikorski and Hieter²³                |
| pRS415: CEN/ARS, LEU2                 |
| Sikorski and Hieter²³                |
| pRS416: CEN/ARS, URA3                 |
| Sikorski and Hieter²³                |
| p1379: CEN/ARS, URA3, MET15           |
| Hatakeyama and De Virgilio²⁶         |
| p1770: CEN/ARS, HIS3, MET15           |
| Varlakhanova et al.⁵¹                |
| CU1776: CEN/ARS, URA3, GFP-CPS1       |
| Odorizzi et al.²¹                    |
| CU4615: CEN/ARS, LEU2, 3xHA-TOR1-I1954V |
| Stracka et al.⁵⁷                     |
| CU5125: CEN/ARS, URA3, P⁎nop1-FAB1-1-330aa-GFP |
| This study                           |
| CU5126: CEN/ARS, URA3, P⁎nop1-FAB1-1-330aa-GFP |
| This study                           |
| p2976: CEN/ARS, URA3, PADH1-GFP-SCH9¹⁻¹₈³ |
| This study                           |
| p2976: CEN/ARS, URA3, PADH1-GFP-SCH9¹⁻¹₈³ |
| This study                           |
| p3613: CEN/ARS, HIS3, P⁎vac8-EEA1(human)¹₂₅₇⁻¹₄₁₇⁻yEmRF-P-TOR1 |
| This study                           |
| p3693: CEN/ARS, HIS3, PADH1-GFP-SCH9¹⁻¹₈³ |
| This study                           |
| p3694: CEN/ARS, HIS3, P⁎ADH-GFP-SCH9¹₈⁴⁻₉₇³ |
| This study                           |
| p3695: CEN/ARS, HIS3, P⁎ADH-GFP-SCH9¹⁻³⁹³ |
| This study                           |
| p3715: CEN/ARS, HIS3, P⁎ADH-yEmRF-P-SCH9¹⁻¹₈³ |
| This study                           |
| p3745: CEN/ARS, HIS3, P⁎CYC-yEmRF-P-SCH9¹⁻¹₈³ |
| This study                           |
| p3776: CEN/ARS, LEU2, P⁎CYC-GFP-SCH9¹⁻¹₈³ |
| This study                           |
| pRH3488: [pET-28a (+)]-His6-fab1 (1-600) (codon optimized) |
| This study                           |
| pRH3490: [pET-28a (+)]-His6-fab1 (580-1180) (codon optimized) |
| This study                           |
| pRH3491: [pET-28a (+)]-His6-fab1 (1160-1760) (codon optimized) |
| This study                           |
| pRH3492: [pET-28a (+)]-His6-fab1 (1740-2278) (codon optimized) |
| This study                           |
| pMP3668: [pET-28a (+)]-His6-fab1 T206A/S208A/S210A (1-600) (3A) (codon optimized) |
| This study                           |
| pMP3669: [pET-28a (+)]-His6-fab1 S203A/S204A/T206A/S208A/S210A (1-600) (5A) (codon optimized) |
| This study                           |
| p2981: 2μ, HIS3, P⁎vac8-EEA1(human)¹₂₅₇⁻¹₄₁₇⁻GFP-SCH9⁷⁰⁹⁻₈₂⁴ |
| Hatakeyama and De Virgilio²⁶         |
| p3047: 2μ, LEU2, P⁎PRC1-SCH9⁷⁰⁹⁻₈₂⁴-GFP-PHO8¹⁻⁶³ |
| Hatakeyama and De Virgilio²⁶         |
| pJU793: CEN/ARS, URA3, GFP-SCH9       |
| Urban et al.⁵³                       |
| p3296: CEN/ARS, URA3, GFP-SCH9¹⁻¹₈³ |
| This study                           |
| p3297: CEN/ARS, URA3, GFP-SCH9¹⁻₈⁴⁻₃⁹⁷ |
| This study                           |

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RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resource and regents should be directed to and will be fulfilled by the Lead Contact, Christian Ungermann (cu@uos.de).

Materials Availability
Yeast strains and plasmids generated in this study are available on request.

Data and Code Availability
The published article includes all datasets generated or analyzed during this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Yeast strains, plasmids, and media
Yeast strains and plasmids are listed in Key Resource table. Yeast strains were grown in yeast extract peptone dextrose (YPD) containing 1% yeast extract, 2% peptone and 2% glucose at 30°C for most growth analyses. For microscopy experiments and spotting assays, yeast strains were grown in synthetic dextrose complete (SDC) media, which contained 6.75 g yeast nitrogen base without amino acids, 20 g glucose, and 0.8 g complete supplement media (CSM) powder per liter.

METHOD DETAILS

Yeast genetic manipulation and molecular biology
S. cerevisiae strains used are listed in Resource Table. Genetic manipulations were made by homologous recombination of PCR fragments as described previously.75,76 Yeast cells were grown in YPD medium overnight and then diluted to 0.1 OD600. They were then grown to log-phase, washed with sterile water, and then with 0.1 M lithium acetate. Cells were resuspended in 350 μl 0.1 M lithium acetate, and 50 μl were used for transformation. The transformation mix contains 240 μl 50% PEG, 36 μl 1 M lithium acetate, 25 μl carrier DNA and 50 μl cells. The mix was incubated at 42°C for 45 min and cells were then plated on selection plates. A CRISPR-Cas9 approach was selected to generate FAB1 point mutants.74 For generation of the corresponding Cas9 plasmid (CU5048), the following primers were used: Fw-GGACAGGGATGCGGTCAACGAAGAAGgttttagagctagaaatagcaagttaaaataagg, Rv- CTTCTTCGTTGACCGCATCCCTGTCgatcatttatctttcactgcggag. We transform both plasmid (CU5048) and the corresponding single stranded DNA (ssDNA) templates for homology directed repair (HDR), HDR-Fab1 6A (TAAAATGCGAACAGGAAACTCTTTATCGAAGACACTTCATCGCC) or HDR-Fab1 6D (TAAAATGCGAACAGGAAACTCTTTATCGAAGACACTTCATCGCC), into the used background strains. After transformation, the cells were re-suspended in YPD and grown at 30°C for 1-2 h for recovery before cells were placed onto plates containing G418. Clones containing the correct mutations were identified by sequencing of the FAB1 region. All plasmids are listed in Resource Table.

Light microscopy and image analysis
Cells were grown to log-phase in yeast extract peptone (YP) medium containing glucose (YPD), or synthetic medium supplemented with essential amino acids (SDC). Vacuole membrane was stained with 30 μM FM4-64 for 30 min, followed by washing with medium, and incubation in medium without dye for 1 hr before analysis.77 For luminal staining of vacuoles, cells were incubated in 0.1 mM 7-amino-4-chloromethylcoumarin (CMAC) for 10 min and washed with SDC medium. Images were acquired on an Olympus IX-71 inverted microscope equipped with a 100x NA 1.49 objective, a sCMOS camera (PCO), an InsightSS1 illumination system and SoftWoRx software (Applied Precision). Images were processed with ImageJ. One representative plane of a z stack is shown unless noted. For quantifications the number of vacuoles were counted in three independent experiments and at least 100 cells were
quantified in each experiment. For determination of colocalization of two proteins, their dot-like signal was quantified as percentage of colocalizing dots.

**Growth test**

Cells were grown to log-phase, washed twice with SDC, and diluted to an optical density 600 (OD600) of 0.25. Serial dilutions (1:10) were spotted onto plates and incubated after 2-4 days.

**In vivo local TORC1 activity assay**

Endosomal and vacuolar TORC1 activities were assayed as previously described.47 The C-terminal portion of Sch9, an established TORC1 substrate, was fused to GFP and an organelle targeting domain: FYVE domain of human EEA1 protein for the endosomal reporter (ET) or the N-terminal domain of Pho8 vacuolar phosphatase for the vacuolar reporter (VT). The strains were transformed with ET/VT reporters and their phosphorylation levels in cell lysate was assessed by western blotting using the phosphospecific anti-Sch9-pThr737 antibodies.

**In vitro kinase assay of Fab1 fragments with purified TORC1**

*In vitro* kinase assays were performed with some variation, essentially as described.79 TORC1 was purified from a Tco89-TEV-TAP-expressing yeast strain grown in YPD and treated for 10 min with cycloheximide. The cells were collected by filtration, rapidly frozen in liquid nitrogen, and subjected to cryogenic disruption with an MM 400 Mixer Mill (Retsch). The obtained powder was resuspended in extraction buffer (50 mM HEPES/NaOH, pH 7.5, 5 mM CHAPS, 400 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 400 mM Pefabloc, Roche complete protease inhibitor EDTA-free). The cleared lysate was incubated with IgG-coupled Dynabeads (Dynabeads M-270 Epoxy; Invitrogen) for 2 h at 4°C. After 5 washes with wash buffer (50 mM HEPES/NaOH [pH 7.5], 5 mM CHAPS, 400 mM NaCl, 0.5 mM DTT) the TORC1 complex was eluted using 2% TEV protease and stored at −80°C after addition of 10% glycerol.

Recombinant His6-tagged Fab1 variants were expressed in Rosetta *E. coli* cells. Bacterial cells were disrupted by sonication in lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM Imidazole, Roche complete protease inhibitor EDTA-free, 0.1% NP40) and proteins were purified through binding to Ni-NTA agarose beads (wash buffer containing 50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 20 mM Imidazole; elution buffer containing 50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 250 mM Imidazole). Eluted fractions were dialysed into 50 mM HEPES, pH 7.5, 150 mM NaCl, 5% glycerol (Slide-A-Lyzer MINI Dialysis Device, 10K MWCO).

*In vitro* kinase reactions were performed in kinase buffer (50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl), with 120 ng of purified His6-tagged Fab1 variants and TORC1 (containing 60 ng Tor1) in 30 µL total volume, started by adding the ATP mix (4.2 mM MgCl2, 300 mM ATP, and 3.3 mCi [γ-32P]-ATP [Hartmann Analytic, SRP-501]), and stopped by adding SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, stained with Sypro Ruby (Sigma) to assess loading, and analyzed using a phosphorimager (Typhoon FLA 9500; GE Healthcare).

**Purification of recombinant Sch91-183-His6**

GST-Sch91-183-His6, which has a TEV protease cleavage site between the GST and Sch91-183, was purified from bacteria (Rosetta strain). Expression was induced by 0.5 mM IPTG overnight at 18°C. Cells were lysed by sonication in lysis buffer (200 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5% glycerol, 1 mM DTT, Roche protease inhibitor cocktail) and clarified by centrifugation. The protein was purified by incubation with glutathione Sepharose beads for 1 h at 4°C, and washed with lysis buffer. Sch91-183-His6 was eluted by incubating the beads with TEV protease in cleavage buffer (50 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 1 mM DTT) for 1 h at 30°C.

**Liposome sedimentation assay**

Liposomes were prepared as previously described with modifications. Liposomes were generated from the lipid mixtures (81.5 mol % dioleoylphosphatidylcholine [Avanti Polar Lipids 850375], 18 mol % dioleoylphosphatidylethanolamine, 0.5 mol % Atto dye [ATTO-TEC AD550-151]) with or without PI(3,5)P2 or PI3P at the indicated concentrations. The lipids were dried by evaporation for 1 h and dissolved in 1 mL of assay buffer (20 mM PIPES-KOH [pH 6.5], 120 mM KCl) supplemented with 5% w/v sucrose to a final lipid concentration of 2 mM. The liposome suspension was freeze/thawed for 8 times in liquid nitrogen and at 55°C, respectively. For the binding assay, 0.5 µg of Sch91-183-His6 was pre-cleaned in assay buffer by centrifugation at 20,000 g for 20 min at 4°C. The supernatant containing Sch91-183-His6 was mixed with 0.54 mM (final concentration) liposomes and incubated at room temperature for 15 min. The liposomes were then centrifuged (20,000 g for 20 min at 4°C), and the supernatant (unbound fraction) was separated from the pellet (liposome-bound fraction). Samples were TCA-precipitated and analyzed via SDS-PAGE and subsequent immunoblot analysis using the anti-His Tag antibodies (SIGMA-ALDRICH, Germany).

**Mass spectrometry sample preparation and data analysis**

MS samples were basically generated as described.47 Briefly, for *in vivo* analyses yeast strains were grown in SILAC medium containing non-labeled or labeled arginine and lysine variants, L-arginine-15N6-13C6 (Arg10) and L-lysine-15N2-13C6 (Lys8) (Sigma-Aldrich). Cells were treated with or not with 200 ng/mL rapamycin for 30 min, and trichloroacetic acid (TCA)-treated cell pellets (50 mg) of both labels were mixed, and stored at −80°C for further use. For *in vitro* analyses, purified TORC1 was incubated with the Fab1 (1-600) fragment in the presence of 1 mM MnCl2 for 30 min as described above. As a negative control, purified TORC1 was inhibited with 6 µM wortmannin for 30 min. *In vitro* kinase assays of purified Fab1 variants and TORC1 were performed on 10 kDa MW-cutoff filters (Pall) as described.47
Proteins were reduced by 1 mM DTT, alkylated by 5 mM iodoacetamide and digested by Lys-C (Lysyl Endopeptidase, WAKO) for 4 h, in case of in vivo analyses, and trypsin (Promega) overnight. Phosphopeptides were enriched by TiO₂ beads (GL Sciences) essentially as described and analyzed by LC-MS/MS using a QExactive HF-X mass spectrometer coupled to an EasyLC 1200 nanoflow-HPLC (Thermo Scientific). MS raw files were analyzed using MaxQuant (version 1.6.2.10), and a Uniprot full-length S.cerevisiae database (March, 2016) containing common contaminants such as keratins and enzymes used for in-gel digestion. Fixed modification was carbamidomethylcysteine, variable modifications were protein amino-terminal acetylation, serine-, threonine- and tyrosine-phosphorylation, and oxidation of methionine. The MS/MS tolerance was set to 20 ppm and three missed cleavages were allowed using trypsin/P as enzyme specificity. Peptide, site, and protein FDR based on a forward-reverse database were set to 0.01, minimum peptide length was set to 7, the minimum score for modified peptides was 40, and minimum number of peptides for identification of proteins was set to one, which must be unique. MaxQuant results were analyzed using Perseus.

QUANTIFICATION AND STATISTICAL ANALYSIS

Co-localization between two proteins
The number of co-localizing dot-like structure between Fab1 and other proteins (Figures 1C and 3), or Ivy1 (Figure 1A) and other proteins were counted, which were divided by the total numbers of the Fab1 dot-like or Ivy1 dot-like signal. For statistical analysis, two-tailed, unpaired Student’s t tests were used, and data is presented as mean ± SD.

Vacuole numbers
The number of the cell containing different amounts of vacuoles was counted in Fab1 mutants (Figure 3B). Two-tailed, unpaired Student’s t tests were used, and data is presented as mean ± SD.

Dots structures in cells
The number of dot-like signals for each protein were counted per 100 cells, and the results are shown as graphs (Figures 3H and 6B). Two-tailed, unpaired Student’s t tests were used, and data is presented as mean ± SD. The number of cells containing Sch91-183 dots were counted and the relative distribution is shown in Figure 5H (no comparative statistical analysis was performed here) and Figures 5G and 5I (Two-tailed, unpaired Student’s t tests were used for statistical analysis).

Western blot
Western blot bands were analyzed by ImageJ, and the quantities were calculated from the relative intensities. For statistical analysis, two-tailed, unpaired Student’s t tests were used, and data is presented as mean ± SD.