Control of vacuole membrane homeostasis by a resident PI-3,5-kinase inhibitor

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Lysosomes have an important role in cellular protein and organelle quality control, metabolism, and signaling. On the surface of lysosomes, the PIKFyve/Fab1 complex generates phosphatidylinositol 3,5-bisphosphate, PI-3,5-P$_2$, which is critical for lysosomal membrane homeostasis during acute osmotic stress and for lysosomal signaling. Here, we identify the inverted BAR protein Ivy1 as an inhibitor of the Fab1 complex with a direct influence on PI-3,5-P$_2$ levels and vacuole homeostasis. Ivy1 requires Ypt7 binding for its function, binds PI-3,5-P$_2$, and interacts with the Fab1 kinase. Colocalization of Ivy1 and Fab1 is lost during osmotic stress. In agreement with Ivy1’s role as a Fab1 regulator, its overexpression blocks Fab1 activity during osmotic shock and vacuole fragmentation. Conversely, loss of Ivy1, or lateral relocalization of Ivy1 on vacuoles away from Fab1, results in vacuole fragmentation and poor growth. Our data suggest that Ivy1 modulates Fab1-mediated PI-3,5-P$_2$ synthesis during membrane stress and may allow adjustment of the vacuole membrane environment.

Significance

The lysosome-like vacuole is the main organelle to degrade membrane proteins and organelles and, thus, provides amino acids, but also ions to the cytosol for cellular survival. Maintenance of vacuole membrane integrity is thus important for cellular adaptations. The vacuole contains several protein complexes on its surface to maintain the vacuole functional, and one such complex is a lipid kinase named Fab1 (of PIKFyve in human cells). Fab1 is part of a protein complex that produces a phosphorylated lipid, PI-3,5-P$_2$. Other proteins bind PI-3,5-P$_2$ and can fragment the vacuole to balance volume vs. membrane during stress. We now identify Ivy1 as a protein that binds Fab1 and controls its activity.

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resistant to vacuole fragmentation (Fig. 2 A and B). Moreover, overexpression using the *TEF* promoter did not impair Ypt7 function as vacuoles remained round under these conditions. As a direct demonstration, we co-cultivated wild type and cells overexpressing *ivy1* in a flow chamber and rapidly exposed cells to high salt. Wild-type cells responded in seconds with vacuole fragmentation, whereas *ivy1*-overexpressing cells maintained round vacuoles (Fig. 2C). This observation was independent of the strain used for tracking vacuole morphology.

Surprisingly, normal round vacuoles have been observed in *ivy1*Δ cells (24, 26). We speculated that *ivy1* might carry out an essential function in vacuole membrane homeostasis, and existing *ivy1*Δ mutant strains might carry suppressors masking the phenotype of the deletion. To test this, we mated two wild-type strains that were transformed with a centromeric plasmid with a *URA3* marker coding for *IVY1*. We deleted *IVY1* in those cells, sporo-

Further deletion of a segment of seven residues close to the end of the *ivy1* I-BAR domain abolished vacuole localization (Fig. 1 A, c and d). Within this targeting region, we mutated two prominent positively charged residues (RK) to alanine. The resulting RK-AA mutant was cytosolic (Fig. 1 A, c), and we thus addresed effects on Ypt7 and PIP lipid binding affinities. Using established Rab protein binding assays (25), recombinant *ivy1* RK-AA mutant was not able to bind GST-Ypt7 in vitro, whereas wild-type *ivy1* was specifically retained by Ypt7-GTP (Fig. 1 B). In contrast, *ivy1* and the RK-AA mutant protein retained PIP lipid binding properties. Purified *ivy1* and the RK-AA mutant bound to PI-3-P and PI-3,5-P₂ in liposome flotation assays, whereas binding to phosphatidic acid, PI, or PI-4-P was negligible (Fig. S1A). Together, these results point to a critical role for the I-BAR domain in *ivy1* vacuolar localization and suggest that the RK-AA mutant protein is specifically impaired in Ypt7 binding rather than generally misfolded (Fig. S1B).

To determine whether Ypt7 binding or vacuole localization is critical for *ivy1* function, we relocalized the RK-AA mutant into vacuoles via a GFP-specific chromobody (CB) that was attached to the vacuolar *Eg01* protein (Fig. S1C). Although the RK-AA mutant was now on vacuoles (Fig. S1C), it was not able to complement the previously reported vacuolar morphology defect or synthetic growth defect of *ivy1Δ vma16Δ* mutant cells on rapamycin, an inhibitor of TORC1 (Fig. S1 C and D) (24). Thus, Ypt7 binding and *ivy1* function are intimately linked.

**Ivy1 Affects Vacuole Membrane Homeostasis.** As *ivy1* binds PI-3,5-
P₂ and redistributes along vacuolar membranes during alterations in PI-3,5-P₂ concentrations (Fig. S1A) (24), we explored its role in PI-3,5-P₂-mediated vacuole fragmentation as a response to osmotic stress. To test if *ivy1* levels affect the response of cells to high salt, we overexpressed the protein and scored vacuole fragmenta-

**Ivy1 Interacts Dynamically with Fab1 on Vacuoles.** We next asked if *ivy1* acts on Fab1. Initially, we used the hyperactive *fab1*Δ allele, which produces fragmented vacuoles (14) (Fig. 3 A and B),
When Ivy1 was overproduced, the vacuole fragmentation was largely suppressed, suggesting a direct effect of Ivy1 on Fab1 activity. Ivy1 did so even in the absence of Fig4, supporting a model where Ivy1 inhibits Fab1 instead of activating Fig4 (Fig. S1 F and G). We measured the PI-3,5-P₂ levels and detected higher levels of PI-3,5-P₂ in the fab1Δ allele, which increased even further under hypertonic conditions. However, Ivy1 overproduction strongly reduced this increase in PI-3,5-P₂ levels (Fig. 3C). The most plausible explanation would be a direct interaction between Ivy1 and Fab1. We used a bifluorescence complementation approach and attached the two halves of YFP, VN and VC, to Ivy1 and Fab1. We observed YFP-positive dots on vacuoles, similar to the native localization of Ivy1, whereas no signal was detectable between Vac8 and Ivy1 regardless of the combination (Fig. 3D and Fig. S2E). As a second approach, we tested for copurification of Ivy1 and Fab1. When cells expressing GFP-tagged Fab1 or Vac8 were subjected to anti-GFP pull down, we observed a clear interaction of Fab1-GFP with Ivy1 (Fig. 3E). Supporting this, mkate-tagged Ivy1 largely colocalized with Fab1-mNeonGreen on vacuoles; however, such a colocalization was not observed between Ivy1 and Vac8 (Fig. 3 F and G). If Ivy1 inhibits Fab1, we would expect that Ivy1 would separate from Fab1 during salt stress, when Fab1 activity increases. We monitored cells when exposed to salt stress as before (Fig. 2C), but now compared the relative distribution of Ivy1 and Fab1 (Fig. 3 H and I). In agreement with our hypothesis, Ivy1 separated from Fab1 within seconds as soon as cells were exposed to high salt, whereas it remained associated with Fab1 without salt stress (Fig. 3 H and I). This observation suggests that Ivy1 is a local and dynamic inhibitor of Fab1 activity on vacuoles.

**Lateral Positioning of Ivy1 Is Critical for Localized Fab1 Inhibition.** The dot-like positioning of Ivy1 suggests a locally confined inhibition of Fab1. We wondered if we could test this by redirecting Ivy1 to different microcompartments of the vacuole. For this, we attached the chromobody to GFP to several proteins that have been either colocalized with Ivy1 (Ego1, Fab1) or not (the V-ATPase subunit Vph1) (24, 32) (this work). CB tagging of these proteins or GFP tagging of Ivy1 did not affect vacuole morphology (Fig. 4 A and F), nor did it alter vacuole function during osmotic stress (Fig. 4E) or normal growth (Fig. S3D). We tagged Ivy1 with GFP in cells carrying CB-tagged proteins and monitored vacuole morphology. When Ivy1-GFP was expressed in the Fab1-CB background, Ivy1 was found in dot-like structures. However, upon exposure to high salt, vacuole fragmentation was blocked, even when Ivy1 was not overexpressed (Fig. 4 B and E). In contrast, when Ego1 was GFP-tagged in the presence of Fab1-CB, vacuoles fragmented as in wild type, indicating that Fab1 functionality is not generally impaired by linking it to GFP-tagged protein (Fig. S3B). These findings agree with our in vivo observation that Ivy1 needs to separate from Fab1 under these conditions to release local inhibition of the kinase (Fig. 3H).

Strikingly, we observed the same vacuole fragmentation when wild-type Ivy1-GFP was coexpressed with Ego1-CB or Vph1-CB. This fragmentation phenotype was restored, when we introduced a plasmid coding for untagged Ivy1 (Fig. 4 C and F), indicating that the fragmentation is a consequence of the lateral relocation of Ivy1. The observed fragmentation was not due to an interference with the fusion machinery since vacuoles became round after water treatment (Fig. 4D). To test if fragmentation is the result of linking Ivy1 to another protein complex, we tagged either Ego1 or the retromer subunit Vps35 with GFP in the Vph1-CB background. Both proteins localized to the vacuole but did not alter vacuole morphology (Fig. S3C). To test how relocation affects physiology, we monitored growth of cells with laterally mistargeted Ivy1 on plates. Strikingly, cells with Ego1-targeted Ivy1 or Vph1-targeted Ivy1 grew very slowly (Fig. 4G), in agreement with the observed growth defect of the ivy1Δ cells (Fig. 2 F and H). We conclude that Ivy1 regulates Fab1 and maintains a functional vacuole during osmotic stress and normal growth.

**Discussion**

Here, we uncover Ivy1 as a sensor of vacuole membrane integrity that controls cell functionality during osmotic stress. Ivy1 binds directly to the vacuolar Fab1 complex and inhibits its activity. During hypertonic stress, Ivy1 relocates away from Fab1 on the
Fig. 4. Lateral relocalization of Ivy1 affects vacuole morphology and cell physiology. (A) Functionality of tagged subunits. Fab1, Ego1, and Vph1 were tagged C-terminally with a chromobody that specifically binds GFP. Vacuoles were FM4-64 stained and analyzed by fluorescence microscopy. (B) Effect of Ivy1 attachment to Fab1. Cells carrying Fab1-CB and expressing Ivy1-GFP were monitored as in A under normal and hypertonic conditions. BF, bright field. (C) Alteration of vacuole morphology upon lateral relocalization of Ivy1. Ivy1-GFP was expressed in cells carrying Ego1-CB and Vph1-CB, and vacuole morphology was analyzed as in A and B. (D) Fusion competence of fragmented vacuoles. Cells expressing Ivy1-GFP and Vph1-CB were FM4-64 stained and incubated in a flow chamber. At indicated times, the medium was replaced by H2O and cells were monitored by fluorescence microscopy. (E) Quantification of vacuole morphology of indicated cells when exposed to hypertonic stress. Results are mean ± SD. n = 3 (48 cells). (F) Quantification of vacuole fragmentation as observed in B–D. Results are mean ± SD. n = 3 (48 cells). (G) Relocalization of Ivy1 impairs growth. Serial dilutions of indicated strains were spotted on SDC and imaged after 2 or 4 d at 30 °C. (H) Model of Ivy1 function. ∗∗P < 0.01, ∗∗∗P < 0.005, n.s., nonsignificant. (Scale bars: 5 μm.)

vacuole, relieving Fab1, which then produces PI-3,5-P2 as a prerequisite of vacuole fragmentation (Fig. 4H). Supporting this, overproduction of Ivy1 blocks vacuole fragmentation, corrects the hyperactive fab1* allele, and suppresses PI-3,5-P2 production during hyperosmotic stress. This important role of Ivy1 can be mirrored if we laterally relocalize Ivy1 with the chromobody attached to selected vacuolar proteins, indicating that lateral localization into microcompartments on the vacuolar membrane is of functional importance. Furthermore, Ivy1Δ mutants present fragmented vacuoles and reduced growth.

How does Ivy1 respond and detect membrane stress? We can only speculate on its precise function. Ivy1 has a central I-BAR domain, which is critical for its function (24). It can oligomerize on membranes in vitro, yet requires Ypt7 for its localization (24) and function. It also binds two signature lipids of the vacuole, PI-3-P and PI-3,5-P2. It has all required properties of an organelle membrane sensor that detects alterations in membrane packing or its homeostasis. While such a function remains to be shown, the lateral relocalization of Ivy1 away from Fab1 during hyperosmotic stress strongly favors this idea. This possible function of Ivy1 as a membrane sensor would be reminiscent of the function of the plasma membrane Slm1 in yeast, which activates the
TORC2 complex and promotes the synthesis of sphingolipids (33). Ivy1, like Slim1, could directly translate alterations in membrane organization into a signaling process that corrects for this defect. Future experiments need to test if such a model applies also to other lipid metabolic enzymes. SV-Harb has recently identified that controls PI-3-P levels on endosomes by inhibiting the PI-3-P kinase complex, suggesting that local inhibition of lipid kinase complexes may be a common scheme (34).

Modulation of Fab1 by Ivy1 adds to the complexity of its regulation. Fab1 is part of a complex, which includes the Fg4 PI-3-5-phosphatase, the adapter Vac14, the membrane protein Vac7, and the propeller Atg18 (20, 21). While deletion of single subunits of the complex reveals roles for each subunit on Fab1 localization and its resulting activity, it has remained a challenge to determine their direct roles due to complex regulatory networks. One striking example is Atg18, where the deletion causes both very high Fab1 activity and large vacuoles (21–23). In this regard, the analysis of Ivy1 marks an advance because we are able to modulate vacuole morphology depending on Ivy1 expression and localization, and can directly detect reduced Fab1 activity upon Ivy1 overexpression. Our data agree with a direct binding of Ivy1 to Fab1 as a local inhibitor, although we cannot exclude other interactions within the complex. It is possible that Ivy1 directly controls the available PI-3-P pool around Fab1 and only makes this available when Fab1 activity is required, although the PI-3-P levels were unchanged upon Ivy1 overexpression. It is noteworthy that vacuole membranes segregate into distinct domains during starvation or upon alteration of its ergosterol content (32, 35, 36). Ivy1 segregates into these domains during heat stress or starvation (24, 32). The lateral separation of Ivy1 and Fab1 may take advantage of possible transient domain formations during osmotic stress.

Why has Ivy1 function been overlooked? In our initial analyses, we uncovered Ivy1 as a membrane modulator and Ypt7 interacting and observed a response of Ivy1 to membrane stress. However, the deletion showed no apparent morphology defect. Only in combination with a V-ATPase deletion, vacuole membranes expanded strongly without further volume increase (24). Our genetic analysis of Ivy1 now reveals that ivy1Δ cells are suppressed by another mutation. Upon segregation, ivy1Δ cells grew extremely slow and had fragmented vacuoles (Fig. 2G), although we could not measure PI-3-5-P2 levels due to the slow growth (Fig. 2J), and the easy acquisition of suppressors. Identification of the suppression mechanism will provide additional insights into the control of vacuolar membrane homeostasis. Importantly, we also observed fragmentation of vacuoles when we deleted Ivy1 (Fig. S2 A and B). Ivy1 function is thus far more critical for vacuole biogenesis and yeast cell survival than anticipated.

Lysosomal function depends on PI-3-5-P2 homeostasis, as revealed by the strong neurological phenotypes upon loss of the PIKfyve kinase and Fg4 phosphatase activities in metazoans (12). Both the V-ATPase and the TORC1 complex are modulated by the lipid environment (6, 7, 37), and cells lacking the Fab1 complex have growth defects. A precise understanding of PI-3-5-P2 function on vacuoles will be important for our further understanding of vacuole biogenesis in general.

Materials and Methods

Yeast Genetic Manipulation and Molecular Biology. Saccharomyces cerevisiae strains used are listed in Table S1. Genetic manipulations were made by homologous recombination of PCR fragments as described previously (38, 39). Ivy1 point mutants were generated by QuikChange mutagenesis. Truncations were generated using primers aligning at the desire region and introduced a restriction site. PCR product was digested and ligated into plasmids. All plasmids are listed in Table S2.

Light Microscopy and Image Analysis. Cells were grown to log-phase in yeast extract peptone (YP) medium containing glucose (YPD), galactose (YPG), or synthetic medium supplemented with essential amino acids (SDC). Vacuole marker memories were obtained by incubating a 30 μM FM4-64 for 30 min, followed by washing with medium, and incubation in medium without dye for 1 h before analysis (40). For luminal staining of vacuoles, cells were incubated in 0.1 mM 4-chloromethylcoumarin (CMAC) for 10 min and washed with SDC. Images were acquired on an Olympus IX-71 inverted microscope equipped with a 100× N.A. 1.49 objective, an sCMOS camera (PCO), an InsightSSi illumination system, and SoftWoRx software (Applied Precision). Images were processed with ImageJ. One representative plane of a z-stack is shown unless noted.

 Purification of Recombinant Proteins. Escherichia coli BL21 (DE3) Rosetta cells were grown to log-phase with the IVY1 or YPT7/IVPS21 plasmids were grown to optical density 600 (OD600) of 0.8. Expression was induced with 0.5 mM IPTG overnight at 16 °C. Cells were harvested and lysed in 50 mM Tris HCl, pH 7.5, 150 mM NaCl, and 1 mM protease inhibitor mixture (1× ; 0.1 mg/mL leupeptin, 1 μM o-phenanthroline, 0.5 mg/mL pepstatin A, 0.1 mM Pefabloc). Lysates were centrifuged 15 min at 30,000 × g, cleared supernatant was added to Ni-NTA beads for His-tagged protein or to GSH-beads for GST-tagged protein, followed by incubation for 1 h at 4 °C. Ni-NTA beads were washed with 25 mL of buffer containing 0.02 M imidazole. His-Ivy1 was eluted from beads with buffer containing 0.3 M imidazole. GST-tagged proteins were eluted with buffer containing 15 mM reduced glutathione. Proteins were desalted into 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1× protease inhibitor mixture, 5% (vol/vol) glycerol buffer using a Slide A Dialysis Cassette, 10,000 molecular weight cutoff (Thermo Scientific), snap frozen, and stored at −80 °C until further use.

Growth Test. Cells were grown to log-phase, washed twice with SDC, and diluted to an OD600 of 0.25. Serial dilutions (1:10) were spotted onto plates and imaged after 2–4 d. For growth curves, cells were grown to logarithmic phase in SDC, washed twice either with SDC or SDC plus the drug to be tested, and diluted to an OD600 of 0.1. Cells were grown overnight at 30 °C in a 96-well plate, and OD was measured using a plate reader (Molecular Devices).

Liposome Flotation Assay. A film of dried lipids was prepared by chloroform evaporation (41). Once dried, the film was resuspended in HK buffer (50 mM Hepes/KOH, pH 7.2, 120 mM KAc). The suspension was subjected to five freeze-thaw cycles and afterward extruded through polycarbonate filters (pores 0.4, 0.2, 0.05, 0.03 μm). The following vacuole-like composition was used (in mol%): dioleoylphosphatidylcholine (DOPC; 51.9), dioleoylphosphatidylethanolamine (DOPE; 18), soy phosphatidylinositol (SoyPI, 18), cardiolipin (1.6), ergosterol (1), dioleoylphosphatidylcholine (DOPC; 51.9), dioleoylphosphatidylethanolamine (DOPE; 18), soy phosphatidylinositol (SoyPI, 18), cardiolipin (1.6), ergosterol (1), rh-DHPE (1.5), Posphoethanolamine (rH-DHPE; 1.5). For the PA-containing liposomes: DOPC (49.9), DOPE (18), SoyPI (18), dioleoylphosphatidic acid (PA, 2), cardiolipin (1.6), ergosterol (8), DAG (1), rH-DHPE (1.5). For the PI-3-P liposomes: DOPC (50.9), DOPE (18), SoyPI (18), cardiolipin (1.6), ergosterol (8), DAG (1), rH-DHPE (1.5), PI-3-P (1). For the PI-3,5-P2 liposomes: DOPC (50.9), DOPE (18), SoyPI (18), cardiolipin (1.6), ergosterol (8), DAG (1), rH-DHPE (1.5), PI-3,5-P2 (1). For the flotation, proteins were incubated with liposomes in 150 μM of HKM buffer (HK buffer with 1 mM MgCl2) at room temperature for 10 min. Suspension was adjusted to 30% sucrose by mixing 100 μL of 75% (wt/vol) sucrose solution in HKM buffer, overlaid with 200 μL containing 25% sucrose and 50 μL of sucrose-free HKM. Samples were centrifuged in a swinging rotor (SW40) for 1 h at 100,000 × g. Top fractions were collected (80 μL) and analyzed by Western blotting.
Phosphoinositide Analysis. Analysis of cellular phosphoinositide levels was performed as previously described (44) with the following modifications. Midlog cells (5 OD600 equivalents) grown in yeast nitrogen base media were harvested, washed in inositol free media (IFM), incubated for 10 min in inositol-free media, and labeled with myo-[2-3H]inositol (PerkinElmer) for 30 min. Following inositol labeling, cultures were split and further incubated in IFM or IFM containing 1.2 M NaCl for 15 min. Cells were precipitated in 4.5% perchloric acid and lysed by vortexing with glass beads. Cell lysates were washed in 100 mM EDTA, phospholipids were decylated, and further processed as previously described (44). Samples were dried and resuspended in H2O. Identification and quantitation of 3H-labeled glycerophosphoinositols was performed by HPLC (HPLC) using a Series 200 HPLC system (PerkinElmer), a partisphere SAX column (HiChrom), an infnow 150TR radiomatic detector (PerkinElmer), TCNav software, and TotalChrom workstation (PerkinElmer). Data for individual phosphoinositide species data are shown as percentages of the total number of counts detected for normalization.

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