PIKfyve-ArPIKfyve-Sac3 CORE COMPLEX: CONTACT SITES AND THEIR CONSEQUENCE FOR Sac3 PHOSPHATASE ACTIVITY AND ENDOCYTIC MEMBRANE HOMEOSTASIS

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The PtdIns(3,5)P2 metabolizing enzymes, the kinase PIKfyve and the phosphatase Sac3, constitute a single multiprotein complex, organized by the PIKfyve regulator ArPIKfyve and its ability to homomerize. We previously established that PIKfyve is activated within the triple PIKfyve-ArPIKfyve-Sac3 (PAS) core. These data assign an atypical function for the phosphatase in PtdIns(3,5)P2 biosynthesis, thus raising the question whether Sac3 retains its PtdIns(3,5)P2 hydrolyzing activity within the PAS complex. Herein, we address the issue of Sac3 functionality by a combination of biochemical and morphological assays in triply transfected COS cells using a battery of truncated or point mutants of the three proteins. We identified the Cpn60_TCP1 domain of PIKfyve as a major determinant for associating the ArPIKfyve-Sac3 subcomplex. Neither Sac3 nor PIKfyve enzymatic activities affected the PAS complex formation or stability. Using the well-established formation of aberrant cell vacuoles as a sensitive functional measure of localized PtdIns(3,5)P2 reduction, we observed a mitigated vacuolar phenotype by kinase-deficient PIKfyveK1831E if its ArPIKfyve-Sac3 binding region was deleted, suggesting reduced Sac3 access to, and turnover of PtdIns(3,5)P2. In contrast, PIKfyveK1831E that displays intact ArPIKfyve-Sac3 binding triggered a more severe vacuolar phenotype if coexpressed with ArPIKfyve-wt-Sac3wt but minimal defects when coexpressed with ArPIKfyve-wt and phosphatase-deficient Sac3D488A. These data indicate that Sac3 assembled in the PAS regulatory core complex functions as an active PtdIns(3,5)P2 phosphatase. Based on these and other data, presented herein, we propose a model of domain interactions within the PAS core and their role in regulating the enzymatic activities.

The seven phosphorylated derivatives of phosphatidylinositol (PtdIns), called collectively PIs, are eukaryotic membrane-anchored signaling molecules that orchestrate diverse cellular processes, including intracellular membrane trafficking (1-6). PtdIns(3,5)P2, a low-abundance PI comprising as little as 0.8% of total PIs in mammalian cells, mediates essential aspects of endocytic membrane homeostasis (7). Although mechanistic details remain to be elucidated, experimental evidence indicates that PtdIns(3,5)P2 may coordinate fission and fusion events in the multivesicular endosomal system of mammalian cells (8,9). Consistent with these roles, perturbations in PtdIns(3,5)P2 production impair several intracellular trafficking pathways, both constitutive and regulated, that emanate from or traverse the early endosomes (10-13). In line with the requirement for PtdIns(3,5)P2 in maintaining proper balance between membrane removal (fission) and membrane insertion (fusion), disrupted function of PIKfyve, the sole enzyme for PtdIns(3,5)P2 synthesis, is phenotypically manifested by endosome vesicle swelling and endomembrane vacuolation seen in a number of mammalian cell types (7). As unraveled recently, PIKfyve is engaged in an unusual physical interaction with the phosphatase Sac3 that turns over PtdIns(3,5)P2, forming a common endogenous complex (the PAS core complex) organized by the PIKfyve activator ArPIKfyve (9,14). The ternary association, scaffolded by ArPIKfyve homomerization, activates the PIKfyve kinase as...
evidenced by recent data for reduced PIKfyve activity upon disintegration of the PAS core (14). However, whereas the assembly of the three proteins in the PAS core is critical for PIKfyve activation and regulated PtdIns(3,5)P2 production, whether the same complex is a functional platform for Sac3 enzymatic activity is currently unknown.

PIKfyve, ArPIKfyve and Sac3 are large, evolutionarily conserved proteins, encoded by single-copy genes from yeast to humans. They all incorporate a range of functional domains (7). In the case of PIKfyve, there is an N-terminally positioned FYVE finger that targets the protein to PtdIns(3)P-enriched endosome membranes (15). Next is the DEP domain still with an uncharacterized function. The middle part of the molecule (residues 560–1438) is occupied by two domains: Cpn60_TCP1, with sequence similarity to molecular chaperonins, and a CHK homology region, with conserved C, H, and K residues uniquely displayed by the PIKfyve orthologs. The region of conserved Lys is homologous to spectrin repeats. At the C-terminus is the catalytic domain, responsible for the three PIKfyve kinase activities, i.e., synthesis of PtdIns(3,5)P2, PtdIns(5)P and phosphoproteins, including phospho-PIKfyve (16,17). The aberrant endomembrane vacuolar phenotype has been first observed upon ectopic expression of kinase-deficient PIKfyve with a point mutation in the predicted ATP-binding K1831 of the catalytic domain (18). Similar defects have been confirmed thereafter by siRNA-mediated silencing and pharmacological inhibition of PIKfyve in different mammalian cell types (13,19). All these maneuvers, however, while preserving the enzyme’s proper intracellular localization (15), affect all three PIKfyve kinase activities. That the aberrant vacuolar phenotype is due to abrogated PtdIns(3,5)P2 synthesis is evidenced by its appearance in cells expressing PtdIns(3,5)P2-deficient, but not PtdIns(5)P-deficient, PIKfyve point mutants and its subsequent reversal upon exogenous delivery of PtdIns(3,5)P2 (17). Importantly, aberrant endomembrane vacuoles are no longer seen if PtdIns(3,5)P2-deficient PIKfyve K1831E is mislocalized by disruption of its FYVE finger (18). Clearly, these data corroborate the conclusion that if properly localized PIKfyve mutants fail to produce PtdIns(3,5)P2, they can then trigger dominant-negative changes in mammalian cells, phenotypically manifested by endomembrane vacuoles. One puzzling observation awaiting clarification is the apparent inability of a Cpn60_TCP1-deletion PIKfyve mutant to induce endomembrane vacuoles despite its proper intracellular localization and lack of in vitro lipid kinase (18). Considering the partner Sac3 phosphatase collaborating in parallel with PIKfyve in triggering the endomembrane defects by reducing localized PtdIns(3,5)P2 levels, here we examined the interacting regions of the three proteins and their functional significance to the endomembrane homeostatic mechanism. We report here that the Cpn60_TCP1 domain of PIKfyve is a major determinant in recruiting the ArPIKfyve-Sac3 subcomplex. A PIKfyve mutant truncated in this domain is not only incapable of binding the ArPIKfyve-Sac3 subcomplex but also unable to induce aberrant endomembrane vacuoles even if harboring the kinase-dead K1831E mutation. Concordantly, the PIKfyveK1831E point mutant whose binding to ArPIKfyve-Sac3 is intact largely loses its ability to vacuolate cells when coexpressed with phosphatase-deficient Sac3D488A and ArPIKfyve but exacerbates the defective vacuolar phenotype when coexpressed with active Sac3WT and ArPIKfyve. These data indicate that Sac3 relays hydrolyzing activity from the PAS core, greatly contributing to the endomembrane defects associated with the PIKfyve inactivation and PtdIns(3,5)P2 deficiency.

EXPERIMENTAL PROCEDURES

cDNA Constructs - The pCMV5- or pEGFP-based deletion and point mutants of mouse PIKfyveS tagged with Myc- or HA-epitopes, including PIKfyveWT, PIKfyveK1831E, PIKfyveAFYVE and PIKfyveACpm+ were generated previously (16,18,20). The new mPIKfyveS constructs are: PIKfyveK1831EACpm+, with a deletion of the entire Cpn60_TCP1 domain and a portion upstream of CH homology (deleted residues 560-1231), and harboring the kinase-deficient point mutation at K1831; PIKfyveAC-Cpm and PIKfyveAC-Cpm+, with deleted N- (deleted...
residues 560-749) and C-terminal halves (deleted residues 807-1032) in the Cpn60_TCP1 domain, respectively; PIKfyve^ΔFΔDΔCpn, with deleted FYVE, DEP and Cpn60_TCP1 domains (deleted residues 1-929); PIKfyve^ΔFΔKin^+, with deleted FYVE finger, kinase domain and additional sequences upstream of the latter (deleted residues 1-199 and 1439-2052); PIKfyve^ΔSpecΔKin^+, with deleted C-terminus encompassing the CH homology domain, spectrin repeats and further downstream sequences including the kinase domain (deleted residues 1262-2052). Generation of these constructs is detailed in Suppl. Material 1. The pCMV5-based cDNA constructs of human ArPIKfyveWT tagged with Myc- or HA-epitopes were described previously (21). Generation of the pEGFP-based constructs of human ArPIKfyve [pEGFP-C2-HA-ArPIKfyveWT, pEGFP-C2-ArPIKfyve(298-782), pEGFP-C2-HA-hArPIKfyve(1-511) and pEGFP-C1-hArPIKfyve(523-782)] was detailed elsewhere (14). The human Sac3 constructs, pEGFP-C3-Sac3^WT, pEGFP-C3-Sac3^DK88A, pEF-BOS-Myc-Sac3 and pCMV5-HA-Sac3^WT were described elsewhere (9). The new hSac3 point and deletion mutants are: pCMV5-HA-hSac3D488A; pEGFP-C3-Sac3(1-315); pEGFP-C3-Sac3(1-574); pEGFP-C3-Sac3(388-907); pEGFP-C3-Sac3(478-907) and pEGFP-C3-Sac3(610-907). Their construction is detailed in Suppl. Material 2. The new constructs were confirmed by restriction mapping and immunoblotting in transfected cells.

Antibodies - Rabbit polyclonal antibodies against PIKfyve (R-7069), ArPIKfyve (WS047) and Sac3 proteins were characterized previously (9,21,22). They were used as protein A (R-7069) and affinity-purified forms (WS047) or as a crude antiserum (anti-Sac3). Polyclonal anti-HA (R4289, a gift by Dr. Mike Czech) was used as Protein A-purified IgG. Anti-Myc monoclonal antibody was from 9E10.2 hybridoma cells (ATCC). Anti-GFP polyclonal (Ab290) and goat anti-EA1 (N-19) were from AbCam and Santa Cruz Biotechnology, respectively.

Cell cultures and transfections - COS7 cells were cultured and transiently transfected with the indicated cDNAs by Lipofectamine 2000 or Lipofectamine (Invitrogen) for immunoprecipitation and immunofluorescence microscopy analyses, respectively, under conditions described in previous studies (14,18).

Immunofluorescence and Light Microscopy – Transfected COS-7 cells grown on coverslips were subjected to immunofluorescence microscopy analysis 12 and/or 24 h post-transfection as described previously (8,18). Permeabilized cells were stained with the primary and secondary antibodies indicated in the figure legends. Coverslips were mounted on slides using Slow Fade Antifade Kit. Cells were viewed in a Nikon Eclipse TE200 inverted microscope equipped with a Plan Apo 60x1.4 Ph3DM oil objective and 3 standard fluorescence channels (i.e., green for GFP, red for anti-Myc/Alexa568, and blue for anti-Sac3/Alexa350 signals). Images were captured with a SPOT RT slider charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI) and processed using SPOT 3.2 and Adobe Photoshop 6.0 software. Colocalization with EEA1 was investigated by confocal microscopy (model 1X81, Olympus) by a 60x UplanApo lens. Images were captured by a cooled charge-coupled device 12-bit camera (Hamamatsu) as described previously (8,9).

Baculoviral Vectors, Recombinant Protein Expression/Purification and In vitro Binding – Generations of GST-His6-mPIKfyve in baculoviral expression vector pAcGHLT-A and that of His6-hArPIKfyve, in bacterial expression vector pRSETb, were described previously (16,21). The baculoviral expression vector pFASTBac-GST-hSac3 was a kind gift by Dr. Takenawa. His6-ArPIKfyve and Sac3 were simultaneously expressed using a modular baculovirus-based system specifically designed for eukaryotic multiprotein expression (23) as detailed in Suppl. Material 3. For the in vitro binding assays, GST-PIKfyve or His6-PIKfyve (~15 ng protein) purified and immobilized on GSH- or Ni-NTA agarose as described elsewhere (16) was incubated for 2-16 h with His6-ArPIKfyve or GST-Sac3, respectively, produced and purified separately. Immobilized GST-PIKfyve was incubated for 2 h with the His6-ArPIKfyve-Sac3 subcomplex, purified from infected Sf21 cells on Ni-NTA column. Beads were washed following previously published protocols (24). The reactions were analyzed by SDS-PAGE and immunoblotting.
**Immunoprecipitation and Immunoblotting** – Fresh cell lysates, collected in RIPA\(^+\) buffer [50 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl, 1% Nonidet P-40, 0.5% Na deoxycholate and 1x protease inhibitor cocktail (1 mM phenylmethylsulphonylfluoride, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ml pepstatin and 1 mM benzamidine)] were precleared (20,000 x \(g\), 15 min, 4°C) and subjected to immunoprecipitation 24 h post-transfection. Control immunoprecipitates with nonimmune rabbit or mouse IgG were run in parallel. Immunoprecipitations were carried out for 16 h at 4°C, with protein A-Sepharose CL-4B added in the final 1.5 h of incubation. Immunoprecipitates were washed five times with RIPA + buffer and then processed by western blotting. Immunoblotting with the antibodies indicated in the figure legends was performed subsequent to protein separation by SDS-PAGE (typically on 6% gels) and electrotransfer onto nitrocellulose membranes as described previously (14,21). A chemiluminescence kit (Pierce) was used to detect the horseradish-peroxidase-bound secondary antibodies.

**Lipid and Protein Kinase Activity In Vitro Assays** - PIKfyve lipid and protein kinase activities were measured in parallel using PIKfyve immunoprecipitates derived from transfected cells. For the lipid kinase assay, washed PIKfyve immunoprecipitates were subjected to 15 min incubation at 37°C in an assay buffer (50 mM Tris, pH 7.4, 2.5 mM MgCl\(_2\) and 2.5 mM MnCl\(_2\)) supplemented with 50 µM ATP, \([γ\text{-}^32\text{P}]\text{ATP} (12.5 \, \mu\text{Ci})\) and 100 µM PtdIns (from soybean, Avanti Polar Lipids Inc) as detailed elsewhere (14,22). Lipids were extracted and analyzed by silica-gel TLC using an acidic solvent system (2M acetic acid/propanol, 1:2 v/v). Both PtdIns(5)P and PtdIns(3,5)P\(_2\) synthesized products were monitored simultaneously as we detailed elsewhere (14,22). PIKfyve protein kinase was measured by the PIKfyve autophosphorylation activity in a reaction mix composed of \([γ\text{-}^32\text{P}]\text{ATP} (5 \, \mu\text{Ci}), 25 \, \mu\text{M ATP}, 24 \, \mu\text{M MgCl}_2\) and 5 mM MnCl\(_2\), in 50 mM Hepes, pH 7.4, and carried out for 30 min at 25°C as described elsewhere (12,16). Generated lipid or protein products were detected by autoradiography and quantified by radioactive counting of the scraped silica spots or cut radioactive band.

**Other Methods** - Protein concentration was determined by the bicinchoninic acid protein assay kit (Pierce). Protein levels were quantified from the intensity of the immunoblot bands with a laser scanner (Microtek) and UNSCAN-IT software (Silk Scientific). Several films of different exposure times were quantified to assure the signals were within the linear range. Data are expressed as mean ± SEM. Statistical analysis was performed by either one- or two-tailed Student’s \(t\) test.

**RESULTS**

**ArPIKfyve and Sac3 associate with each other via their C-termini independently of the Sac3 phosphatase activity** - As established previously, PIKfyve interacts efficiently with ArPIKfyve and Sac3 only when the two proteins are together, whereas ArPIKfyve and Sac3 form an easily detectable heteromeric subcomplex without PIKfyve (14). That ArPIKfyve and Sac3 interact directly with each other in a manner independent of PIKfyve or other proteins was also confirmed herein in Sf21 insect cells infected with baculoviruses expressing His\(_{6}\)-ArPIKfyve and Sac3 from a Cre-loxP-mediated fused plasmid. Purification of His\(_{6}\)-ArPIKfyve on Ni-NTA agarose resin reproducibly copurified Sac3 (Suppl. Fig. 1). Therefore, to begin characterizing the contact sites in the PAS core complex, we first examined the molecular interaction of the ArPIKfyve-Sac3 subcomplex. We generated two overlapping GFP-based constructs of the Sac3 N- and C-terminal halves (Fig. 1A) and examined their efficiency to interact with Myc-ArPIKfyveWT by coimmunoprecipitation analyses in doubly transfected COS cells. As illustrated in Fig. 1B, whereas the N-terminal half of Sac3 (residues 1-574) coimmunoprecipitated with Myc-ArPIKfyve\(_{WT}\) by coimmunoprecipitation analyses in doubly transfected COS cells. As illustrated in Fig. 1B, whereas the N-terminal half of Sac3 (residues 1-574) coimmunoprecipitated with Myc-ArPIKfyve\(_{WT}\) only weakly, the C-terminal Sac3 fragment (residues 388-907) displayed the wild-type binding efficiency. With a goal to determine the minimal region that supports the interaction with ArPIKfyve, we next assessed shorter C-terminal fragments of Sac3 (Fig. 1A,B). Sac3(478-907) associated with an efficiency similar to that of Sac3(388-907) or Sac3\(_{WT}\), whereas the
Sac3(610-907) peptide fragment displayed 85±8% of the maximal binding (Fig. 1A,B). Sac3D488A, that harbors a point mutation in the phosphatase domain to abrogate the phosphatase activity displayed the Sac3WT-association efficiency (Fig. 1A,B). These data indicate that Sac3 interacts with ArPIKfyveWT independently of its phosphatase activity and a C-terminal fragment of ~430 aa is required to mimic the efficiency of the Sac3WT association with ArPIKfyve.

To determine the ArPIKfyve region interacting with Sac3 we examined two nearly overlapping GFP-based constructs of the ArPIKfyve N- and C-terminal region for their interaction with Myc-Sac3WT by coimmunoprecipitation in doubly transfected COS cells (Fig. 1C). Notably, only the fragment of the ArPIKfyve C-terminal region spanning residues 523-782 was recovered with Myc-Sac3WT (Fig. 1D). The association of the N-terminal region was <10% of that seen with the ArPIKfyveWT binding to Sac3WT (Fig. 1D). These data indicate that the C-terminal region of ArPIKfyve spanning residues 523-782 interacts with Sac3.

**Sac3 interacts with an ArPIKfyve homodimer** - As revealed recently, ArPIKfyve forms a homodimer (or a higher-order homooligomer) that scaffolds the PAS core (14). Intriguingly, the same C-terminal fragment that interacts with Sac3 is engaged in the ArPIKfyve homomerization (14). To better understand how the ArPIKfyve-Sac3 subcomplex is organized, we examined if ArPIKfyve could concurrently interact with Sac3 and with itself. To test this, we expressed simultaneously two distinctly tagged versions of ArPIKfyveWT together with Myc-Sac3WT. We used Myc-ArPIKfyveWT and GFP-HA-ArPIKfyveWT because they exhibit markedly different electrophoretic mobilities making their unambiguous detections possible. Importantly, both Myc-Sac3WT and Myc-ArPIKfyveWT were recovered in the HA-ArPIKfyveWT immunoprecipitates (Fig. 1E). The ArPIKfyveWT fragment neither homodimerized nor associated with Sac3WT to a significant extent, as evidenced by the lack of Sac3WT or ArPIKfyveWT immunoreactive bands coimmunoprecipitated with anti-HA ArPIKfyveWT (Fig. 1E). Together, these data indicate that ArPIKfyve-ArPIKfyve and ArPIKfyve-Sac3 interactions proceed simultaneously through the same region of ArPIKfyve spanning residues 523-782. Intriguingly, this region, identified by database searches as “Domain with unknown function”, incorporates 4 out of the predicted 5 coiled-coil motifs in ArPIKfyve (Fig. 1C), conducive in forming coiled-coil helical backbone for structural stability of multi-protein interactions (25). Because Sac3 does not form homooligomeric structures (14,26), seen also with the yeast counterpart (14,26), these data are consistent with the notion that the subcomplex incorporates ArPIKfyve and Sac3 at a molar ratio 2 (or higher) to 1.

**ArPIKfyve and Sac3 N-termini interact with PIKfyve** - We next sought to determine the regions in ArPIKfyve-Sac3 interacting with PIKfyve. As found previously in ectopically transfected COS cells, PIKfyve efficiently interacts with ArPIKfyve and Sac3 only when the two are together (14). This conclusion was corroborated herein by the data from in vitro reconstituted binding of the three recombinant proteins, expressed in and purified from insect or bacterial cells. Thus, GST-PIKfyve immobilized on GSH-agarose did not pull down His6-ArPIKfyve to a significant degree (Fig. 2A). Likewise, His6-PIKfyve immobilized on Ni-NTA agarose did not pull down GST-Sac3 (not shown). However, if the His6-ArPIKfyve/Sac3 subcomplex, purified from infected Sf21 on Ni-NTA agarose was incubated with purified GST-PIKfyve immobilized on GSH-beads both His6-ArPIKfyve and Sac3 were readily pulled down (Fig. 2A). Therefore, to determine how PIKfyve associates with the ArPIKfyve-Sac3 subcomplex we triply transfected COS cells with constructs of appropriately tagged PIKfyveWT, ArPIKfyveWT, and C-terminal fragments of Sac3, or PIKfyveWT, Sac3WT and C-terminal fragments of ArPIKfyve. Coimmunoprecipitation was carried out with antibodies against the tags in ArPIKfyveWT or Sac3WT. Levels of retrieved PIKfyveWT were quantified relatively to those found with the ArPIKfyve-Sac3 wild-types. As illustrated in Fig. 2B, Sac3WT(610-907) and ArPIKfyveWT poorly associated with PIKfyveWT as judged by the low HA-PIKfyveWT amounts coimmunoprecipitated with anti-Myc-ArPIKfyveWT. Likewise, a longer Sac3
fragment, spanning residues 388-907 did not bring about greater levels of HA-PIKfyve WT retrieved in Myc-ArPIKfyve WT immunoprecipitates (Fig. 2B,C). As expected, however, complexes between ArPIKfyve WT and the Sac3 C-terminal fragments were readily formed at the efficiency similar to that seen with the Sac3 wild-type (Fig. 2B,C). This observation indicates that even though a complex between ArPIKfyve and C-terminal Sac3 is formed, PIKfyve is not efficiently retrieved, consistent with the requirement of the Sac3 N-terminal region (1-388) in the PAS core formation. Likewise, ArPIKfyve-Sac3 complexes made of Myc-Sac3WT and GFP-ArPIKfyve(523-782) or GFP-ArPIKfyve(288-782) did not efficiently retrieve HA-PIKfyveWT (quantified in Fig. 2C), indicating the ArPIKfyve N-terminal 1-288 residues are required for an efficient PIKfyve incorporation within the PAS complex. These observations further indicate that the PAS complex could not be formed in the absence of ArPIKfyve or Sac3 N-termini, even though a stable subcomplex via their C-termini can still be formed.

PIKfyve Cpn60_TCP1 and CHK homology are essential in interacting with the ArPIKfyve-Sac3 subcomplex - To examine how PIKfyve associates with the ArPIKfyve-Sac3 subcomplex in the PAS core we generated several GFP-HA or HA-based truncated mutants of PIKfyve (Fig. 3A). Their efficiency to interact with the wild-type ArPIKfyve-Sac3 subcomplex was examined relative to that of PIKfyve WT by coimmunoprecipitation in triply transfected COS cells. We began characterizing the binding efficiency of a truncated mutant with deleted Cpn60_TCP1 domain and N-terminal parts of the CHK homology (deleted residues 560-1231; Fig. 3A). The CHK homology (residues 1151-1461), shared by all PIKfyve orthologs, harbors conserved Cys (6 vs. Fab1) or His (4 vs. Fab1), positioned at the N-terminal half (residues 1155-1327), and Lys (11 vs. Fab1), positioned at the C-terminal half of the homology (1295-1461). Sequence analyses by modular databases identify the region of conserved Lys as Spectrin repeats (residues 1367-1464; Fig. 3A), a module that supports assembly in multiprotein complexes involved in cytoskeletal architecture and signal transduction (27). Therefore, we herein refer to the N-terminal and C-terminal part of the CHK homology as CH and Spectrin repeats, respectively (Fig. 3A). Intriguingly, for reasons awaiting clarification, the Δ560-1231 mutant (herein as ΔCpn+) was known for quite some time to be devoid of lipid kinase activity (22) despite the intact lipid kinase domain (Fig. 3A). This peculiar observation points to the Cpn60_TCP1 and/or CH homology domains as a plausible candidate region for binding the ArPIKfyve-Sac3 subcomplex, thereby activating PIKfyve kinase activity. This suggestion was corroborated herein by coimmunoprecipitation analyses, shown in Fig. 3B, in which we detected only insignificant amounts of Myc-ArPIKfyve WT and Myc-PIKfyveSac3 WT (<5%; Fig. 3A) in HA-PIKfyve ΔCpn+ immunoprecipitates. Mutants with shorter truncations in the Cpn60_TCP1 domain, namely Δ560-749 (herein as ΔN-Cpn) and Δ807-1032 (herein as ΔC-Cpn) were also with markedly reduced ability of retrieving Myc-ArPIKfyve WT and Myc-PIKfyveSac3 WT (Fig. 3A,C), indicative for the presence of binding determinants on both the N- and C-terminal halves of the Cpn60_TCP1 domain. In contrast, the FYVE-finger deletion mutant interacted with ArPIKfyve-Sac3 at the wild-type efficiency, as judged by the similar levels of Myc-ArPIKfyve WT and Myc-Sac3 WT immunoreactive bands recovered in immunoprecipitates of HA-PIKfyveΔFYVE vs. HA-PIKfyve WT (Fig. 3A,B).

To confirm the critical role of the Cpn60_TCP1/CH homology domains in binding the ArPIKfyve-Sac3 complex, we sought to reconstitute the triple association in ectopically transfected COS cells. We assessed the efficiency at which ArPIKfyve WT and Sac3 WT coimmunoprecipitated with a PIKfyve fragment spanning residues 1-1260. This mutant, referred to herein as PIKfyveΔSpecΔKin+, harbors the region of Cpn60_TCP1 and CH deleted in the ΔCpn+ mutant, but the C-terminal part downstream of the CH homology encompassing the Spectrin repeats and the catalytic domain is eliminated (Fig. 3A). Surprisingly, the PIKfyve ΔSpecΔKin+ mutant was incapable of reproducing the efficiency of the PAS wild-type associations as evidenced by only the 25% potency of PIKfyveΔSpecΔKin+ vs. PIKfyve WT immunoprecipitates to retrieve ArPIKfyve WT.
and Sac3\textsuperscript{WT} (Fig. 3\textit{A,D}). Likewise, a larger fragment that incorporates the entire CH/Spectrin repeats homology along with the Cpn60\_TCP1 domain, referred to herein as \textDelta F\textDelta K\textDelta D, was unable to coimmunoprecipitate Myc-\textDelta F\textDelta K\textDelta D WT and Myc-Sac3\textsuperscript{WT} to an extent seen with PIKfyve\textsuperscript{WT} (Fig. 3\textit{A,E}). These data indicate that the region encompassing Cpn60\_TCP1 and upstream of CH is a necessary binding determinant yet alone it is insufficient to associate with the ArPIKfyve-Sac3 subcomplex as efficiently as PIKfyve wild-type.

The PIKfyve kinase domain, but not activity, is required for interaction with the ArPIKfyve-Sac3 subcomplex - The observation that a peptide fragment of the N-terminal and middle region of PIKfyve, incorporating the Cpn60\_TCP1 domain and upstream of the CH homology is insufficient to mimic the PIKfyve\textsuperscript{WT} association with ArPIKfyve-Sac3 suggests the C-terminal region downstream of the CH homology is also playing a role in the PAS core formation and stabilization. To test this, we examined the interaction of a truncated mutant harboring nearly the entire PIKfyve sequence minus the C-terminal kinase domain (Fig. 3\textit{A}). As illustrated in Fig. 3\textit{A,F}, there was significantly less Myc-ArPIKfyve\textsuperscript{WT} and Myc-Sac3\textsuperscript{WT} retrieved in immunoprecipitates of PIKfyve\textsuperscript{ΔCpn} vs. PIKfyve\textsuperscript{WT}, suggesting interaction sites for ArPIKfyve-Sac3 are present within the PIKfyve kinase domain. Noteworthy, while the catalytic domain is apparently important for efficient PAS core formation, the PIKfyve kinase activity itself is not. This was evidenced herein by the observed wild-type efficiency of the lipid/protein kinase-deficient HA-PIKfyve\textsuperscript{K1831E} mutant to coimmunoprecipitate Myc-ArPIKfyve\textsuperscript{WT} and Myc-Sac3\textsuperscript{WT} (Fig. 3\textit{A,F}). Consistent with the requirement for the catalytic domain in productive ternary-complex formation, a PIKfyve C-terminal fragment spanning residues 930-2052 (referred to as \textDelta F\textDelta D\textDelta Cpn), interacted to some extent with ArPIKfyve-Sac3 as judged by the detection of Myc-ArPIKfyve\textsuperscript{WT} and Myc-Sac3\textsuperscript{WT} in HA-PIKfyve\textsuperscript{ΔF\textDelta D\textDelta Cpn} immunoprecipitates (Fig. 3\textit{A,G} and not shown). It should be emphasized however that either of the two overlapping PIKfyve halves (residues 1-1260 and 930-2052) produced only \textasciitilde 1/4 of the PIKfyve wild-type association with ArPIKfyve-Sac3 (Fig. 3\textit{A}), suggesting ArPIKfyve/Sac3-induced alterations in the PIKfyve conformation to optimally accommodate the ArPIKfyve-Sac3 subcomplex. Together, these data are consistent with the notion that ArPIKfyve-Sac3 first docks at the Cpn60\_TCP1/CH region, which induces conformational changes in PIKfyve to uncover additional binding sites stabilizing the ternary associations of the PAS core.

No phenotypic defects by PIKfyve\textsuperscript{K1831E} if its ArPIKfyve-Sac3 binding region is eliminated - Having identified the Cpn60\_TCP1/CH region as a major determinant in associating with ArPIKfyve-Sac3, we next assessed the outcome of the lost ArPIKfyve-Sac3 binding to the genuine PIKfyve characteristics, such as lipid or protein kinase activities, intracellular localization and the effect on endomembrane homeostasis. Intriguingly, the PIKfyve\textsuperscript{ΔCpn} mutant was devoid of measurable \textit{in vitro} lipid kinase activity as observed previously (22) and confirmed herein under longer exposure times (4 days) of the autoradiograms (Fig. 4\textit{A}, quantified in Fig. 3\textit{A}). However, PIKfyve\textsuperscript{ΔCpn} displayed \textit{in vitro} protein kinase activity comprising 20-25% of that determined for PIKfyve\textsuperscript{WT} (Fig. 4\textit{A}), unlike the PIKfyve\textsuperscript{K1831E} mutant that lacked any lipid or protein kinase activities (16). These data suggest that associated ArPIKfyve-Sac3 is essential for PtdIns(3,5)P\textsubscript{2} and PtdIns(5)P synthesis but less critical for the protein kinase activity of PIKfyve. This conclusion is also substantiated by data demonstrating that if PAS-associated ArPIKfyve levels are reduced by siRNA-mediated knockdown or detergent stripping, the PIKfyve lipid kinase activity is markedly inhibited whereas the autokinase activity remains unaltered (12,21).

Given this lack of activity, one would assume that, like PIKfyve\textsuperscript{K1831E} and other PtdIns(3,5)P\textsubscript{2}-deficient mutants such as PIKfyve\textsuperscript{K1999E} or PIKfyve\textsuperscript{K1999/2000E} (17,18), expressed PIKfyve\textsuperscript{ΔCpn} will induce the typical endomembrane vacuolation defects. Oddly however, defective endomembrane morphology was not seen in COS cells due to PIKfyve\textsuperscript{ΔCpn}...
(18). Considering the inability of PIKfyveΔCpn+ to impair endomembrane homeostasis resulted from residual PtdIns(3,5)P2-synthesis, here we characterized the behavior of a PIKfyveK1831E mutant with truncated Cpn+ region (residues 560-1231). Like PIKfyveΔCpn+ (Fig. 3B) the PIKfyveK1831EΔCpn+ mutant did not associate with ArPIKfyve-Sac3 (no shown but quantified in Fig. 3A). Like PIKfyveK1831E, PIKfyveΔCpn+ was devoid of lipid kinase activity (Fig. 4A, Suppl. Fig. 2, quantified in Fig. 3A). Remarkably, however, unlike PIKfyveK1831E that unconditionally triggers aberrant endomembrane defects (18), PIKfyveK1831EΔCpn+ failed to induce endomembrane swelling and vacuolation as apparent from the phase-contrast images of PIKfyveK1831EΔCpn+-expressing cells (Fig. 4B). Defective cell morphology was undetectable even after prolonged PIKfyveK1831EΔCpn+ expression (48 h, not shown), conditions under which PIKfyveK1831E expression further exacerbates the vacuolar defects (18). Likewise, PIKfyveK1831EΔCpn+-dependent endomembrane vacuoles were not seen upon coexpression with Sac3WT and ArPIKfyveWT, alone or in combination (not shown).

To assess whether the inability of PIKfyveK1831EΔCpn+ to imbalance endomembrane homeostatic mechanism and trigger endomembrane vacuoles is related to possible mislocalization of the mutant, we treated the transfected cells with wortmannin. As with PIKfyveK1831E (8), wortmannin rendered the PIKfyveK1831EΔCpn+-vesicular pattern diffuse, consistent with localization of the PIKfyveK1831EΔCpn+ mutant to PtdIns(3)P-enriched endosomes (Fig. 4B). Similarly to PIKfyveK1831E (8), the GFP-PIKfyveK1831EΔCpn+ positive vesicles displayed substantial colocalization (~40%) with the EEA1 endosomal marker (Fig. 4C) and nearly completely overlapped the PIKfyveK1831E-immunofluorescence signals (not shown). Together, these data indicate that if kinase-deficient PIKfyveK1831E is incapacitated for binding ArPIKfyve-Sac3, it does not affect endomembrane homeostasis despite the correct localization and dead kinase activity.

We also examined the lipid kinase activity of other PIKfyve truncated mutants (Suppl. Fig. 2) that displayed intact kinase domain but reduced ArPIKfyve-Sac3 binding and inspected the mutant’s ability to induce vacuolar defects. Quantitative summary of these observations from at least three independent experiments for each construct is presented in Fig. 3A. The comparative analysis allows two important conclusions. First, impeded binding to the ArPIKfyve-Sac3 subcomplex is associated with markedly reduced lipid kinase activity of the mutants (Suppl. Fig. 2 and Fig. 3A). We have to point out, however, that in addition to reduced levels of bound ArPIKfyve-Sac3 subcomplex resulting in kinase inhibition (12,14,21), conformational changes due to the deletions contributing to the drastic loss of the lipid kinase activity could not be ruled out. Second, if the mutants fail to bind ArPIKfyve-Sac3 with efficiency as high as that of PIKfyveWT, they are unable to trigger vacuolar defects despite drastically reduced (>30-fold) lipid kinase activity and an intact localization pattern determined by the intact FYVE domain.

Sac3WT exacerbates the vacuolar defects by PtdIns(3,5)P2-deficient PIKfyve mutants with intact Sac3 binding - The above result that expression of PIKfyveK1831EΔCpn+, in contrast to PIKfyveK1831E, is not dominantly interfering suggests that associated ArPIKfyve-Sac3, and, particularly, the PtdIns(3,5)P2-hydrolyzing activity of Sac3 contribute a great deal to the dominant-negative effect of the PtdIns(3,5)P2-deficient point mutants on the endomembrane morphology. As we elaborated previously, the dominant-negative phenotype of kinase-deficient PIKfyveK1831E is a complex, gradually developing process that affects different endosomal types depending on the duration of expression (8,18). At earlier stages of COS cell transfection (9-15 h), the PIKfyveK1831E-positive vesicles are enlarged, but translucent vacuoles are not visible. The latter emerge 15-24 h post-transfection, first at the perinuclear region and then in the whole cell. The vacuoles, with or without PIKfyveK1831E on the limiting membrane, progressively increase in size and decrease in number as a result of fusion (8,18). Therefore, to assess the role of Sac3 activity in induction and progression of the
PIKfyve<sub>K1831E</sub> dominant-negative effect, we examined the phenotypic changes in COS cells triply transfected with PIKfyve<sub>K1831E</sub>, ArPIKfyve<sub>WT</sub> and Sac3 in either phosphatase-active (Sac3<sup>WT</sup>) or -deficient forms (Sac3<sup>D488A</sup>), both forming PAS complexes with similar efficiencies (Fig. 1A,B). Cells were monitored by immunofluorescence microscopy at two time points: 10-12 h and 22-24 h post-transfection. Intriguingly, the cell phenotype under triple expression of eGFP-PIKfyve<sub>K1831E</sub>, Myc-ArPIKfyve<sub>WT</sub> and HA-Sac3<sub>WT</sub> was markedly different compared to that under expression of PIKfyve<sub>K1831E</sub> alone. Thus, the early phase of endosome vesicle swelling seen under single eGFP-PIKfyve<sub>K1831E</sub> expression was not manifested (Fig. 5A). Rather, the PIKfyve<sub>K1831E</sub>/ArPIKfyve<sub>WT</sub>/Sac3<sub>WT</sub> transfected cells displayed large translucent vacuoles within the whole cell as early as 10-12 h post-transfection, which persisted 24 h post-transfection (Fig. 5A,B). By contrast, cells expressing eGFP-PIKfyve<sub>K1831E</sub>, Myc-ArPIKfyve<sub>WT</sub> and HA-Sac3<sup>D488A</sup> displayed neither dilated endosomes at the early phase nor translucent vacuoles in the later phase to a substantial degree (Fig. 5A,B). Noteworthy, no significant Sac3-related phenotypic changes were seen if eGFP-PIKfyve<sub>K1831E</sub> was coexpressed with Sac3<sub>WT</sub> or Sac3<sup>D488A</sup> in the absence of ArPIKfyve<sub>WT</sub> (Fig. 5B).

As a further test of our hypothesis for Sac3 functioning as an active phosphatase in the PAS complex, we examined the effect of Sac3<sup>WT</sup> vs. Sac3<sup>D488A</sup> on cell morphology under expression of the PIKfyve<sub>K2000E</sub> point mutant. As opposed to Lys-1831 or Lys-1999, Lys in position 2000 was previously characterized to be partially engaged in PtdIns(3,5)P<sub>2</sub> coordination, resulting in only ~40% decreased in vitro PtdIns(3,5)P<sub>2</sub> synthesis by PIKfyve<sub>K2000E</sub> (17). At this level of activity, PIKfyve<sub>K2000E</sub>, unlike PIKfyve<sub>K1831E</sub> or PIKfyve<sub>K1999E</sub>, does not induce vacuolar defects in transfected COS cells (17). Remarkably, coexpression of Sac3<sub>WT</sub> and ArPIKfyve with PIKfyve<sub>K2000E</sub> triggered profound cell vacuolation, whereas coexpression of Sac3<sup>D488A</sup>-ArPIKfyve did not substantially affect the PIKfyve<sub>K2000E</sub>-dependent cell morphology. Quantitation of the PIKfyve<sub>K2000E</sub>-induced vacuolar phenotype due to Sac3<sub>WT</sub> vs. Sac3<sup>D488A</sup> from 2 independent transfection experiments is presented in Fig. 5C. Taken together, the results illustrated in Figs. 4 and 5 are consistent with the conclusion that Sac3 functions as a PtdIns(3,5)P<sub>2</sub> phosphatase in the context of the PAS complex.

**ArPIKfyve<sup>Ct</sup> that disassembles the PAS complex alleviates the phenotypic defects by PIKfyve<sub>K1831E</sub>** – As revealed recently, ArPIKfyve homomerization mediated by the ArPIKfyve C-terminal domain scaffolds the PAS complex (14). Therefore, as a final verification of our hypothesis that Sac3 functions as a phosphatase when associated with PIKfyve, we examined if disassembly of the PAS complex by ArPIKfyve<sup>Ct</sup> (14) alleviates the phenotypic defects induced by PIKfyve<sub>K1831E</sub> in transfected COS cells. As illustrated in Fig. 5D, expressed Myc-PIKfyve<sub>K1831E</sub> was significantly less potent in triggering formation of aberrant vacuoles if cells coexpressed ArPIKfyve<sup>Ct</sup>. Quantitation of 2 separate experiments counting ~250 transfected cells revealed that coexpressed ArPIKfyve<sup>Ct</sup> diminished the number of vacuolated cells due to PIKfyve<sub>K1831E</sub> by 42±4%. These data further indicate that the dominant phenotype of aberrant swollen vacuoles is due to two superimposed events: first, disrupted PIKfyve-catalyzed PtdIns(3,5)P<sub>2</sub> synthesis at endosomal microdomains determined by the PIKfyve FYVE finger and, second, Sac3-dependent hydrolysis localized at the PtdIns(3,5)P<sub>2</sub> synthetic sites through association of the ArPIKfyve-Sac3 subcomplex with PIKfyve.

**DISCUSSION**

We have recently reported that in native mammalian cells, the PtdIns(3,5)P<sub>2</sub>-metabolizing enzymes PIKfyve kinase and Sac3 phosphatase are organized in a common complex, the PAS core, that includes also the PIKfyve regulator ArPIKfyve (9,14). Heterologous expression in mammalian cell systems had further revealed that the three proteins are both necessary and sufficient to form and maintain a stable PAS complex, and that ArPIKfyve and Sac3 are mutually dependent for an efficient association with PIKfyve (14). The integrity of the PAS core...
complex has been found critical for PIKfyve activation, indicating an unusual requirement for the Sac3 phosphatase in PtdIns(3,5)P₂ synthesis (14). Thus, given Sac3’s role in PtdIns(3,5)P₂ biosynthesis and the presence of a PIKfyve-independent ArPIKfyve-Sac3 subcomplex, an outstanding question awaiting clarification is whether the formation of the PAS complex is permissible with Sac3 functioning as a phosphatase or whether its hydrolyzing activity is inhibited upon association. The answer to this question becomes even more prominent in light of the documented ~3.5-fold decrease of cellular PtdIns(3,5)P₂ in a Sac3 knockout mouse model (28), thus raising doubts whether intracellular Sac3 functions as a PtdIns(3,5)P₂-phosphatase or whether it just facilitates the PtdIns(3,5)P₂ synthetic arm. To this end, in this study we initiated a detailed biochemical characterization of Sac3’s mode of interacting with ArPIKfyve and PIKfyve, and used the well-established phenomenon of an aberrant vacuolar phenotype as a functional readout for reduced PtdIns(3,5)P₂ localized levels (13,17,19) and, hence, Sac3 activity. Our observation that elimination of the Cpn60_TCP1 domain in PIKfyve resulted in nearly total loss of bound ArPIKfyve and Sac3 is consistent with the notion that this region plays a major role in associating the ArPIKfyve-Sac3 subcomplex (Fig. 3A-C). In contrast, neither the PIKfyve kinase activity nor the Sac3 phosphatase activity played any role in the PAS core formation or stability, as evidenced by the wild-type associations of the activity-deficient point mutants PIKfyveK1831E and Sac3D488A (Figs. 1 and 3A,F). Intriguingly, deletion of the ArPIKfyve-Sac3 binding region mitigated the potency of the kinase-dead PIKfyveK1831E-truncated mutant to dominantly interfere with the endomembrane homeostasis and trigger vacuolar formation even when coexpressed with Sac3WT and/or ArPIKfyveWT, consistent with reduced Sac3 access to, and turnover of PtdIns(3,5)P₂ (Fig. 4). Concordantly, PIKfyveK1831E with an intact Cpn60_TCP1 domain and wild-type binding to ArPIKfyve-Sac3 (Fig. 3A) induced drastically exacerbated vacuolar defects when coexpressed with ArPIKfyveWT-Sac3WT but a less severe phenotype if coexpressed with ArPIKfyveWT-Sac3D488A (Fig. 5A,B). Finally, the PIKfyveK1831E-dependent vacuolar defects were alleviated by disassembly of the ternary complex with ArPIKfyveWT (Fig. 5D). These data are consistent with the conclusion that endogenous Sac3, assembled in the PAS core, is an active PtdIns(3,5)P₂ phosphatase. Thus, our results provide the first experimental evidence that the intracellular PAS core complex relays two opposing enzymatic activities: PtdIns(3,5)P₂ synthesis (14) and PtdIns(3,5)P₂ turnover (this study).

Our data also enlightened the paradoxes associated with the aberrant endomembrane vacuolar phenotype, revealing that defective PtdIns(3,5)P₂ synthesis by PIKfyve at proper PtdIns(3)P-enriched endosomes (15,18) only partially disrupts the endomembrane homeostatic mechanism. We demonstrate here that the disequilibrium between PIKfyve-catalyzed PtdIns(3,5)P₂ synthesis and Sac3-dependent PtdIns(3,5)P₂ hydrolysis at endosomal locales determined by the PIKfyve FYVE finger, triggers full expression of aberrant gross vacuoles. Therefore, the PtdIns(3,5)P₂-deficient PIKfyve mutants, including K1831EΔCpn+, ΔCpn+, ΔN-Cpn, ΔC-Cpn, ΔKin or others, which do not bind Sac3 at the wild-type efficiency (Fig. 3A) yet localize properly (Fig. 4B,C), fail to induce endomembrane vacuoles. In contrast, the PtdIns(3,5)P₂-deficient mutants K1831E, K1999E and K1999/2000E with preserved binding to Sac3 (and to ArPIKfyve for that matter) readily do that in a manner reversible by PIKfyveWT expression (11,17,18). Concordantly, a PIKfyve point mutant, K2000E, partially deficient in PtdIns(3,5)P₂ synthesis but with intact ArPIKfyve-Sac3 binding triggers vacuolar phenotype only if coexpressed with Sac3WT (Fig. 5C). This conclusion is also supported by data with PIKfyve pharmacological inhibition, where aberrant vacuoles are seen in all cells shortly after treatment (19), consistent with active Sac3 present in the endosome-localized PAS core. In contrast, siRNA-mediated PIKfyve silencing that concurrently eliminates active Sac3 from PIKfyve-specified locales, is a less potent maneuver in triggering endosome defects even if PIKfyve is efficiently knocked down (13,19).

Of particular importance in this study is the characterization and quantitative evaluation
of the triple associations with a battery of deletion and point mutants (Figs. 1-3). These data lend new mechanistic insight allowing us to propose an in-depth model of the PAS complex interacting domains and their significance for the enzymatic activities (Fig. 6). In addition to the major role of Cpn60_TCP1/CH in the association with the ArPIKfyve-Sac3 complex discussed above, this model is based on several other observations and conclusions: (i). Sac3 and ArPIKfyve associate with each other through their C-termini (Fig. 1A-D); (ii) Sac3 C-terminus associates with at least two ArPIKfyve copies (hence, ArPIKfyve$_n$-Sac3, where $n \geq 2$), all of them interacting through their C-termini (Fig. 1E); (iii) a stable subcomplex made of ArPIKfyve$^\text{WT}$ and Sac3 C-terminus, or vice versa, does not efficiently bind PIKfyve, indicating both ArPIKfyve- and Sac3-N-termini stabilize the PAS core complex (Fig. 2); (iv) the PIKfyve FYVE finger is irrelevant in the PAS complex formation (Fig. 3A,B); (v) a PIKfyve fragment spanning residues 1-1260 that includes the entire Cpn60_TCP1 and CH homology domains has only 25-30% of the wild-type binding to ArPIKfyve-Sac3. In fact, neither of the two overlapping PIKfyve halves reaches half of the wild-type interaction with the ArPIKfyve-Sac3$^\text{WT}$ subcomplex, conceivable with conformational changes to expose other PIKfyve interacting domains (Fig. 3A,D,G); (vi) a PIKfyve fragment downstream of Cpn60_TCP1, including the kinase domain, interacts with the ArPIKfyve-Sac3 subcomplex, whereas a fragment with a deleted kinase domain, ΔKin, exhibits reduced binding to ArPIKfyve-Sac3, consistent with additional contact sites for ArPIKfyve$_n$-Sac3 binding within the kinase domain (Fig. 3); (vii) interaction sites between the Sac3 N-terminus and ArPIKfyve are likely to be exposed after the ArPIKfyve$_n$-Sac3 docking to PIKfyve because Sac3$^\text{WT}$ and PIKfyve$^\text{WT}$ do not interact under pair-wise analyses (14). Thus, taking into consideration this experimental evidence, we propose a model whereby an ArPIKfyve$_n$-Sac3 subcomplex, formed by the C-termini of each subunit first docks at the Cpn60_TCP1/CH homology region of PIKfyve that is localized on endosomal PtdIns(3)P via its FYVE finger (Fig. 6). We suggest that this induces a conformational change in PIKfyve to allow additional PAS-stabilizing interactions that engage a significant portion of the PIKfyve C-terminal region, including the Spectrin repeats and kinase domain, on one hand, and the N-termini of ArPIKfyve$_n$-Sac3, on the other. Because the triple association activates PIKfyve (14), recruited Sac3 in the PAS complex ensures the PtdIns(3,5)P$_2$ homeostatic control by rapid turnover counterbalancing locally elevated PtdIns(3,5)P$_2$.

Two recent studies have now confirmed the triple assembly of mammalian PIKfyve, ArPIKfyve and Sac3 with the respective yeast orthologs Fab1, Vac14 and Fig4 (26,30). Despite some discrepancies between the two reports, these new observations are of considerable significance as they establish evolutionary conservation of the PAS ternary assembly, as we previously predicted (7). Thus, based on biochemical data for the triple associations in yeast (26,30) and those reported by us in mammalian cells (9,14) or presented herein, it could be concluded that the organization of mammalian and yeast PAS complexes share many similar characteristics. These include: mutual dependence of ArPIKfyve/Vac14 and Sac3/Fig4 for a productive association with PIKfyve/Fab1; no direct interaction between PIKfyve/Fab1 and Sac3/Fig4; requirement for the PIKfyve/Fab1 chaperonin-like domain in the ArPIKfyve/Vac14 - Sac3/Fig4 interaction (Fig. 3); ArPIKfyve/Vac14 homodimerization; independence of the ternary assembly from the enzymatic activities of either the kinase or the phosphatase (Figs. 1 and 3); impeded PIKfyve/Fab1 activity if Cpn60_TCP1 domain is mutated (Fig 4); membrane localization of the ternary complex via the PIKfyve/Fab1 FYVE finger domain. However, certain differences are also evident, the most apparent one being the affinity of the interactions. Although affinity constants are yet to be determined, the triple complex in mammalian cell systems might be of higher affinity. This conclusion is corroborated by the biochemical detection of the PAS complex with the mammalian endogenous proteins under stringent conditions of RIPA-buffer detergents (9), whereas that with the yeast native proteins remains to be seen. Even under genetic manipulations, the biochemical demonstration of the yeast triple complex...
requires as much as 13 tandem epitope-tag copies on Fab1 and mild conditions of a single detergent at a low concentration (26). Therefore, perhaps the identification of the yeast triple assembly was initially unsuccessful (31-33) and lagged behind that in mammalian cells (9,21).

The demonstration that the PAS core relays not only PIKfyve-catalyzed synthesis but also Sac3-catalyzed breakdown of PtdIns(3,5)P2 is indicative of a pivotal need for a tight control of the PtdIns(3,5)P2 homeostatic mechanism and strict coordination of the antagonistic enzymatic activities. Concordantly, in multicellular organisms such as Drosophila melanogaster or Caenorhabditis elegans, PtdIns(3,5)P2 is essential for life as evidenced by the embryonic lethality of the PIKfyve-null mutants, in parallel with undetectable PtdIns(3,5)P2 levels (29,34). A still unresolved question is how important PtdIns(3,5)P2 is for the life of mammals, given PIKfyve-null mice are still unavailable. Transgenic models of Sac3 and ArPIKfyve are reported, but they do not provide a coherent relationship between the reduced PtdIns(3,5)P2 and lethality. Thus, ArPIKfyve knockout mice displaying 50% of the normal PtdIns(3,5)P2 intracellular levels die 1-2 days after birth, whereas the Sac3 mouse model lives to the age of ~6 weeks with only ~25% of normal PtdIns(3,5)P2 (28,35). As ArPIKfyve and Sac3 are expected to affect the same subcellular PtdIns(3,5)P2 pool produced by PIKfyve, this discrepancy is currently unclear. Additional functional inputs by ArPIKfyve (36) could not be excluded.

In conclusion, the data herein provide new mechanistic and functional insight about the domain organization of the PAS complex and its consequence to Sac3 function as a phosphatase. Perturbation of Sac3 phosphatase activity favors PtdIns(3,5)P2 synthesis over turnover, an observation that might be of therapeutic interest for processes that depend on elevated PtdIns(3,5)P2 (4,12,37). An important challenge for future studies is the nature of the extracellular stimuli and molecular mechanisms that coordinate the kinase and phosphatase activities within the same complex to assure proper performance of endosomal operations.

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FOOTNOTES

The abbreviations used are: PtdIns, phosphatidylinositol; PI, phosphoinositide; PIKfyve, Phosphoinositide Kinase for position five containing a five finger domain; ArPIKfyve, Associated regulator of PIKfyve; Sac3, Sac domain-containing phosphatase 3; GFP, green fluorescence protein; eGFP, enhanced green fluorescence protein; HA, hemagglutinin; GST, glutathione S-transferase; GSH, glutathione; NTA, nitrilo-triacetic acid; TLC, thin layer chromatography; PAS, PIKfyve-ArPIKfyve-Sac3.

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LEGENDS TO FIGURES

FIGURE 1. ArPIKfyve and Sac3 associate with each other through their C-termini. (A) Sac3 deletion and point mutants presented schematically relative to the Sac domain structure, predicted by Swiss-Prot and Smart databases (E-value cutoff=1). Shown is a quantitative summary of their interaction with ArPIKfyve WT based on coimmunoprecipitation in COS7 cells, exemplified in B. (B) Cells cotransfected with cDNAs of Myc-ArPIKfyve WT and either eGFP-Sac3 WT, eGFP-Sac3(1-315), eGFP-Sac3(1-574), eGFP-Sac3(388-907), eGFP-Sac3(478-907), eGFP-Sac3(610-907) or eGFP-Sac3(D488A) were analyzed by immunoprecipitation (IP) with anti-Myc or anti-GFP antibodies as indicated. (C) ArPIKfyve deletion mutants presented schematically relative to the ArPIKfyve domain architecture predicted by Pfam databases (E-value cutoff =1) and COILS. Shown is quantitation of their interaction with Sac3 WT without or with differently tagged ArPIKfyve WT, and measured by coIP as shown in D and E. (D) Cells cotransfected with cDNAs of Myc-Sac3 WT plus either eGFP-HA-ArPIKfyve WT, eGFP-HA-ArPIKfyve WT or eGFP-ArPIKfyve WT were analyzed by IP with anti-Myc antibody. (E) Cells doubly transfected with cDNAs of Myc-ArPIKfyve WT plus eGFP-HA-ArPIKfyve WT (a control for ArPIKfyve homomerization) or triply transfected with cDNAs of Myc-ArPIKfyve WT, Myc-Sac3 WT and either eGFP-HA-ArPIKfyve WT or eGFP-HA-ArPIKfyve WT, were analyzed by IP with anti-HA antibodies. Shown are chemiluminescence detections of a representative experiment for each of B, D and E panels out of three to six independent determinations with SEM <10% of the mean value, quantitatively scored in A and C. Mutants scored as “-“ displayed <10% of the wild-type association.

FIGURE 2. ArPIKfyve and Sac3 N-terminal regions interact with PIKfyve. (A) In vitro associations of purified recombinant GST-PIKfyve, His6-ArPIKfyve and Sac3. GST-PIKfyve or GST control purified from infected Sf9 cells and immobilized on GSH-agarose beads was incubated with bacterially produced His6-ArPIKfyve or His6-GDI2 (as a control) both purified on Ni-NTA agarose (left panels) or with His6-ArPIKfyve/Sac3 subcomplex, purified on Ni-NTA agarose from infected Sf21 cells (right panels) as indicated. Blots, cut in the middle, were probed with anti-PIKfyve (upper halves) or anti-ArPIKfyve and anti-Sac3 antibodies (lower halves) with a stripping step between the latter two. Shown are representative blots, illustrating that efficient associations occur only with the three purified recombinant proteins. *, depicts incompletely stripped His6-ArPIKfyve. (B) COS7 cells were triply transfected with cDNAs encoding Myc-ArPIKfyve WT, HA-PIKfyve WT and either eGFP-Sac3 WT, eGFP-Sac3(388-907) or eGFP-Sac3(610-907). Fresh RIPA + lysates were subjected to IP with anti-Myc antibody and after washes in the same buffer, IPs were analyzed by SDS-PAGE and immunoblotting with anti-GFP, anti-Myc and anti-HA antibodies, with a stripping step in between. Shown are chemiluminescence detections of a representative transfection experiment out of three independent determinations with SEM <10% of the mean value, quantified in B. (C) A schematic diagram of the PIKfyve domain structure, predicted by Pfam (E-value cutoff=1) or BLAST databases, and a quantitative summary of the PIKfyve interaction with the complexes of ArPIKfyve WT-Sac3 WT, ArPIKfyve WT-Sac3(610-907), ArPIKfyve WT-Sac3(388-907), ArPIKfyve(523-782)-Sac3 WT and ArPIKfyve(298-782)-Sac3 WT.

FIGURE 3. PIKfyve regions associating with the ArPIKfyve WT-Sac3 complex. (A) Presented are the PIKfyve truncated mutants and a quantitative summary of their interactions with ArPIKfyve WT-Sac3 WT.
subcomplex measured by coimmunoprecipitation in COS7 cells (shown in B-G), the in vitro lipid kinase activity, determined herein (see Fig. 4A and Suppl. Fig. 2) or in indicated References and their ability to induce vacuolar defects, determined herein (see Fig. 4B and data not shown) or in indicated References. CHK homology (residues 1155-1461) harbors conserved C and H, within its N-terminal half (residues 1155-1327), and conserved K, within its C-terminal half (residues 1295-1461) that is homologous to Spectrin repeats. (B-G) COS7 cells were triply transfected with Myc-Sac3 WT, Myc-ArPIKfyve WT and the following PIKfyve constructs: HA-PIKfyve WT, HA-PIKfyve ΔCpn+. eGFP-HA-PIKfyve ΔCpn+ or eGFP-HA-PIKfyve ΔN-Cpn (C); HA-PIKfyve WT or HA-PIKfyve ΔSpec+ or eGFP-HA-PIKfyve ΔSpec+ (D); eGFP-HA-PIKfyve WT or eGFP-HA-PIKfyve ΔFAkin+ (E); HA-PIKfyve WT, HA-PIKfyve K1831E or HA-PIKfyve ΔKin (F); eGFP-HA-PIKfyve WT or eGFP-HA-PIKfyve ΔPDΔCpn (G). Anti-HA IPs from fresh RIPA + lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA, anti-Myc and anti-GFP antibodies, with a stripping step in between. Shown are chemiluminescence detections from representative transfection experiments for each of the panels out of three to nine independent determination quantified in A (mean±SEM). Levels of the CoIP proteins were quantified relative to their total expression amounts (seen in inputs) and then normalized for the immunoprecipitated levels of the indicated PIKfyve construct relative to PIKfyve WT, which was scored as 100%. A strong triple interaction was observed with PIKfyve WT, PIKfyve K1831E and PIKfyve ΔFYVE. The association is independent of whether PIKfyve carries GFP-HA- or HA-tags, but note the different electrophoretic mobility due to the different tag size.

FIGURE 4. PIKfyve K1831E without the ArPIKfyve-Sac3-binding region is not dominantly interfering. (A) Fresh RIPA + lysates, derived from COS cells singly transfected with cDNAs of HA-PIKfyve WT, HA-PIKfyve ΔCpn+, HA-PIKfyve K1831E or with the empty vector underwent IP (in duplicate) with anti-HA antibodies. Washed IPs were subjected to assays for lipid kinase activity or autophosphorylation. Shown are autoradiograms of a plate with TLC-separated radiolabeled lipids and of the autokinase reaction resolved by SDS-PAGE and transferred onto a membrane, subsequently immunoblotted (IB) with anti-HA antibodies. Shown is a representative experiment out of five to eight with similar results. The lipid kinase activity is quantified by radioactive counting of the scraped silica and is shown in Fig. 3A (B) COS7 cells were transfected with cDNAs of eGFP-HA-PIKfyve K1831E or eGFP-HA-PIKfyve ΔCpn+. Twenty hours post-transfection cells were treated with or without wortmannin (100 nM/20 min/37°C, conditions that in COS cells do not induce wortmannin-dependent aberrant morphology; Ref. (18) and then fixed in 4% formaldehyde. Fluorescence and phase-contrast images of transfected cells were captured by a Nikon Eclipse TE200 microscope (Apo 60×/1.40 Ph3DM oil objective) and processed as detailed in Experimental Procedures. Shown are: typical images of the defective vacuolar phenotype induced by PIKfyve K1831E (panels a and b); lack of vacuoles by HA-PIKfyve K1831EΔCpn+ seen in 94±5% (mean±SEM) of transfected cells (panels c and d); wortmannin-sensitivity of the HA-PIKfyve K1831EΔCpn+ punctate staining observed in 88±7% (mean±SEM) of treated transfected cells (panels e and f). (C) Cells were transfected with eGFP-HA-PIKfyve K1831EΔCpn+ cDNA and 24 h post-transfection processed for immunofluorescence microscopy (Olympus 1X81) with anti-EEA1. Confocal images (panels a and b) were processed by deconvolution analysis. The merge (panel c) of the two images indicates ~40% colocalization of GFP-HA-PIKfyve K1831EΔCpn+ with EEA1 as reported with PIKfyve K1831E (8). Bar, 10 μm

FIGURE 5. ArPIKfyve WT-Sac3 WT exacerbates, whereas ArPIKfyve WT-Sac3 D488A prevents the endomembrane defects by Sac3-binding PIKfyve mutants defective in PtdIns(3,5)P 2 synthesis. (A) COS7 cells were triply cotransfected with cDNAs of eGFP-HA-PIKfyve K1831E, Myc-ArPIKfyve WT, and either HA-Sac3 WT or HA-Sac3 D488A, the phosphatase-deficient point mutant. Twenty-four hours post-transfection cells were fixed in 4% formaldehyde and stained consecutively for Sac3 (anti-Sac3 IgG) followed by Alexa350 anti-rabbit secondary antibody and ArPIKfyve (anti-Myc monoclonal antibody followed by Alexa568 anti-mouse secondary antibody). Fluorescence and phase-contrast images were
captured by a Nikon Eclipse TE200 microscope (Apo 60x/1.40 Ph3DM oil objective) using 3 standard filter sets – green for eGFP, red for Alexa568, and blue for Alexa350. Note the presence of multiple vacuoles (panels a-d) under coexpression of enzymatically active Sac3\textsuperscript{WT} and their absence under coexpression of inactive Sac3\textsuperscript{D488A} (panels e-h), quantified in B. Bar, 10 μm. (B) Quantification of the aberrant vacuolar phenotype in COS7 cells expressing eGFP-HA-PIKfyve\textsuperscript{K1831E} alone or in double and triple combinations with the indicated constructs. Cells showing clear translucent cytoplasmic vacuoles 12- and 24-h post-transfection were scored as positive. Data collected from examining >100 transfected cells per combination in 3 separate transfection experiments are presented as a percentage of the total number of inspected cells (mean±SEM; *, \( p<0.01 \) or more vs. eGFP-PIKfyve\textsuperscript{K1831E}). In all double combinations with eGFP-HA-PIKfyve\textsuperscript{K1831E} the aberrant vacuolar phenotype was seen to the same extent as in cells expressing eGFP-HA-PIKfyve\textsuperscript{K1831E} alone. (C) Quantitation of the vacuolar phenotype in COS7 cells expressing pEGFP-HA-PIKfyve\textsuperscript{K2000E} alone or in a triple combination with ArPIKfyve\textsuperscript{WT}-Sac3\textsuperscript{WT} or ArPIKfyve\textsuperscript{WT}-Sac3\textsuperscript{D488A} scored by the presence of translucent cytoplasmic vacuoles 24-h post-transfection. Data are collected from examining at least 100 transfected cells per combination and are presented as a percentage of the total number of transfected cells (mean±SEM). Note that eGFP-HA-PIKfyve\textsuperscript{K2000E}, partially deficient in PtdIns(3,5)\( P_2 \) synthesis was incapable of inducing vacuoles when expressed alone or with Sac3\textsuperscript{D488A}-ArPIKfyve\textsuperscript{WT} but readily triggered vacuolar defects when coexpressed with Sac3\textsuperscript{WT}-ArPIKfyve\textsuperscript{WT}. (D) ArPIKfyve\textsuperscript{Ct} alleviates the phenotypic defects by PIKfyve\textsuperscript{K1831E}. COS7 cells were transfected with cDNAs of Myc-PIKfyve\textsuperscript{K1831E} and 3 h later, with eGFP-ArPIKfyve\textsuperscript{Ct}. Twenty hours post-transfection cells were fixed and stained with anti-Myc monoclonal antibody and Alexa568 anti-mouse secondary antibody. Fluorescence (panels a and b) and phase-contrast images (panel c) were captured by a Nikon Eclipse TE200 microscope (Apo 60x/1.40 Ph3DM oil objective). Coexpressed ArPIKfyve\textsuperscript{Ct} diminished the number of PIKfyve\textsuperscript{K1831E}-vacuolated cells by 42±4%; \( p<0.001 \) (based on observing 250 doubly transfected cells in 2 separate experiments with consecutive transfections). Bar, 10 μm.

FIGURE 6. Model for the interacting domains of the three proteins and regulated enzymatic activities in the PAS complex. PIKfyve associates with PtdIns(3)\( P_2 \)-enriched endosome membranes via its FYVE finger, which is independent of the ArPIKfyve-Sac3 subcomplex. Without bound ArPIKfyve-Sac3, PIKfyve relays submaximal activity due to kinase-unfavorable conformation. The ArPIKfyve\textsubscript{Ct}-Sac3 complex, formed through an association of the C-termini of an ArPIKfyve dimer (or higher-order homooligomer; hence ArPIKfyve\textsubscript{n} where \( n \geq 2 \)) and the C-terminus of a Sac3 monomer, docks to the PIKfyve Cpn60_TCP1 and the CH homology domains. This induces conformational changes in PIKfyve to uncover binding sites at the catalytic domain, which associate with the N-termini of ArPIKfyve\textsubscript{n} and Sac3. These interactions stabilize a productive PAS core and allow PIKfyve to acquire “activated” conformation. This transiently increases local synthesis of PtdIns(3,5)\( P_2 \) that serve as recognition sites for PtdIns(3,5)\( P_2 \) effectors. The local increase in PtdIns(3,5)\( P_2 \) is counterbalanced by Sac3, which when incorporated in the PAS core retains its activity for PtdIns(3,5)\( P_2 \) hydrolysis. Neither Sac3 nor ArPIKfyve are dispensable for a productive PAS core complex in the absence of the either of the two N-termini even though a stable subcomplex via the two C-termini is formed.
Fig. 1

A

PIKfyve binding region

ArPIKfyve binding region

ASSOCIATION WITH ArPIKfyve^{WT}

Sac1 homology 92-430
Sac phosphatase 154-547
Low complexity 742-771

WT

- +++++

- +

++++

++++

+++ D488A ++++
|          | Input | IP anti-Myc |          | Input | IP anti-Myc |
|----------|-------|-------------|----------|-------|-------------|
| Myc-ArPLKf3yve<sup>WT</sup> | +     | +           | +        | +     | +           |
| GFP-Sac<sup>3</sup>W<sup>T</sup> | +     | -           | +        | -     | -           |
| GFP-Sac<sup>3</sup>1-574<sup>WT</sup> | -     | +           | -        | +     | -           |
| GFP-Sac<sup>3</sup>1-315<sup>WT</sup> | -     | +           | -        | +     | -           |

|          | Input | IP anti-Myc |          | Input | IP anti-Myc |
|----------|-------|-------------|----------|-------|-------------|
| Myc-ArPLKf3yve<sup>WT</sup> | +     | +           | +        | +     | +           |
| GFP-Sac<sup>3</sup>W<sup>T</sup> | +     | -           | +        | -     | -           |
| GFP-Sac<sup>3</sup>388-907<sup>WT</sup> | -     | -           | -        | +     | -           |
| GFP-Sac<sup>3</sup>610-907<sup>WT</sup> | -     | +           | -        | -     | +           |

|          | Input | IP anti-Myc |          | IP anti-GFP |
|----------|-------|-------------|----------|-------------|
| Myc-ArPLKf3yve<sup>WT</sup> | +     | +           | +        | +           |
| GFP-Sac<sup>3</sup>D488A<sup>WT</sup> | +     | -           | -        | +           |
| GFP-Sac<sup>3</sup>W<sup>T</sup> | -     | -           | -        | +           |
| GFP-Sac<sup>3</sup>W<sup>T</sup>Sac<sup>3</sup>D488A | -     | -           | -        | +           |
### Fig. 1

#### C

- **PIKfyve binding region**
- **Sac3 binding region**
- **ArPIKfyve binding region**

| ASSOCIATION WITH: | Sac3<sup>WT</sup> | ArPIKfyve<sup>WT</sup>-Sac3<sup>WT</sup> |
|-------------------|------------------|-----------------------------------|
| WT                | ++++             | ++++                              |
| Nt                | -                | -                                 |
| Cl                | ++++             | Not tested                        |

- **H** Heat repeats (6-41; 89-125; 130-152; 171-207; 212-248; 438-474)
- **DUF** Domains with unknown function
- **▲** Coiled-coils (6-48; 523-546; 592-612; 648-674; 748-765)

#### D

|          | Input | IP anti-Myc |
|----------|-------|-------------|
| Myc-Sac3<sup>WT</sup> | +     | + + +       |
| GFP-ArPIKfyve<sup>WT</sup> | +     | + - -       |
| GFP-ArPIKfyve<sup>Nt</sup> | -     | + - -       |
| GFP-ArPIKfyve<sup>Ct</sup> | -     | - - +       |
| GFP-ArPIKfyve<sup>WT</sup> |        |             |
| GFP-ArPIKfyve<sup>Nt</sup> |        |             |
| GFP-ArPIKfyve<sup>Ct</sup> |        |             |
| IgG      |        |             |
| Myc-Sac3 |        |             |
### Fig. 1

|                | Input | IP anti-HA |
|----------------|-------|------------|
| Myc-Sac3<sup>WT</sup> | −     | +          |
| Myc-ArPIKfyve<sup>WT</sup> | +     | +          |
| GFP-HA-ArPIKfyve<sup>WT</sup> | +     | −          |
| GFP-HA-ArPIKfyve<sup>Nt</sup> | −     | +          |

**Myc-Sac3**

**Myc-ArPIKfyve**

**GFP-HA-ArPIKfyve**

**GFP-HA-ArPIKfyve**

**Myc-Sac3**

**Myc-ArPIKfyve**

**GFP-HA-ArPIKfyve**

**GFP-HA-ArPIKfyve**
Fig. 2

| Beads:                      | Pull-down |
|-----------------------------|-----------|
| GST-PIKfyve                 | + + +     |
| GST                          | - - -     |
| GST-PIKfyve                  |           |
| His-ArPIKfyve               |           |
| His-GDI-2                   | - + -     |
| His-ArPIKfyve               | + - +     |
| GST-PIKfyve                 |           |
| His-ArPIKfyve               |           |
| Sac3                        |           |
| His-ArPIKfyve/Sac3          | + - +     |
|          | Input | IP anti-Myc |
|----------|-------|-------------|
| Myc-ArPIKfyve<sup>WT</sup> | +     | +           |
| HA-PIKfyve<sup>WT</sup>      | +     | +           |
| GFP-Sac3<sup>WT</sup>         | −     | +           |
| GFP-Sac3<sup>388-907</sup>    | −     | −           |
| GFP-Sac3<sup>610-907</sup>    | −     | −           |

**Fig. 2**

![Western blot images](http://www.jbc.org/)

**HA-PIKfyve**

**GFP-Sac3<sup>WT</sup>**

**GFP-Sac3<sup>388-907</sup>**

**GFP-Sac3<sup>610-907</sup>**

**Myc-ArPIKfyve**
| B   | Input | IP anti-HA |
|-----|-------|------------|
| Myc-Sac3<sup>3T</sup> | +     | +          |
| Myc-ArPIKfyve<sup>WT</sup> | +     | +          |
| HA-PIKfyve<sup>WT</sup> | +     | +          |
| GFP-HA-PIKfyve<sup>WT</sup> | +     | +          |
| Myc-Sac3 | +     | +          |
| Myc-ArPIKfyve | +     | +          |
| HA-PIKfyve<sup>WT</sup> | +     | +          |
| ΔCpn+       | +     | +          |

| C   | Input | IP anti-HA |
|-----|-------|------------|
| Myc-Sac3<sup>3T</sup> | +     | +          |
| Myc-ArPIKfyve<sup>WT</sup> | +     | +          |
| HA-PIKfyve<sup>WT</sup> | +     | +          |
| HA-PIKfyve<sup>WT</sup>ΔCpn | +     | +          |
| GFP-HA-PIKfyve<sup>WT</sup>ΔCpn | +     | +          |
| Myc-Sac3 | +     | +          |
| Myc-ArPIKfyve | +     | +          |
| HA-PIKfyve<sup>WT</sup>ΔCpn | +     | +          |
| ΔCpn+       | +     | +          |

| D   | Input | IP anti-HA |
|-----|-------|------------|
| Myc-Sac3<sup>3T</sup> | +     | +          |
| Myc-ArPIKfyve<sup>WT</sup> | +     | +          |
| HA-PIKfyve<sup>WT</sup> | +     | +          |
| HA-PIKfyve<sup>WT</sup>ΔSpec<sup>Δ</sup> | +     | +          |
| Myc-Sac3 | +     | +          |
| Myc-ArPIKfyve | +     | +          |
| HA-PIKfyve<sup>WT</sup>ΔSpec<sup>Δ</sup> | +     | +          |
| ΔSpecΔKin++ | +     | +          |

| E   | Input | IP anti-HA |
|-----|-------|------------|
| Myc-Sac3<sup>3T</sup> | +     | +          |
| Myc-ArPIKfyve<sup>WT</sup> | +     | +          |
| HA-PIKfyve<sup>WT</sup> | +     | +          |
| GFP-HA-PIKfyve<sup>WT</sup> | +     | +          |
| GFP-HA-PIKfyve<sup>WT</sup>ΔDelta<sup>Δ</sup>Cpn | +     | +          |
| Myc-Sac3 | +     | +          |
| Myc-ArPIKfyve | +     | +          |
| GFP-HA-PIKfyve<sup>WT</sup>ΔDelta<sup>Δ</sup>Cpn | +     | +          |
| ΔDeltaΔKin++ | +     | +          |

| F   | Input | IP anti-HA |
|-----|-------|------------|
| Myc-Sac3<sup>3T</sup> | +     | +          |
| Myc-ArPIKfyve<sup>WT</sup> | +     | +          |
| HA-PIKfyve<sup>WT</sup> | +     | +          |
| HA-PIKfyve<sup>WT</sup>ΔDelta<sup>Δ</sup>Cpn | +     | +          |
| Myc-Sac3 | +     | +          |
| Myc-ArPIKfyve | +     | +          |
| HA-PIKfyve<sup>WT</sup>ΔDelta<sup>Δ</sup>Cpn | +     | +          |
| ΔDeltaΔKin++ | +     | +          |

| G   | Input | IP anti-HA |
|-----|-------|------------|
| Myc-Sac3<sup>WT</sup> | +     | +          |
| Myc-ArPIKfyve<sup>WT</sup> | +     | +          |
| GFP-HA-PIKfyve<sup>WT</sup> | +     | +          |
| GFP-HA-PIKfyve<sup>WT</sup>ΔDelta<sup>Δ</sup>Cpn | +     | +          |
| Myc-Sac3 | +     | +          |
| Myc-ArPIKfyve | +     | +          |
| GFP-HA-PIKfyve<sup>WT</sup>ΔDelta<sup>Δ</sup>Cpn | +     | +          |
| ΔΔΔΔΔCpn | +     | +          |
Fig. 4

B

- Wortmannin

\[ K^{1831} \quad K^{1831}\Delta Cpn^+ \]

+ Wortmannin

\[ K^{1831}\Delta Cpn^+ \]

GFP-PIKfyve

Phase

\[ a \quad c \quad e \]

\[ b \quad d \quad f \]

Scale bar: 20 μm
Fig. 4

C

$K^{\Delta Cpn+}$ EEA1 Merge

(a) (b) (c)
Fig. 5

A

GFP-PIKfyve$^{K1831}$  Myc-ArPIKfyve$^{WT}$  HA-Sac3  Phase

a  b  c  d

WT

e  f  g  h

D$^{488}$
Fig. 5

B

Time post-transfection

12 h  24 h

Vacular phenotype (% positive cells)

GFP-PIKfyve\textsuperscript{K1831E}  +  +  +  +  +  +  +  +  +  +  +
Myc-ArPIKfyve\textsuperscript{WT}  –  +  +  –  +  +  –  –  –  –  +  –
HA-Sac3\textsuperscript{WT}  –  +  –  –  +  –  –  +  –  –  +  –
HA-Sac3\textsuperscript{D488A}  –  –  +  –  –  +  +  –  –  –  +  –
Fig. 5

C

Vacuolar phenotype (% positive cells)

| Construct                  | + | + | + |
|----------------------------|---|---|---|
| GFP-PIKfyve<sup>K2000E</sup> |   |   |   |
| Myc-ArPIKfyve<sup>WT</sup>  | – | + | + |
| HA-Sac3<sup>WT</sup>         | – | + | – |
| HA-Sac3<sup>D488A</sup>     | – | – | + |
Fig. 5

D

Myc-PIKfyve<sup>K1831</sup>  GFP-ArPIKfyve<sup>523-782</sup>  Phase

(a)  (b)  (c)
PIKfyve-ArPIKfyve-Sac3 core complex: Contact sites and their consequence for Sac3 phosphatase activity and endocytic membrane homeostasis
Ognian C. Ikonomov, Diego Sbrissa, Homer Fenner and Assia Shisheva

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