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PIKfyve: PARTNERS, SIGNIFICANCE, DEBATES AND PARADOXES

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Abstract

Key components of membrane trafficking and signaling machinery in eukaryotic cells are proteins that bind or synthesize phosphoinositides. PIKfyve, a product of an evolutionarily conserved single-copy gene has both these features. It binds to membrane phosphatidylinositol (PtdIns)3P and synthesizes PtdIns(3,5)P₂ and PtdIns5P. Molecular functions of PIKfyve are elusive but recent advances are consistent with a key role in the course of endosomal transport. PIKfyve dysfunction induces endosome enlargement and profound cytoplasmic vacuolation, likely as a result of impaired normal endosome processing and membrane exit out of endosomes. Multicellular organisms with genetically impaired function of PIKfyve or that of the PIKfyve protein partners regulating PtdIns(3,5)P₂ homeostasis display severe disorders, including embryonic/perinatal death. This review describes recent advances on PIKfyve functionality in higher eukaryotes, with particular reference to biochemical and genetic insights in PIKfyve protein partners.

Keywords

PIKfyve; PtdIns3,5P₂; PtdIns5P; endosome fusion; endosome fission; Sac3; ArPIKfyve; endosomal trafficking; GLUT4

1. Introduction

Highly specific or more promiscuous kinases and phosphatases catalyze the reversible phosphorylation of phosphatidylinositol (PtdIns) at positions 3,4, and/or 5 of the inositol headgroup to generate seven phosphoinositide (PI) species, *i.e.*, PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,4)P₂, PtdIns(3,5)P₂, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃. Although present in mammalian cells only in minute quantities, and in the case of PtdIns(3,4,5)P₃, only upon cell stimulation, PIs are indispensable and versatile membrane-anchored signals that control diverse and essential cellular processes, including intracellular membrane trafficking, signaling, cytoskeletal reorganization, DNA synthesis and cell cycle (Shisheva, 2003; Roth, 2004; DiPaolo and De Camilli, 2006; Balla, 2006; Takenawa and Itoh, 2006; Lindmo and Stenmark, 2006). Whereas many details underlying the cellular and molecular mechanisms are yet to be determined, the activity of the PI-metabolizing enzymes has to be synchronized in time and space because each of the seven PIs displays a unique steady-state intracellular compartmentalization. At these locations, PIs recruit/activate downstream protein effectors that display PI-binding domains, including PH (Plekstrin Homology), FYVE finger (Fab1p,

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YOTB, Vac1p and EEA1), ENTH (Epsin N-Terminal Homology), PX (Phox homology), GRAM (Glucosyltransferases, Rab-like GTPase Activators and Myotubularins) and PHD (Plant Homeo Domain). Intriguingly, several PI-metabolizing enzymes harbor binding modules specific for the substrate of their catalytic activity, consistent with the critical requirement for a rapid recruitment of the enzyme to the appropriate membrane and a subsequent retention at this membrane until the substrates of the enzyme action are consumed. PIKfyve, an evolutionarily conserved large protein and a product of a single-copy gene from yeast through plants to mammals, is perhaps one of the best examples of a PI-binding/PI-synthesizing enzyme; it associates with membrane PtdIns3P, synthesizing PtdIns(3,5)P₂ and PtdIns5P. Since its identification about nine years ago (Shisheva et al., 1999; Ikonomov and Shisheva, 1999), a growing body of morphological, biochemical and genetic evidence suggests that PIKfyve controls pleiotropic cell functions, the whole complexity of which we are just beginning to recognize. Through its PtdIns(3,5)P₂-synthesizing activity, PIKfyve is intimately involved in the regulation of endomembrane homeostasis and likely affects several aspects of endosome processing in the course of endocytic cargo transport. The PtdIns5-synthesizing activity of PIKfyve appears to regulate F-actin remodeling. Recent work has revealed that PIKfyve associates with several protein partners and given its intrinsic protein kinase activity, in addition to the lipid kinase activity, it is conceivable that there are other functional outputs, their nature being yet unknown. Whereas PIKfyve knockout or transgenic mouse models are thus far unavailable, the potential fundamental significance of the PIKfyve-dependent functions to human physiology is intimated by the embryonic lethality of *Drosophila* and *C. elegans* PIKfyve null mutants (Nicot et al., 2006; Rusten et al., 2006). Here, I review the current status of our knowledge about PIKfyve functionality in higher eukaryotes, giving particular emphasis to PIKfyve's protein partners. Aspects of the orthologous system in yeast are discussed only for comparative purposes; the reader is referred to recent reviews on the yeast system (Efe et al., 2005; Michell et al., 2006; Dove and Johnson, 2007).

2. PIKfyve: overview and update

Mammalian PIKfyve is a member of an evolutionarily ancient gene family of PtdIns(3,5)P₂-synthesizing enzymes that are large proteins, represented by a single-copy gene in most, if not all, species with sequenced genomes (Fig. 1). Mouse PIKfyve, named so after its function and domain structure (PhosphoInositide Kinase for five position containing a Fyve finger) was the first among the PtdIns(3,5)P₂-synthesizing enzymes in higher eukaryotes to be identified. It was cloned from a mouse F442A adipocyte library through a screen for transcripts that, like the GLUT4 transporter, are enriched in fat and muscle (Ikonomov and Shisheva, 1999). The mouse gene (ID: 18711) is located on chromosome 1 and is comprised of 42 exons. Three alternatively spliced forms of mouse PIKfyve, which differ by the presence or absence of exon 4 (33 nt) and/or exon 12' (168 nt; corresponds to human exon 11) are identified thus far: PIKfyveS (2041 residues), PIKfyveL (2052 residues) and a longer form of 2108 residues (Shisheva et al., 1999; Dondapati and Shisheva, unpublished). The human PIKfyve clone has been recently assembled based on the human EST database and the generated PCR product is found to encode a protein of 2098 amino acids (Cabezas et al., 2006). The human genomic sequence (ID: 200576) is located on chromosome 2 (2q34) and is comprised of 41 exons; the 33 nt corresponding to mouse exon 4 being absent.

In vitro assays with immunopurified PIKfyve reveal that the enzyme phosphorylates the D-5 position in PtdIns and PtdIns3P to make PtdIns5P and PtdIns(3,5)P₂, respectively (Sbrissa et al., 1999). Cell studies support PIKfyve-catalyzed synthesis of both lipid products (Fig. 1). Thus, overexpression and knockdown of PIKfyve in several mammalian cell types increases and decreases, respectively, the accumulated levels of ³²P-PtdIns(3,5)P₂ (Ikonomov et al., 2001; Sbrissa and Shisheva, 2005; Sbrissa et al., 2007). Likewise, the mass level of PtdIns5P is higher in a HEK293 cell line stably expressing PIKfyve^{WT} and lower in a HEK293 cell line

stably expressing the dominant-negative kinase-deficient PIKfyve^{K1831E} mutant, as compared to control cells (Sbrissa et al., 2002^b). These data indicate that PIKfyve activity underlies the intracellular biosynthesis of both PtdIns(3,5)P₂ and PtdIns5P. No information is currently available to implicate other kinases in intracellular synthesis of these lipids, although PtdIns(3,5)P₂ production by PI3K-dependent phosphorylation of PtdIns5P is highly likely. PtdIns5P may also be produced by turnover of higher phosphorylated PIs. Experimental evidence thus far implicates the action of myotubularin and myotubularin-related phosphatases in PtdIns5P production from PtdIns(3,5)P₂ (Walker et al., 2001; Tronchere et al., 2004; Robinson and Dixon, 2006). The relative contribution of PIKfyve vs. myotubularins to the intracellular PtdIns5P pool is, however, unclear. The intracellular levels of both PtdIns5P and PtdIns(3,5)P₂ are low, ranging from undetectable to up to 5% of cellular PIs (Ikononov et al., 2001; Sbrissa et al., 2002^b). However, they both could be modulated under certain stimuli, implying that specific regulation of PIKfyve lipid kinase activity could contribute to the dynamic changes in these lipids.

Adding to the complexity in understanding PIKfyve functionality is the fact that in addition to being a lipid kinase, PIKfyve also displays a protein kinase activity. *In vitro* phosphorylation on Ser is documented for several exogenous substrates, including PIKfyve itself (Sbrissa et al., 2000; Ikononov et al., 2003^a). Because PIKfyve lipid substrates largely inhibit the *in vitro* PIKfyve autophosphorylation, it is speculated that in a cell context the PIKfyve lipid kinase activity is regulated by autophosphorylation (Sbrissa et al., 2000). Indeed, endogenous PIKfyve is found phosphorylated but the proportion of self-phosphorylated PIKfyve vs. total phospho-PIKfyve is unclear and needs thorough examination. Thus, the experimental data collected to date are consistent with the notion that PIKfyve relays both lipid- and protein-phosphorylated signals to control complex and distinct cellular functions.

3. PIKfyve domains: functional clues

PIKfyve harbors several functional domains conserved across a great evolutionary distance (Fig. 1). The N-terminally positioned FYVE finger domain serves as a major determinant for localizing PIKfyve to cell membranes containing PtdIns3P. This is evidenced by fluorescence microscopy studies demonstrating that, unlike PIKfyve^{WT}, mutants with a truncated FYVE domain do not associate with membranes (Shisheva et al., 1999). Consistently, cell treatment with the PI3K inhibitor wortmannin renders PIKfyve^{WT} cytosolic (Sbrissa et al., 2002^a; Rutherford et al., 2006). Concordantly, a GST fusion of the PIKfyve FYVE domain selectively binds PtdIns3P-enriched liposomes in *in vitro* assays, whereas a FYVE domain-truncated mutant does not (Sbrissa et al., 2002^a). Adjacent to the FYVE finger domain is the DEP domain (Dishevelled, Egl10 and Pleckstrin), an evolutionarily late acquisition by PIKfyve molecules of higher eukaryotes (after the fruit fly), which is absent in yeast or plant PIKfyve (Shisheva, 2001). In other proteins, this domain contributes to protein targeting and stability but its function in the context of PIKfyve is unknown and should be addressed in future studies. This same stretch may also be engaged in DNA-binding, forming a “winged helix”. This observation may shed light on recent findings for a PIKfyve-assisted EGF receptor trafficking to the nucleus in carcinoma cell lines (Kim et al., 2007) and deserves further investigation. The central part in PIKfyve is occupied by the Cpn60_TCP1 (HSP chaperonin_T-complex protein 1) homology found in cytoplasmic CCT (chaperonins containing TCP1). Yeast two hybrid screens with this domain have identified several candidate PIKfyve protein partners (see below), indicating that the chaperonin-like domain is heavily engaged in regulatory interactions. Some of these interactions are apparently essential to set in motion the PIKfyve lipid kinase activity because deletion of the Cpn60_TCP1 domain abolishes the lipid kinase activity as measured *in vitro* (Sbrissa et al., 1999). Intriguingly, the Cpn60_TCP1 domain-deletion mutant (Δ 560–1231) displays unaltered protein kinase activity making it unlikely major structural deteriorations of the molecule (Sbrissa and Shisheva, unpublished). Rather,

this result suggests differential regulation of the lipid and protein kinase activities of PIKfyve. Adjacent to the Cpn60_TCP1 domain is a homology spanning ~300 residues, which is found uniquely in the PIKfyve orthologs. It includes multiple well-conserved Cys, His and Lys residues contributing to the relatively high pI of this stretch. In mammalian PIKfyve enzymes, parts of this region display sequence similarities to spectrin repeats that are found in some cytoskeletal proteins. There is no information about a plausible function of this homology yet it is worth noting that a subfraction of PIKfyve is biochemically recovered with cytoskeletal elements (Shisheva et al., 2001). The catalytic domain is positioned at the C-terminus of PIKfyve and displays sequence similarities with the catalytic domains in PI4Ks and PI5Ks but not with those in PI3Ks and PtdIns4Ks (Shisheva, 2003). This domain is responsible for the three PIKfyve activities, *i.e.*, PtdIns(3,5)P₂, PtdIns5P and phosphoprotein(s) synthesis, and mutation in the predicted ATP-binding Lys at position 1831 abolishes them all (Sbrissa et al., 2000). However, unlike with other kinases, the PIKfyve catalytic domain alone is devoid of activity. Thus, immunopurified GFP-fusion peptide (1684–2052) that encompasses the entire catalytic domain, like the Cpn60_TCP1-deletion mutant, has no measurable lipid kinase activity (Sbrissa and Shisheva, unpublished). Together, these features indicate a complex molecular organization of PIKfyve, with potentially multiple regulatory interactions to spatially and temporally integrate several possible signals, emanated by PIKfyve.

4. What PIKfyve does and how it does it

Given the several functional outputs of PIKfyve activity, it is conceivable that the enzyme controls diverse cellular processes. The best characterized role of PIKfyve is within the endosomal/endocytic system, first recognized by the ability of the kinase-deficient PIKfyve^{K1831E} mutant to induce progressively exacerbated cytoplasmic vacuolation in many cell types, including COS, HeLa, HEK293, fibroblasts and CHO (Ikononov et al., 2001). The K1831E mutation, however, abrogates all three enzymatic activities of PIKfyve (*i.e.*, to phosphorylate PtdIns, PtdIns3P and itself, Sbrissa et al., 2000). That the cytoplasmic vacuolation is a result of a defect in PtdIns(3,5)P₂-synthesis is evidenced by the inability of a point mutant, selectively defective in PtdIns5P synthesis but displaying nearly normal protein kinase or PtdIns(3,5)P₂-synthesizing activities, to vacuolate COS cells (Ikononov et al., 2002^a). The specific role of PtdIns(3,5)P₂ in the endomembrane vacuolation is further substantiated by the findings that PtdIns(3,5)P₂, but not PtdIns5P or other PIs, microinjected into vacuolated COS cells reverses the defective phenotype back to normal (Ikononov et al., 2002^a).

Phase-contrast and immunofluorescence microscopy indicate that the deteriorations of the endomembrane morphology due to dominant-negative kinase-deficient PIKfyve mutants is a complex, gradually developing process that affects different endosomal types depending on the duration of mutant expression (Ikononov et al., 2001; Ikononov et al., 2003^b; Ikononov et al., 2006). At earlier stages of COS cell transfection (9–15 h), translucent vacuoles are not visible but the PIKfyve^{K1831E}-positive vesicles are enlarged and, unlike the wild type, are largely positive for early-endosomal markers EEA1 and Rab5 (Ikononov et al., 2006). This early endosome enlargement is followed by appearance of translucent vacuoles that progressively increase in size and decrease in number, likely as a result of fusion. They may or may not contain PIKfyve^{K1831E} on the limiting membrane, but the perinuclearly positioned vacuoles are positive for cation-independent mannose-6-phosphate receptor (CI-MPR) (Ikononov et al., 2001; Ikononov et al., 2003^b). Because the bulk of steady-state MPR in kidney cells is found on late endosomes, and less so on early endosomes and TGN (Griffiths et al., 1990; Rohn et al., 2000; Lin et al., 2004), these data suggest that the translucent vacuoles originate from the late endocytic compartment (Ikononov et al., 2001; Ikononov et al., 2003^b). Similar defects are recently reported under siRNA-mediated PIKfyve depletion in HeLa cells, where different sizes of vacuoles, positive for distinct endosomal markers, are

observed (Rutherford et al., 2006). The small diameter vacuoles display early endosomal characteristics, whereas the large vacuoles are of late endocytic origin. These data support the conclusion that suppressed PIKfyve-catalyzed PtdIns(3,5)P₂ synthesis in mammalian cells induces an imbalance in endosomal membrane traffic, resulting in endosome enlargement and profound vacuolation. Importantly, cellular studies documenting remarkably similar enlargement of compartments along the endo-lysosomal system in the *S. cerevisiae*, *C. elegans* or *Drosophila* PIKfyve mutants (Odorizzi et al., 2000; Nicot et al., 2006; Rusten et al., 2006) are consistent with an evolutionarily conserved role of PtdIns(3,5)P₂ in endosome-related functions. The main difference among the species studied so far is related to the identity of the endosomal compartment where PtdIns(3,5)P₂ function is required. Whereas in lower organisms, *i.e.*, *S. cerevisiae* and *C. elegans*, the loss of Fab1/PIKfyve function affects the later stages of the endocytic pathway at the level of the lysosomes, in the higher eukaryotes (fruit fly and mammals) the defects affect earlier compartments in the endocytic pathway (Table 1).

Although there is little doubt that PIKfyve dysfunction induces endosome enlargement and cytoplasmic vacuolation in cells of all species examined thus far, the underlying cellular and molecular mechanisms are less clear. Endosomes represent a heterogeneous membrane system, which function as crossroads in traffic from and to plasma membrane, TGN and lysosomes (Fig. 2). Two studies based on either expression of dominant-negative kinase-deficient PIKfyve^{K1831E} or depletion of PIKfyve protein in mammalian cells report delayed fluid-phase transport at a post-early-endosomal step or impaired early endosome-to-TGN retrograde traffic (Ikonov et al., 2003^b; Rutherford et al., 2006). By contrast, receptor internalization, recycling, trafficking to lysosomes or degradation, and cargo sorting in the biosynthetic pathway are not affected to a significant degree (Ikonov et al., 2003^b; Rutherford et al., 2006). These data, together with the unconditional gross enlargement of mammalian early endosomes observed under these conditions, are consistent with a rate-limiting function of the PIKfyve-catalyzed PtdIns3P-to-PtdIns(3,5)P₂ conversion in the course of cargo exit from early/post-early endosomes to other destinations.

Trafficking from early endosomes occurs by cargo-loaded vesicle intermediates that arise by still unresolved mechanisms of budding/detachment from early endosomes, early endosome maturation or both (Gruenberg and Stemmark, 2004; Rink et al., 2005). Regardless of debates about their biogenesis, there is a consensus that endosomal transport intermediates are a subpopulation of carrier vesicles or maturing endosomes with characteristics distinct from both early and late endosomes, allowing for their isolation. Employing this feature, recent studies directly examine the role of PIKfyve and its protein partners (see below) in early endosome plasticity by *in vitro* reconstitution assays. These data indicate that PIKfyve triggers formation/fission (or maturation) of transport intermediates from early endosomes, negatively regulating the fusion events in the endosomal system (Ikonov et al., 2006, Sbrissa et al., 2007). It is likely that the PIKfyve function in endosome fission and fusion mechanistically underlie the constraints in the trafficking pathways traversing endosomes and the subsequent endosome swelling and cytoplasmic vacuolation. Ultrastructural studies in mammalian cells also indicate PIKfyve's role in endosomal intraluminal invaginations, a process ultimately coupled with receptor sorting for degradation, but this, oddly enough, proceeds in a way that does not affect EGFR degradative sorting (Ikonov et al., 2003^b). Though many questions and details remain to be answered and clarified, the available data indicate that PIKfyve and its product PtdIns(3,5)P₂ participate in several aspects of endosome operations, affecting some but not all endosomal trafficking pathways.

5. And where is PIKfyve?

Whereas the cytosolic PIKfyve represents the majority of the total PIKfyve (Shisheva et al., 2001), it is the membrane-associated subfraction that is a key to PIKfyve function in

endomembrane homeostasis. Fluorescence microscopy in transfected COS cells demonstrating that the PIKfyve^{K1831E} mutant with a deleted FYVE domain, thus unable to associate with membranes, also fails to induce endosome dilation and cytoplasmic vacuolation, supports this conclusion (Ikonomov et al., 2001). A substantial amount of work has been focused on identifying the PIKfyve membrane residence in mammalian cells. The existing anti-PIKfyve antibodies generated independently in different laboratories are unsuitable for detecting the low abundance endogenous protein by immunofluorescence or electron microscopy in many cell types, and as a result, the residence place of native PIKfyve remains largely enigmatic (Shisheva et al., 2001; Ikonomov et al., 2006; Cabezas et al., 2006; Rutherford et al., 2006; Shisheva and James, unpublished). A successful immunofluorescence microscopy detection of endogenous PIKfyve is reported only in 3T3L1 adipocytes, where discrete peripheral punctae resembling endosomes are documented (Shisheva et al., 2001). Whereas no morphological details are available for compartment identification, the biochemical data from equilibrium sedimentation in this cell type are inconsistent with recycling/early endosomes being the principal localization sites for endogenous PIKfyve (Shisheva et al., 2001). Rather, the majority of the protein co-fractionates with the denser bulk of membranes, cytoskeletal elements and insoluble proteinaceous aggregates. Additional information about PIKfyve localization comes from subsequent work by heterologous cell expression, which yields somewhat discordant observations. In COS cells, expressed PIKfyve^{WT} partially colocalizes with CI-MPR, found predominantly on late endosomes in kidney cell types (Griffiths et al., 1990; Rohn et al., 2000), but not with early endosomal markers EEA1 and Rab5^{WT}, or with fluorescent transferrin that labels recycling endosomes (Shisheva et al., 2001; Ikonomov et al., 2006). In other cell types such as HeLa, expressed PIKfyve^{WT} is found predominantly on EEA1-positive early endosomes (Cabezas et al., 2006; Rutherford et al., 2006). Closer microscopic inspection, however, reveals that in this cell type PIKfyve^{WT} occupies an early-endosome microdomain distinct from that occupied by EEA1 and Hrs, indicating different steady-state localization of these molecules (Cabezas et al., 2006). In addition to variations due to the cell type and levels of protein overexpression, the discordant information about the localization of ectopically expressed PIKfyve^{WT} among different studies might also be related to the enzymatic activity itself. This conclusion is supported by recent fluorescence microscopy data in transfected COS cells revealing that, unlike PIKfyve^{WT}, the kinase-deficient PIKfyve^{K1831E} point mutant (or other kinase-deficient truncated forms) accumulates on EEA1/Rab5-positive early endosomes in a PtdIns3P-dependent manner (Ikonomov et al., 2006). It is thus suggested that PIKfyve dynamically cycles as a function of its enzymatic activity and changes in the membrane PtdIns3P-to-PtdIns(3,5)P₂ ratio (Ikonomov et al., 2006). A model has been recently proposed, whereby the PtdIns3P-enriched microdomain at early endosomes is viewed as a major entry site for the cytosolic pool of PIKfyve (Fig. 2). Driven by its catalytic activity, PIKfyve rapidly exits early endosomes to other compartments by a mechanism that involves local membrane remodeling in two ways: decreasing PtdIns3P and increasing PtdIns(3,5)P₂ (Ikonomov et al., 2006). This model predicts that a subfraction of PIKfyve localizes to membranes in a PtdIns3P-independent manner but, even in this case, this ought to be PI3K-dependent because cell treatment with wortmannin renders a diffuse (cytosolic) staining for PIKfyve (Sbrissa et al., 2002^a; Rutherford et al., 2006). Thus, the current data allow the conclusion that membrane PIKfyve^{WT} is largely associated with the endosomal system and localizes to various degrees on early endosomes, MVBs, late endosomes, and most likely, the TGN, with variations depending on the cell type, the level of protein expression and the rate of PtdIns3P-to-PtdIns(3,5)P₂ conversion. Morphological identity and definition of the residence compartments for endogenous PIKfyve remain to be determined and this work is much needed.

6. PIKfyve in PtdIns5P biosynthesis: Pros and Cons

There is a continuing debate about the contribution of PIKfyve to the intracellular PtdIns5P pool. Steady-state PtdIns5P represents only a minor fraction of PIs, with levels from undetectable to 5% of total PIs, depending upon the cell type (Shisheva, 2003). PtdIns5P detection by conventional HPLC analysis is challenging due to its similar HPLC elution characteristics with that of the abundant PtdIns4P (~45% of total PIs). Given these limitations, it was not surprising that PtdIns5P was the last of the seven PIs to be discovered (Rameh et al., 1997). Dynamic changes in the levels of this lipid were possible to be quantified only after a new assay had been developed in Irvine's lab, which enzymatically quantifies the PtdIns5P mass levels and thus overcomes the inherent limitations associated with the HPLC direct detection (Morris et al., 2000). By mass assay, it has been demonstrated that HEK293 cell lines stably expressing PIKfyve^{WT} or dominant-negative kinase-deficient PIKfyve^{K1831E} mutant display about 2-fold higher and 2-fold lower mass levels of PtdIns5P, respectively, compared to the parental cell line (Sbrissa et al., 2002^b). The cell studies, together with the documented ability of PIKfyve to readily convert PtdIns to PtdIns5P *in vitro*, support the contention that PIKfyve-catalyzed biosynthesis contributes to the cellular PtdIns5P pool (Sbrissa et al., 1999; Sbrissa et al., 2002^b). Experimental evidence refuting PIKfyve-directed biosynthesis of PtdIns5P is the early complementation analyses finding HPLC-undetectable PtdIns5P in a *Afab1* yeast strain ectopically expressing PIKfyve^{WT} (McEwen et al., 1999). As a result, some researchers favor the PtdIns(3,5)P₂ catabolism by myotubularins as a principal, if not exclusive, pathway for PtdIns5P generation (Walker et al., 2003; Tronchere et al., 2004; Michell et al., 2006). While myotubularin-catalyzed hydrolysis of PtdIns(3,5)P₂ could be one source for PtdIns5P, additional considerations and experimental evidence support the notion that the PIKfyve-dependent biosynthetic arm is likely a major route for the intracellular PtdIns5P. Thus, even when up-regulated, the intracellular levels of PtdIns(3,5)P₂ are by several orders of magnitude lower than PtdIns, making the biosynthesis of PtdIns5P from PtdIns highly probable. Functional studies show that stress fiber breakdown, induced selectively by elevation of PtdIns5P, could be reproduced by ectopic expression of PIKfyve^{WT} in CHO cells (Sbrissa et al., 2004^b). It should also be taken into consideration that the PIKfyve^{WT} expression levels in the initial study of functional complementation in *Afab1* cells could not have been sufficient to drive PtdIns5P biosynthesis. Consistent with this prediction, previous conclusions from McEwen et al. (1999) related to PIKfyve inability to rescue the hyperosmotic elevation of PtdIns(3,5)P₂ in the *Afab1* yeast strain, have recently been revised based on more effective expression of PIKfyve^{WT} (Michell et al., 2006). Moreover, recent advances in PIKfyve studies revealing the enzyme activity being dependent on physically associated partners ArPIKfyve and Sac3 suggest that the latter are certainly inadequately complemented, both qualitatively and quantitatively, in the yeast experimental setting expressing solely PIKfyve^{WT}. Intriguingly, no information is currently available about steady-state PtdIns5P in yeast based on the mass level assay, proven to be far more sensitive than conventional HPLC (Sbrissa et al., 2002^b; Sbrissa et al., 2004^b). PtdIns5P naturally occurs in cells of both plant and animal kingdom and, given the ability of yeast Fab1 to make PtdIns5P from PtdIns *in vitro* (McEwen et al., 1999), the presence of PtdIns5P in yeast is highly likely. Thus, PtdIns5P biosynthesis by PIKfyve is supported by solid experimental evidence and the early observations in yeast by no means refute this biosynthetic arm in mammalian cells. On the contrary, these data are compatible with the conclusion that PIKfyve, in addition to synthesizing PtdIns(3,5)P₂ contributes, at least in part, to the PtdIns5P intracellular pool. This implies that the source of the PtdIns5P intracellular pool is not only by hydrolysis of higher phosphorylated PIs; as PtdIns3P or PtdIns4P, PtdIns5P is also biosynthesized from highly abundant PtdIns.

7. PIKfyve protein partners: functional significance

PIKfyve functions are likely regulated and mediated by a number of physically associated or functionally related up-stream regulators and downstream effectors, the identity and precise roles of which are far from being clear and are under intensive investigation. These efforts have led to the identification and characterization of several proteins as PIKfyve partners, including the ArPIKfyve regulator, the Sac3 phosphatase, the p40 transport factor and the SKD1 AAA ATPase. Below I discuss each of these proteins in the light of their link and significance to PIKfyve's predicted functions. An excellent up-to-date review focuses on potential downstream effectors of the PIKfyve/Fab1 lipid products (Michell et al., 2006) and will not be detailed here.

Partners in PtdIns(3,5)P₂ production: ArPIKfyve and Sac3

The founding member of the PtdIns(3,5)P₂-producing enzymes is yeast Fab1 (Yamamoto et al., 1995). It is not only structurally (Fig. 1) but also functionally related to PIKfyve. This is evidenced by the enlarged yeast vacuoles (a functional equivalent of mammalian lysosomes) under *FAB1* inactivation and concomitant depletion of PtdIns(3,5)P₂, highly reminiscent of the enlarged endosomes and cytoplasmic vacuoles due to PIKfyve dysfunction (Gary et al., 1998; Cooke et al., 1998; McEwen et al., 1999; Ikononov et al., 2001). It is therefore conceivable that PIKfyve and Fab1 share common regulatory mechanisms. One activator of Fab1 and the PtdIns(3,5)P₂ pathway in yeast is the evolutionarily conserved protein Vac14 (Bonangelino et al., 2002; Dove et al., 2002). Its mammalian counterpart has been recently identified and characterized as a *bona fide* PIKfyve activator (Sbrissa et al., 2004^a). PIKfyve and Vac14 physically associate in mammalian cells as evidenced by coimmunoprecipitation assays with endogenous or overexpressed proteins, hence the name ArPIKfyve, for Associated regulator of PIKfyve (Sbrissa et al., 2004^a; Sbrissa and Shisheva, 2005). Physical interaction is not confirmed for the yeast counterparts (Bonangelino et al., 2002; Dove et al., 2002) but this likely reflects detection difficulties rather than a true lack of such. Because cell loss of ArPIKfyve is associated with depletion in PtdIns(3,5)P₂, whereas ectopic expression of ArPIKfyve results in gain of PtdIns(3,5)P₂, with reciprocal changes in PtdIns3P in both cases, there is little doubt that ArPIKfyve, as PIKfyve, increases intracellular PtdIns3P-to-PtdIns(3,5)P₂ conversion in mammals (Sbrissa et al., 2004^a; Sbrissa and Shisheva, 2005). Intriguingly, *in vitro* studies indicate that ArPIKfyve is also required for PIKfyve-dependent PtdIns5P synthesis but not autophosphorylation, consistent with the differential regulation of the PIKfyve protein kinase vs. lipid kinase activities (Sbrissa et al., 2004^a; Ikononov et al., 2007). However, the regulatory mechanism and mode of the PIKfyve-ArPIKfyve physical interaction are unknown and, most likely, are quite complex, because a third member of the PIKfyve-ArPIKfyve heterooligomer, the Sac-domain containing Sac3 phosphatase, has been recently identified (Sbrissa et al., 2007). Sac3 is the mammalian counterpart of Fig4, found to directly interact with Vac14 in yeast, catalyzing PtdIns(3,5)P₂ turnover (Dove et al., 2002; Gary et al., 2002; Rudge et al., 2004). Likewise, in mammalian cells, an ArPIKfyve-Sac3 subcomplex can be formed independently of PIKfyve, and Sac3 depletion elevates slightly PtdIns(3,5)P₂ (Sbrissa et al., 2007). Nonetheless, biochemical fractionation and co-immunoprecipitation analyses in mammalian cells indicate that the three proteins, *i.e.*, PIKfyve, ArPIKfyve and Sac3 form a common complex, which regulation, stoichiometry and, possibly, other constituents remain to be seen. Overexpression of Sac3^{WT} in COS cells or ablation of ArPIKfyve in HEK293 cells result in endosome enlargement and render cells susceptible to formation of translucent vacuoles, reminiscent of the morphological changes seen by perturbations of PIKfyve (Sbrissa et al., 2004^a; Sbrissa et al., 2007). These data indicate that both ArPIKfyve and Sac3 regulate PIKfyve/PtdIns(3,5)P₂ function in the endosomal system (Fig. 2). This prediction finds further support by data from an *in vitro* reconstitution assay documenting that formation/fusion (or maturation) of transport intermediates from early

endosomes correlates with PtdIns(3,5)P₂ production (Sbrissa et al., 2007). Thus, expression of dominant-negative kinase-deficient PIKfyve^{K1831E} or Sac3^{WT}, and knockdown of PIKfyve or ArPIKfyve - all conditions decreasing PtdIns(3,5)P₂ in favor of PtdIns3P - inhibit formation of transport intermediates from early endosomes. Conversely, depletion of the Sac3 phosphatase or expression of PIKfyve^{WT}, both conditions increasing PtdIns(3,5)P₂ levels, have the opposite effect. It is thus proposed that PtdIns(3,5)P₂ produced on early endosomes drives membrane removal in the course of traffic progression to other compartments (Sbrissa et al., 2007). The association of PIKfyve, ArPIKfyve and Sac3 in a oligomeric complex, whose characteristics remain to be examined, most likely reflects the need for efficient regulatory coupling between PtdIns(3,5)P₂ synthesis and turnover on early endosomes. Such a mechanism will secure coordination of endosome fusion and fission, events thought to underlie formation of a temporally dynamic network of endosomal structures during endosome transport progression.

Partners in endosome-to-TGN traffic – the Rab9 effector p40

A yeast two-hybrid screen with the conserved Cpn60_TCP1 domain of PIKfyve has identified p40 as a PIKfyve partner (Ikononov et al., 2003^a). p40 is a transport factor predicted to directly interact with Rab9 and stimulate the late endosome-to-TGN transport of CI-MPR (Diaz et al., 1997). The p40 sequence is comprised almost entirely of six internal kelch repeats that likely form a four-stranded β -sheet corresponding to a single blade of a β -propeller. p40-PIKfyve interaction is apparent by GST-pull-down assays but not by coimmunoprecipitation in native cells, indicating that the association may be transient and/or unstable (Ikononov et al., 2003^a). That p40 may function as a downstream effector of the PIKfyve lipid products has been suggested by the biochemical observations for a profound release of membrane p40 into the cytosol in a HEK293 cell line stably expressing the dominant-negative kinase-deficient PIKfyve^{K1831E}. The p40 sequence displays two clusters of basic residues, potentially PI-binding candidates. Moreover, the predicted β -propeller structure of p40 resembles that found in a group of conserved proteins with PtdIns(3,5)P₂-binding specificity, including yeast Svp1 and mammalian WIPI49, considered to be PtdIns(3,5)P₂ effectors in membrane retrieval from the yeast vacuole and mammalian endosomes, respectively (Dove et al., 2004; Jeffries et al., 2004). Despite these features, interaction of p40 with PtdIns(3,5)P₂ or PtdIns5P is not confirmed by a liposome binding assay (Sbrissa et al., 2005). Instead, p40 is found to be an *in vitro* substrate of the PIKfyve protein kinase activity (Ikononov et al., 2003^a). It remains to be seen whether the PIKfyve-dependent phosphorylation of p40 takes place in intact cells and, if yes, what its outcome is for the endosome-to-TGN transport. Elucidation of the mechanistic link between PIKfyve and p40 becomes particularly significant in the light of recent findings that replication of HIV and other enveloped viruses is dependent on intact late endosome-to-TGN core trafficking machinery, including Rab9, p40, PIKfyve and TIP47 (Murray et al., 2005). Thus, PIKfyve, p40, or their interaction may emerge as important targets for inhibiting viral assembly and certainly deserves future investigation.

There are no obvious homologs of p40 and Rab9 in *S. cerevisiae*. In yeast, the endosome-to-TGN membrane retrieval is achieved by a subset of evolutionarily conserved proteins that assemble in a complex, called retromer. Members of the retromer complex are now identified in mammalian cells (Pfeffer, 2004; Seeman, 2005) but how they relate to the function of Rab9 or p40 in the context of endosome-to-TGN transport remains to be determined. Intriguingly, two members of the mammalian retromer *i.e.*, SNX1 and SNX2 bind PtdIns(3,5)P₂ *in vitro* along with PtdIns3P (Cozier et al., 2002; Carlton et al., 2005). Therefore, if PIKfyve is mechanistically coupled to the retromer-dependent transport from endosomes to the TGN, it ought to function prior to SNX1 (or SNX2). Noteworthy, knockdown of retromer subunits or WIPI49 in several mammalian cell types does not induce cytoplasmic vacuolation as seen by perturbations in PIKfyve (Jeffries et al., 2004; Carlton et al., 2005; Gullapalli et al., 2006),

indicating that PIKfyve functioning in the endosome-to-TGN-retrieval must be only one aspect of PIKfyve-controlled endosome dynamics.

Partners in degradative sorting

Sorting of cell surface receptors into the intraluminal invaginations of the multivesicular endosomes (MVE) is required for their degradation. Components of the sorting molecular machinery, first identified in yeast and found conserved in mammals, involve three protein complexes, ESCRT I, II and III, which act sequentially in cargo inclusion in the internal vesicles of MVEs (Babst, 2004; Hurley and Emr, 2006; Slagsvold et al., 2006). Intriguingly, PIKfyve and yeast Fab1 are not members of the ESCRT system, yet impaired Fab1 function prevents degradative sorting of some, although not all, cargoes and decreases the number of intraluminal vesicles (Odorizzi et al., 2000). It has been suggested that ESCRTIII in concert with Vps4, a AAA-type ATPase that catalyzes the dissociation of the three ESCRT complexes from endosomes, are involved in mechanical deformation of the membrane to drive the invagination and fission of intraluminal vesicles into MVE. Several lines of experimental evidence and considerations support the notion that this last step may also require PIKfyve-catalyzed PtdIns(3,5)P₂ production. Thus, perturbation in PIKfyve activity leads to a decreased number of intraluminal vesicles on MVE, possibly through affecting fission, fusion or both (Ikonomov et al., 2003^b; Ikonomov et al., 2006; Sbrissa et al., 2007). A functional relationship between the mammalian Vps4 ortholog, SKD1, and PIKfyve has been established consistent with SKD1 activity preceding that of PIKfyve (Ikonomov et al., 2002^c). Like the dominant-negative kinase-deficient PIKfyve^{K1831E}, ectopic expression of a catalytically-inactive dominant-negative form of SKD1 in COS cells induces profound cytoplasmic vacuolation that can be partially suppressed by expression of PIKfyve^{WT} (Ikonomov et al., 2002^c). The notion that PtdIns(3,5)P₂ may function in degradative sorting downstream of ESCRTI and II is also supported by the findings that Vps24, a subunit of the ESCRTIII complex, binds PtdIns(3,5)P₂ and that ectopic expression of a Vps24-deletion mutant with, presumably, impaired SKD1, but not lipid, interactions induces a vacuolation phenotype in COS cells, similar to that seen by PIKfyve dysfunction (Whitley et al., 2003). More details about the functional relationship between PIKfyve and the ESCRT protein system are certainly needed, which might explain the paradox for near normal EGFR degradation under perturbed PIKfyve functionality and aberrant intraluminal invagination of MVE (Ikonomov et al., 2003^b; Rutherford et al., 2006).

8. PIKfyve: friend or foe in insulin responsiveness of GLUT4 translocation and glucose uptake

GLUT4 is the facilitative glucose transporter that mediates the insulin-stimulated uptake of glucose in fat and muscle (Bryant et al., 2002; Watson and Pessin, 2006; Huang and Czech, 2007). In basal states, GLUT4 is intracellular and cycles slowly between the plasma membrane and one or more intracellular compartments. Insulin stimulates GLUT4 translocation to the cell surface by >10-fold, thereby increasing the rate of glucose transport. Despite intensive work, the precise mechanism of the GLUT4 membrane dynamics is still unclear. Current models propose two inter-related vesicular pools for the basal GLUT4 residence: one associated with the endosomal system, which harbors recycling endosomal markers (*i.e.*, GLUT1 or transferrin receptor), and another, referred to as GLUT4-storage compartment (GSC), that lacks endosomal markers but contains the insulin-responsive aminopeptidase (IRAP). Insulin action mobilizes principally the GSC, which is the main reservoir during the initial hormonal effect on glucose uptake in adipose cells. Insulin also mobilizes the recycling pathway and the GLUT4 inter-endosomal traffic away from early endosomes (Foster et al., 2001; Karylowski et al., 2004). Given PIKfyve functioning in endosome processing, its role in the elaborate GLUT4 endosome dynamics is highly probable. Our group and that of Tavaré have investigated how PIKfyve affects insulin-regulated GLUT4 translocation but reached

contradictory conclusions. The first clue that PIKfyve enzymatic activity functions as a positive regulator in insulin responsiveness of glucose metabolism comes from work with the dominant-negative kinase-deficient PIKfyve^{K1831E} mutant, whose expression in 3T3L1 adipocytes blunts insulin-induced GLUT4 surface translocation (Ikononov et al., 2002^b). This conclusion is recently supported by an independent approach that utilizes RNAi-mediated protein silencing. Depletion of PIKfyve and/or ArPIKfyve in 3T3L1 adipocytes dampens insulin responsiveness of glucose uptake and GLUT4 surface translocation, which correlates with the magnitude of the depleted PtdIns(3,5)P₂ intracellular pool (Ikononov et al., 2007). Conversely, gain of insulin responsiveness is seen under ablation of the Sac3 phosphatase that counteracts the PIKfyve action to some extent (Shisheva et al., 2007). Reduced PIKfyve/ArPIKfyve/PtdIns(3,5)P₂ levels arrest the surface translocation of GLUT4/IRAP vesicles, but not that of recycling GLUT1, implicating PIKfyve functionality selectively in insulin action on GLUT4 (Ikononov et al., 2007). A potential mechanism mobilizing PIKfyve and localizing PtdIns(3,5)P₂ production is provided by the biochemical observations for enzyme recruitment to adipocyte inner membranes and phosphorylation on a Ser in a manner stimulated by insulin but the identity of the membrane(s) or kinase(s) await identification (Shisheva et al., 2001; Shisheva, 2003). Insulin-dependent phosphorylation of PIKfyve was subsequently confirmed by Tavaré's group pinpointing the residue S318 as a site phosphorylated by activated Akt (Berwick et al., 2004). Conspicuously, although activating the PIKfyve lipid kinase, this phosphorylation has a negative impact on GLUT4/IRAP surface translocation (Berwick et al., 2004). Thus, in contrast to the conclusions from our studies, the interpretation of Tavaré's data is that PIKfyve enzymatic activity negatively regulates insulin-induced GLUT4 dynamics as highlighted by other authors (Michell, 2004). Careful analyses of the data in Berwick et al. (2004) and in their subsequently published work point to several issues that require re-evaluation of a number of conclusions. Thus, the conclusion about PIKfyve's negative role in GLUT4/IRAP dynamics is based on a mutant lacking the Akt-specific phosphorylation site (S318) but the lipid kinase activity of the PIKfyve^{S318A} mutant has never been tested *in vitro* or in a cell context. Furthermore, the identification of S318 as a major Akt phosphorylation site in PIKfyve now appears incorrect. Paradoxically, recent work that examines the *in vitro* phosphorylation of the same PIKfyve^{S318A} mutant by Akt shows PIKfyve^{S318A} to be as good a substrate as PIKfyve^{WT} (Seeböhm et al., 2007). Clearly, the incomplete data, together with the questionable properties of the PIKfyve^{S318A} mutant used by Berwick et al. (2004) question the conclusion that PIKfyve activity is inhibitory in insulin-induced GLUT4 surface translocation. Rather, they are compatible with our published observations that PIKfyve functions as a positive regulator of insulin responsiveness. It is highly likely that PIKfyve activity stimulates GLUT4 exit from early endosomes to recharge the rapidly depleting GSC during or after insulin challenge. The observed increases of both PtdIns(3,5)P₂ and PtdIns5P (Sbrissa et al., 2004^b, Ikononov et al., 2007) by insulin support such a model but it remains to be mechanistically resolved how the acute gain in these lipids is translated into GLUT4 translocation.

9. Membrane trafficking of ion channels: the SGK1 connection

Recent studies suggest a positive role of PIKfyve in transport systems that are activated by SGK1. SGK1, a member of the AGC subfamily of protein kinases, is related to PKB (Lang and Cohen 2001; Loffing et al., 2006). Its expression is controlled by a large number of stimuli, the most prominent being serum, glucocorticoids, oxidative stress, cytokines and cell volume. Like PKB, SGK1 is activated by phosphorylation in response to PI3K-coupled signals. However, both kinases apparently phosphorylate distinct substrates mediating different functions. SGK1 powerfully activates a subset of ion channels. Intriguingly, channels are found to not be direct substrates of SGK1 in many instances, suggesting an intermediate protein substrate is phosphorylated in an SGK-dependent manner. Two recent studies utilizing a *Xenopus* oocytes expression system propose that PIKfyve plays such a role in the exocytosis

of KCNQ1/KCNE1 potassium channels and Na⁺/glucose transporter SGLT1(SLC5A1) (Seeböhm et al., 2007; Shojaiefard et al., 2007). The assigned phosphorylation site in PIKfyve is again S318 but, unlike with PKB (Berwick et al., 2004), the SGK1 does not phosphorylate the PIKfyve^{S318A} mutant *in vitro* (Seeböhm et al., 2007). It is suggested that this SGK1-dependent phosphorylation activates PIKfyve to produce PtdIns(3,5P)₂ that somehow mobilizes KCNQ1/KCNE1 or Na⁺/glucose transporter to the cell surface (Seeböhm et al., 2007; Shojaiefard et al., 2007). Whereas the proposed up-regulation of PtdIns(3,5)P₂ intracellular levels and gain of cell surface KCNQ1/KCNE1 (or Na⁺/glucose transporter) under the experimental conditions remain to be seen, these data, taken together with that for GLUT4, are in line with the PIKfyve enzymatic activity positively regulating cell surface trafficking mechanisms of certain ion channels and transporter molecules. It is conceivable that PIKfyve-dependent deregulation of ion transport across the plasma membrane could contribute to the endosome swelling, although direct evidence is currently unavailable.

SGK1 proteins are conserved over a long evolutionary distance. Yeast displays two orthologous forms, Ypk1 and Ypk2, both implicated in the internalization of the G-protein coupled receptor Ste2 (Casamayor et al., 1999). No information is currently available to link the Ypk1/Ypk2 activation with the regulation of the Fab1 pathway.

10. Is PIKfyve activity regulated by external stimuli?

Whereas PIKfyve activity is expected to be subject to regulation by extracellular stimuli, this has been convincingly demonstrated only in few cases. A number of external cell stimuli such as insulin, EGF, PDGF or osmolarity have been evaluated by the *in vitro*-measured lipid kinase activity with immunopurified PIKfyve but changes to a significant degree have not been observed (Sbrissa et al., 1999; Sbrissa et al., 2001; Sbrissa et al., 2002^b). Thus, if the enzyme intrinsic activity is regulated, it is possible that the intricate molecular organization of the PIKfyve complex prevents *in vitro*-measurable changes. An alternative regulatory mechanism may involve intracellular re-distribution of PIKfyve without changes in the intrinsic activity. This is supported by biochemical and morphological studies in 3T3L1 adipocytes and PC12 cells where insulin and nicotine stimulation, respectively, cause a recruitment of cytosolic PIKfyve to membrane structures (Shisheva et al., 2001; Osborne et al., 2008). PIKfyve regulation is also deduced by the levels of the lipid products formed subsequent to cell stimulations. Mitogenic signals such as IL-2 or UV light in lymphocytes, the protein kinase C-activating agent PMA in platelets, and EGF stimulation in COS cells have been reported to moderately increase intracellular PtdIns(3,5)P₂ levels (Banfic et al., 1998; Jones et al., 1999; Tsujita et al., 2004). However, none of these studies provide experimental evidence to implicate PIKfyve-catalyzed synthesis and, therefore, it is formally possible that decreased rate of PtdIns(3,5)P₂ turnover could account, partially or entirely, for the effect. Hyperosmolarity, a stimulus that profoundly increases PtdIns(3,5)P₂ in yeast, does not up-regulate this lipid in a number of mammalian cell types, including HEK293, COS and CTLL lymphocytes (Dove et al., 1997; Jones et al., 1999; McEwen et al., 1999; Sbrissa and Shisheva, 2005). One exception is the differentiated 3T3L1 adipocytes, which significantly elevate PtdIns(3,5)P₂ in response to hyperosmotic stress (Sbrissa and Shisheva, 2005). In this case, the effect is shown to directly depend on PIKfyve and ArPIKfyve because the siRNA-mediated depletion of these proteins, separately or together, completely suppresses the hyperosmotic rise in PtdIns(3,5)P₂. Despite further mechanistic details being unavailable, the unprecedented elevation of PtdIns(3,5)P₂ in hyperosmotically-stressed adipocytes may reflect the necessity for a stringent osmoprotective response against water loss, as the cytoplasm in these fat-laden cells comprises only a negligible portion of the total cell volume (Sbrissa and Shisheva, 2005). Under similar analyses, acute insulin does not induce changes in total intracellular PtdIns(3,5)P₂ in this cell type (Sbrissa and Shisheva, 2005). However, an *in vitro* assay in biochemically fractionated cells reveals a significant insulin-dependent increase of PtdIns(3,5)P₂ production on intracellular membranes

(Ikononov et al., 2007). Importantly, the effect directly relates to the presence of ArPIKfyve and PIKfyve because membranes derived from cells depleted of these proteins do not generate PtdIns(3,5)P₂, nor is there an insulin-dependent increment (Ikononov et al., 2007). Increased PtdIns(3,5)P₂ production on intracellular membranes in response to insulin could be partially due to recruited PIKfyve on these membranes, as found in biochemical studies (Shisheva et al., 2001). The membrane identification is undoubtedly an important challenge as this might represent a key regulatory mechanism.

Acute insulin action in insulin-sensitive cell types such as 3T3L1 adipocytes or CHO cells that express the insulin receptor is associated with a wortmannin-insensitive transient increase of PtdIns5P mass levels but whether this is related to PIKfyve activation is unclear (Sbrissa et al., 2004^b). Other stimuli such as cellular stressors, thrombin or PLC-coupled agonists are also shown to affect PtdIns5P mass in different mammalian cell types (Morris et al., 2000; Sbrissa et al., 2002^b; Roberts et al., 2005; Jones et al., 2006). However, as discussed above, the complex enzymology of PtdIns5P makes inconclusive the identity of the enzyme(s) subjected to regulation.

11. Lessons from worms and fruit flies

Recent studies with *C. elegans* and *Drosophila* PIKfyve mutants underscore PIKfyve indispensable functions during differentiation and development (Rusten et al., 2006; Nicot et al., 2006). A complete loss of PIKfyve/Fab1 function in *C. elegans* induces developmental defects characterized by embryonic lethality, whereas partial loss of function leads to growth retardation (Nicot et al., 2006). *Drosophila* null or close to null alleles also die at the pupal stage, displaying an overgrowth phenotype indicating that PIKfyve attenuates the organ and cell sizes (Rusten et al., 2006). In both model organisms, intracellular PtdIns(3,5)P₂ levels are undetectable and the endosome/lysosome structures are grossly enlarged (Table 1). Intriguingly, the fruit fly mutants display normal receptor endosomal processing despite the enlarged multivesicular endosomes, but receptor degradation appears defective. Conspicuously, unlike mutants of ESCRT1 and ESCRT2 subunits, whose defective receptor degradation is manifested by a tumor-like overproliferation of *Drosophila* tissues and increased receptor-mediated signaling, the PIKfyve mutant clones do not display such defects (Gilbert and Moberg, 2006; Rusten et al., 2006). In fact, besides the endosome enlargement and abnormal lysosomal acidification, there is no information about marked morphological aberrations. Thus, the causes for embryonic lethality of the PIKfyve knockout models remain to be clarified.

12. Lessons from humans and mice

Mutation in PIKfyve has been implicated in the cause of Francois-Neetens Mouchettee fleck corneal dystrophy (CFD, MIM 121850), an autosomal dominant human syndrome characterized by the presence of numerous white flecks scattered in all layers of the stroma (Li et al., 2005). Most of the mutations are of a nonsense and frame-shift type that occur in and around the Cpn60_TCP1 domain, predicting termination of the peptide prior to the catalytic domain. Although the PIKfyve protein, its activity or PtdIns(3,5)P₂ levels have not been determined under these conditions, it is expected that the mutations will result in a loss of PIKfyve function. Given the lethality of the PIKfyve null mutations in the model organisms discussed above, the asymptomatic condition of the CFD patients is quite surprising but could be attributed to the presence of one normal allele. Histological findings in CFD indicating that the corneal flecks are swollen keratocytes containing membrane-limited empty vacuoles (Nicholson et al., 1977) are consistent with the phenotypic changes seen under perturbed PIKfyve function.

More severe symptoms are recently seen in patients with mutations in one of the PIKfyve partners - the Sac3 phosphatase that is localized on chromosome 6q21 in humans (Chow et al., 2007). Out of the examined ninety-five patients with hereditary motor and sensory neuropathy resembling forms of the Charcot-Marie-Tooth (CMT) disorder but without identifiable mutations, four unrelated patients appear to carry mutations in the *SAC3* phosphatase gene. In addition to unique mutations in the Sac3 molecule, all four patients carry an I⁴¹-to-T mutation. A corresponding substitution in the yeast ortholog, Fig4^{I59T}, is associated with inability of yeast cells to maximally elevate PtdIns(3,5)P₂ in response to hyperosmotic stress (Chow et al., 2007). This suggests that I41T mutation in the Sac3 phosphatase is a gain of function increasing PtdIns(3,5)P₂ turnover. The authors, however, favor the idea that the I41T is an allele causing loss of function (Chow et al., 2007). The impeded hyperosmotic response in the yeast experimental system expressing Fig4^{I59T} is attributed to impaired mechanisms of PIKfyve activation, postulated to require intact Fig4 phosphatase (Duex et al., 2006; Chow et al., 2007). While this prediction might be correct, information about levels or activity of PIKfyve or its regulator ArPIKfyve under this or other Sac3 mutations is currently unavailable. Other forms of Charcot-Marie-Tooth disorder (types 4B1 and 4B2) are caused by mutations in myotubularin-related MTMR2 and the associated, enzymatically inactive MTMR13, which also turn over PtdIns(3,5)P₂, in addition to PtdIns3P. Disease-causing mutations are inherited recessively, indicating that the loss-of-function alleles lead to reduced phosphatase activity and, hence, higher intracellular PtdIns(3,5)P₂ (or PtdIns3P) (Berger et al., 2002, Robinson and Dixon, 2006), although this is yet to be experimentally resolved. Thus, it is unclear whether the neurological disorders in CMT patients directly or inversely correlate with PtdIns(3,5)P₂ levels.

As in humans, mutations of the mouse *Sac3* gene is linked to polyneurological defects, known as “pale tremor” phenotype (Chow et al., 2007). The *Sac3*-deficient mice exhibit juvenile lethality and display large vacuoles in a number of cell types, indicative of inadequate PtdIns(3,5)P₂ levels. Paradoxically, the elimination of the *Sac3* phosphatase in mice is associated with a loss, rather than the expected gain in PtdIns(3,5)P₂, the reasons for which are to be determined. The status of the remaining two proteins, ArPIKfyve and PIKfyve, may provide some clues but this is yet to be explored. As reported just recently, knockout of the *ArPIKfyve* gene in mice causes perinatal death and produces a pattern of neurodegeneration and neurological defects reminiscent of those seen by the lack of *Sac3* (Zhang et al., 2007). These results further reinforce the functional interaction between the two proteins in regulating PtdIns(3,5)P₂ homeostasis and suggest that abnormal functioning of the PIKfyve-associated protein partners in the endosomal system induces neurodegeneration and neurological defects. Transgenic or knockout mouse models of PIKfyve are much needed and may enlighten some of the paradoxes observed with the *Sac3* mutants.

Concluding remarks

Significant progress in understanding PIKfyve's pleiotropic functions has been made during the last few years. Available data in mammalian cells indicate a critical role of PIKfyve in the proper performance of the endosomal/endocytic system and support a model whereby PIKfyve-catalyzed PtdIns3P-to-PtdIns(3,5)P₂ conversion regulates endosome processing in the course of cargo transport. Trafficking pathways, both constitutive and regulated, which emanate from or traverse early endosomes *en route* to the TGN or later endosomal compartments have been found to be particularly sensitive to dysfunction of PIKfyve. In this regard, the involvement of PIKfyve in HIV virus propagation, *Helicobacter pylori* toxin-induced vacuolation and surface translocation of key transporter molecules may unravel new potential therapeutic targets. *Drosophila* and *C. elegans* null mutants indicate PIKfyve's essential role in the development of these multicellular model organisms and largely support PIKfyve functioning in the endosomal system. Several protein partners interacting with PIKfyve have been

characterized. A finding of particular interest is the PIKfyve physical assembly with its activator ArPIKfyve and the Sac3 phosphatase, which unravel a key requirement for tight regulation of PtdIns(3,5)P₂ synthesis and turnover. Mouse knockout models of ArPIKfyve and Sac3, which exhibit similar postnatal lethality and neuronal loss, further support the functional relation between the proteins. As mediators of PIKfyve activity could be PtdIns5P and phosphoproteins, in addition to PtdIns(3,5)P₂, each of which, in turn, transmits signals to a number of downstream effectors regulating variety of intracellular events, the PIKfyve functionality is likely to be immensely more complex than anticipated. Future research will be able to enlighten the ample number of fundamental functional and mechanistic questions that still remain.

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Figure 1.

Schematic diagram of the domain structure of the evolutionarily conserved mammalian and yeast counterparts engaged in PtdIns(3,5)P₂ metabolism (<http://scansite.mit.edu/cgi-bin/motifscan> and <http://pfam.janelia.org>). The DEP domain region (349–487) is also homologous to “winged helix” DNA-binding domain by <http://supfam.org/SUPERFAMILY> (not shown). CHK-Hom indicates homologous region enriched in Cys, His and Lys. Parts of CHK-Hom show homology to spectrin repeats (not shown). H in ArPIKfyve are heat repeats. Presented are the products of PIKfyve or Fab1, the substrates of Sac3 or Fig4, and the affected PI by ArPIKfyve or Vac14, confirmed both *in vitro* and *in vivo*. See text for further details and references.

Figure 2. General scheme of endocytosis and proposed model for the locus and mode of action of PIKfyve and its physically associated partners

The PIKfyve-ArPIKfyve complex assembles on PtdIns3P-enriched early endosome membranes and catalyzes PtdIns3P-to-PtdIns(3,5)P₂ conversion. This switch triggers formation/detachment or maturation of transport intermediates from early endosomes and likely decreases the rate of endosome fusion due to consumption of PtdIns3P and acquisition of PtdIns(3,5)P₂. PIKfyve-ArPIKfyve complex then recruits Sac3 which turns over PtdIns(3,5)P₂ to PtdIns3P, allowing membrane fusion may occur. The described events may occur on other PtdIns3P-containing endosomal structures (*i.e.*, ECV/MVB or late endosomes). Impaired endosome plasticity controlled by the PIKfyve-ArPIKfyve-Sac3 complex affects exit from early/post-early endosomal structures to other destinations (purple line), evidenced by impaired post-early endosomal traffic of solutes and endosome-to-TGN membrane transport, endosomal carrier vesicles/multivesicular bodies.

Intracellular compartments, membrane trafficking pathways and endosome operations affected by the PIKfyve loss-of function in higher eukaryotes.

Table 1

Organism Event	by PIKfyve DN ^A	Mammals by PIKfyve KD ^B	<i>Drosophila</i> ^C	<i>C. elegans</i> ^D
Compartment morphology				
Early endosomes	Enlarged	Enlarged	Enlarged	Normal
MVB/late endosomes	Enlarged	Enlarged	Enlarged	Slightly enlarged
Lysosomes	Normal	Nd	Nd	Enlarged
Golgi/TGN	Normal	Nd	Nd	Normal
Trafficking pathways				
Endoplasmic reticulum	Nd	Nd	Nd	Normal
Receptor internalization	Functional	Functional	Functional	Nd
Cell surface recycling	Functional	Functional	Nd	Nd
Lysosomal degradation	Functional	Functional	Defective	Functional
Fluid-phase endocytosis	Retarded	Nd	Retarded	Functional
Endosome-to-TGN	Nd	Delayed	Nd	Nd
Biosynthetic pathway	Functional	Nd	Functional	Nd
Lysosome maturation	Nd	Nd	Nd	Defective
Endosome dynamics				
Fusion	↑ (early/late endosomes)	Nd	Nd	↑ (lysosomes)
Fission/maturation	↓ (early endosomes)	Nd	Nd	Nd
Intralumenal vesiculation	↓	Nd	↓ or →	Nd
Acidification	Nd	Nd	Defective	Defective

^A Data from: Ikonomov et al., 2003, Ikonomov et al., 2006;

^B Rutherford et al., 2006;

^C Rusten et al., 2006;

^D Nicot et al., 2006.

Nd, not determined;

DN, dominant-negative;

KD, knockdown.