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Phosphatidylinositol 3,5-bisphosphate: low abundance, high significance

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Abstract

Recent studies of the low abundant signaling lipid, phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$), reveal an intriguingly diverse list of downstream pathways, the intertwined relationship between $PI(3,5)P_2$ and PI5P, as well as links to neurodegenerative diseases. Derived from the structural lipid phosphatidylinositol, $PI(3,5)P_2$ is dynamically generated on multiple cellular compartments where interactions with an increasing list effectors regulate many cellular pathways. A complex of proteins that includes Fab1/PIKfyve, Vac14 and Fig4/Sac3 mediates the biosynthesis of $PI(3,5)P_2$, and mutations that disrupt complex function and/or formation cause profound consequences in cells. Surprisingly, mutations in this pathway are linked with neurological diseases, including Charcot-Marie-Tooth Syndrome and Amyotrophic Lateral Sclerosis. Future studies of $PI(3,5)P_2$ and PI5P are likely to expand the roles of these lipids in regulation of cellular functions, as well as provide new approaches for treatment of some neurological diseases.

INTRODUCTION

Phosphorylated phosphatidylinositol (PIP) signaling lipids play regulatory roles. These low-abundance lipids are produced from phosphatidylinositol (PI), an abundant structural component of membranes, which can be phosphorylated in any combination on positions three, four or five. Highly regulated PIP kinases and phosphatases generate and turn over the resultant seven PIP lipids (Fig. 1).

PIP lipids provide spatial and temporal regulation of complex protein machines. The interconvertibility of PIPs enables rapid changes in the identity of the signaling lipid to dynamically recruit effector proteins to specific membranes at the right time. For example, synthesis of phosphatidylinositol 3-phosphate (PI3P) [1] at a confined region is predicted to assemble a large complex of multiple PI3P binding proteins and their associated binding partners. Notably, the lipid kinase, Fab1, binds PI3P [2] (Fig. 2) and catalyzes the conversion of PI3P to $PI(3,5)P_2$ [1]. Recruitment of Fab1 causes local depletion of PI3P and an increase in the levels of $PI(3,5)P_2$, which releases PI3P binding proteins and recruits a distinct set of $PI(3,5)P_2$ binding proteins.

Since the discovery of $PI(3,5)P_2$ in 1997 [3,4], the number of known $PI(3,5)P_2$ regulated pathways has expanded greatly. Identification of a comprehensive list of these pathways and

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downstream effector proteins will be required to fully understand $PI(3,5)P_2$ signaling. Similarly, stimuli that regulate $PI(3,5)P_2$ levels remain to be identified. Here we assess current knowledge and suggest future directions for the study of this very low abundance lipid.

$PI(3,5)P_2$ is much less abundant than most PIPs, including PI4P and $PI(4,5)P_2$. $PI(3,5)P_2$ is present at about 0.1% and 0.04% of total phosphatidylinositol in yeast and mammalian fibroblasts, respectively. The amount of $PI(3,5)P_2$ is 17-fold and 125-fold less abundant than $PI(4,5)P_2$ in yeast [5] and mammalian fibroblasts [6], respectively. The scarcity of $PI(3,5)P_2$ likely contributed to the twenty-five year delay in its discovery [3,4] relative to PI4P and $PI(4,5)P_2$ [7]. Utilizing dilute perchloric acid to precipitate cells followed by deacylation of lipids significantly improved the yield of glycerol-inositol head-groups and the identification of $PI(3,5)P_2$ over the traditional Folch extraction [4,5].

SYNTHESIS AND TURNOVER OF $PI(3,5)P_2$ IS TIGHTLY CONTROLLED BY A LARGE PROTEIN COMPLEX

In yeast, Fab1 [8] is the sole PI3P 5-kinase [1,9] and Vps34 is the sole PI 3-kinase [10]. Both $PI(3,5)P_2$ and PI3P levels dynamically and transiently change in response to specific stimuli. Prolonged introduction of yeast into hyperosmotic media causes a 20-fold transient elevation of $PI(3,5)P_2$ [3] that lasts for about ten minutes before a precipitous drop to basal levels [11]. Concomitant with the rise in $PI(3,5)P_2$, synthesis of PI3P increases. These data suggest that $PI(3,5)P_2$ and PI3P play early roles in adaptation of yeast to hyperosmotic stress. Similarly, these lipids may regulate adaptation in plants and animals, such as transient responses to hormonal or sensory stimuli.

Fab1, commonly called PIKfyve in mammals, is present in most eukaryotes [12]. In this review, “Fab1” refers to Fab1 in all non-mammalian species and “PIKfyve” to mammals. “Fab1/PIKfyve” refers to the mammalian and non-mammalian enzyme. In yeast and mouse embryonic fibroblasts (MEF), Fab1/PIKfyve provides all of the $PI(3,5)P_2$ [1,6,9,13–18]. Across species, the domain structure is similar (Fig. 2).

The $PI(3,5)P_2$ Synthesis Complex

The dynamic and rapid changes in $PI(3,5)P_2$ observed in yeast suggests that Fab1 is tightly regulated. Moreover, overexpression of Fab1 does not increase $PI(3,5)P_2$ levels [1]. Indeed, Fab1 activity requires formation of a complex of regulatory proteins, including Fig4, Vac14, Vac7 and Atg18.

Fig4, a $PI(3,5)P_2$ 5-phosphatase, catalyzes the turnover of $PI(3,5)P_2$ in yeast [11,19–21]. Unexpectedly, Fig4 is also required for the activation of Fab1/PIKfyve [6,11,19,22]. Mutations in the catalytic site of Fig4 negatively affect both the turnover of $PI(3,5)P_2$ and the elevation in $PI(3,5)P_2$ in response to hyperosmotic stress [19]. In addition to Fig4 catalytic activity, other regions in Fig4 may play a role. Several disease mutations in Fig4 reside in a non-catalytic, N-terminal domain [23]. Analysis of a corresponding mutation (Fig4-I59T) in yeast revealed a defect in hyperosmotic shock induced activation of Fab1 [22]. Analysis of this N-terminal domain may provide insight into how Fig4 activates Fab1/PIKfyve.

Vac14 regulates both Fab1 and Fig4 [5,24] and is required for the synthesis and turnover of $PI(3,5)P_2$ [11]. Vac14, composed of virtually all HEAT repeats (Fig. 2), functions as a scaffold protein that nucleates the formation of a complex including Fab1, Fig4 and other regulators [25,26] (Fig. 3). Vac14 forms dimers or oligomers [24–27]. In the cytoplasm,

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Fig4 and Vac14 interact without Fab1 [25]. There may be additional proteins required to form the complex.

Similarly, mammalian Vac14 (ArPIKfyve) forms a complex with PIKfyve, and Fig4 (Sac3) [26,28–31]. The interaction sites between the yeast and mammalian complexes are likely conserved. The binding site for Fab1 on Vac14 is conserved in the mammalian complex [26,28]. Fig4 binds to Vac14 through the conserved C-terminal region [28]. Additionally, the N-terminal pathogenic point mutation, Fig4-I41T, disrupts the interaction of Fig4 with Vac14 and destabilizes Fig4 [29,32]. These observations raise the possibility that both the N- and C-termini of Fig4 interact with Vac14.

Yeast Vac7, a critical activator of Fab1, has no recognizable motifs; its mode of action is unknown [1,5,11,19,20,33]. Vac7 resides within the Fab1 complex, but is not required for formation or localization of the complex [1,21,26]. This is surprising because Vac7 is the only protein in the complex with a transmembrane domain [5]. Vac7 function is likely conserved in metazoans. However, based on sequence similarity, Vac7 is only present in some fungi. Either, alternative mechanisms activate Fab1/PIKfyve in metazoans, or proteins with functions analogous to Vac7 cannot be identified by BLAST search.

Yeast Atg18, a negative regulator of $PI(3,5)P_2$ levels, resides within the Fab1 complex. Through two adjacent binding sites [34–36], Atg18 binds to $PI(3,5)P_2$ and PI3P [37]. These sites are essential for Atg18 to negatively regulate $PI(3,5)P_2$ levels and for localization of Atg18 on the vacuole [38]. Relief of Fab1 inhibition in an *atg18Δ* strain requires Fab1 activators. Thus, Atg18 likely inhibits the activators rather than acting on Fab1 directly. Metazoans may have unidentified proteins that function similarly to yeast Atg18. The mammalian genes, *WIPI1*, *WIPI2*, *WIPI3* and *WIPI4*, encode proteins with greater than 20% identity to yeast Atg18 [15,39]. WIPI1 and WIPI2, like Atg18, function in autophagy. However, whether they function as negative regulators of $PI(3,5)P_2$ levels has not been tested.

Orchestrating Fab1/PIKfyve activity—At least three mechanisms within the Fab1 complex contribute to the dynamic regulation of $PI(3,5)P_2$. First, the lipid kinase and phosphatase reside within the complex. Second, the Fab1 activator, Vac7, and inhibitor, Atg18, bind overlapping sites on Vac14 and likely compete for access to Fab1. Third, catalytic activity of Fig4 is required for the activation of Fab1. Tight coordination between synthesis and turnover of $PI(3,5)P_2$ likely explains how a sustained stimulus of hyperosmotic shock causes a steep transient increase in $PI(3,5)P_2$ levels.

Other opposing lipid kinases and phosphatases reside in the same complex or have coordinated regulation (reviewed in [40]). MTM1, a lipid 3-phosphatase, resides in a complex with the PI3-kinase, Vps34. Inositol polyphosphate 4-phosphatase is in a complex with a class I PI3-kinase. The added complexity in the Fab1 complex, that the opposing lipid phosphatase has a second role as activator of the lipid kinase, underscores the importance of directly measuring phosphoinositide lipid levels to determine cellular functions of predicted lipid phosphatases.

Comparison of $PI(3,5)P_2$ synthesis in yeast and metazoans—In metazoans, several PI 3-kinases, in addition to Vps34, may produce the PIKfyve substrate, PI3P. Indeed, knockdown of either PIKfyve or PI3K-C2α, but not Vps34, affects TORC1 activity in adipocytes [41]. Thus, in some cases PI3K-C2α may provide the pool of PI3P utilized to generate $PI(3,5)P_2$.

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A major difference between the yeast and mammalian Fab1/PIKfyve complex are the lipid pools that they control. Surprisingly, in MEF cells the PIKfyve complex is required for most of the PI5P and all of the $PI(3,5)P_2$ pool [6,14,17,42]. An independent study concluded that PIKfyve does not contribute to PI5P levels [43]; however, that study assumed that PIKfyve inhibition did not impact the lipids used to standardize the samples.

The relative importance of Vac14 for Fab1/PIKfyve activity differs between the yeast and mammalian complexes. In *vac14Δ* yeast, $PI(3,5)P_2$ levels are reduced at least 10-fold [11,19], while $Vac14^{-/-}$ and $Fig4^{-/-}$ MEF cells reveal a more modest 2-fold reduction in $PI(3,5)P_2$ and PI5P [6,22,42]. Since Vac14 or Fig4 are required for only half of the $PI(3,5)P_2$ pool and PIKfyve is required for the entire pool, PIKfyve either retains partial function in the absence of Vac14 or Fig4 and/or PIKfyve has additional regulators.

Localization of the Vac14, Fab1/PIKfyve and Fig4

In yeast, Fab1, Vac14, Fig4 are found on the limiting membrane of vacuoles and adjacent foci, which are likely endosomes [21,25,26]. In metazoan cells, Fab1/PIKfyve and Vac14 are found on early and late endosomes, lysosomes and in the cytosol [44–50]. Questions remain about how the complex is associated with membranes. Is the FYVE domain of Fab1/PIKfyve sufficient for localization of the complex? Are there other lipid-binding or transmembrane containing subunit(s)?

$PI(3,5)P_2$ is a precursor for PI5P synthesis

$PI(3,5)P_2$ likely serves as a precursor for most of the cellular PI5P pool. The strongest evidence for this hypothesis comes from heterologous expression of PIKfyve in yeast, which greatly increases $PI(3,5)P_2$, decreases its precursor, PI3P. Importantly, the combined total of PI3P, PI5P and $PI(3,5)P_2$ remains constant in the presence or absence of heterologous PIKfyve [6]. If PI5P were generated directly by PIKfyve, then new direct conversion of PI to PI5P would raise the combined total of PI3P, PI5P and $PI(3,5)P_2$. Additionally, transient activation or inhibition of endogenous PIKfyve in fibroblasts, causes $PI(3,5)P_2$ levels to reach a new steady-state faster than PI5P, an outcome consistent with a precursor-product relationship [6].

Generation of PI5P from $PI(3,5)P_2$ requires proteins with 3-phosphatase activity [6], which may be provided by myotubularins (MTMRs) [51]. Indeed, mouse MTMR2 and *Drosophila* MTMR3 function with Fab1/PIKfyve to control PI5P and $PI(3,5)P_2$ [52,53]. That both $PI(3,5)P_2$ and PI5P are embedded in membranes, and cannot freely diffuse, raises the possibility that MTMRs reside within the PIKfyve complex. This would provide rapid access of MTMRs to the newly synthesized $PI(3,5)P_2$.

An alternative hypothesis, that PIKfyve directly generates most of the cellular PI5P, has been recently reviewed [54]. Briefly, there is controversy between independent *in vitro* studies about whether PIKfyve directly generates PI5P [12]. In some studies PIKfyve was immunoprecipitated from cells that express many lipid 3-phosphatases. Tight association of PIKfyve with 3-phosphatases during immunoprecipitation may explain some discrepancies. Strong *in vitro* evidence that PIKfyve can directly generate PI5P comes from studies of PIKfyve expressed from insect Sf9 cells [55]. Development of a general inhibitor of myotubularin function may help resolve whether most of the cellular PI5P pools are generated directly or indirectly by PIKfyve.

That PIKfyve is either directly or indirectly responsible for most of the PI5P in fibroblasts raises questions about whether $PI(3,5)P_2$ and PI5P reside on the same membrane. Localization of Vac14, Fab1/PIKfyve, and Fig4 provide insights into the subcellular

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locations of $PI(3,5)P_2$ in yeast and metazoans. If MTMRs are associated with this complex, then MTMR localization would provide information for the subcellular distribution of PI5P as well (Fig. 4A). However, the presence of Vac14, Fab1/PIKfyve, and Fig4 does not *a priori* indicate enzymatic activity. Thus, development of probes will be critical to determine the spatial and temporal dynamics of $PI(3,5)P_2$ and PI5P.

$PI(3,5)P_2$ BINDING PROTEINS

Based on the pleiotropic defects observed in cells and organisms with defects in PIKfyve activity, most $PI(3,5)P_2$ binding proteins are likely not yet identified. To date, multiple types of motifs as well as novel sequences have been shown to interact with $PI(3,5)P_2$. $PI(3,5)P_2$ binds directly to some WD40 domain containing proteins, including Atg18, Atg21, Hsv2, Tup1 (in yeast) and Raptor (in adipocytes), and regulates their functions *in vivo* [37,41,56]. Additionally, sorting nexin proteins, SNX1 and SNX2 (PX domain) [57,58], Cti6 (PHD domain) [56], clavesin (Sec14 domain) [59] and class II formins (PTEN domain) [60] interact with $PI(3,5)P_2$. TRPML1 and RyR1 also bind $PI(3,5)P_2$ [61,62]. In these latter examples, no lipid binding motif is apparent. Thus, bioinformatic approaches are not sufficient to determine which proteins bind $PI(3,5)P_2$.

Atg18 binds $PI(3,5)P_2$ with a high affinity, in the nanomolar range [37], likely due to tandem lipid binding sites. Lower affinity interactions may be of equal biological significance but are more difficult to detect. Moreover, some effectors require simultaneous interactions with other proteins (reviewed in [63]). For example, the FYVE domain containing protein, EEA1, associates with membranes via simultaneous interaction with PI3P and Rab5 GTPase. The development of strategies to detect relatively low affinity binding will be necessary to identify the full set of $PI(3,5)P_2$ and PI5P effectors.

PATHWAYS REGULATED BY Vac14, Fab1/PIKfyve and Fig4

Mutants deficient in Fab1 or its regulators provide information on its cellular (Table 1) and physiological roles (Table 2). The PIKfyve inhibitors, YM201636 and MF4, have also facilitated studies of cellular functions of PIKfyve [13,64]. However, off-target effects need to be considered [65].

In mammals, loss of PIKfyve function decreases both $PI(3,5)P_2$ and PI5P [6,14,42], thus phenotypes linked to mutations in this pathway may be due to loss of $PI(3,5)P_2$, PI5P or both lipids. In addition, disagreement among studies about whether a specific pathway requires PIKfyve may be due to differences in the extent of decrease of PIKfyve activity. In most cell-based studies some PIKfyve function remains, including mutant cells and RNAi experiments. For example, in $PIKfyve^{\beta geo/\beta geo}$ MEF cells, 5% of the normal levels of PIKfyve provide half of the normal levels of $PI(3,5)P_2$ and PI5P [6]. Here, we present pathways that require PIKfyve. Those known to be directly regulated by $PI(3,5)P_2$, because $PI(3,5)P_2$ protein effectors have been identified, will be indicated. In other cases, the regulatory lipid may either be $PI(3,5)P_2$ or PI5P.

Formation of large vacuoles

A striking feature in $PI(3,5)P_2$ deficient organisms are enlarged vacuoles [8,18,22,33,42,48,64,66–68]. In mutant yeast, the vacuole/lysosome is enlarged. In $Vac14^{-/-}$ and $Fig4^{-/-}$ MEF cells, the vacuoles are heterogeneous, arising from both late endosomes and lysosomes, as well as enlarged autophagosomes [22,42,44,69]. Complete inhibition of PIKfyve causes vacuoles to form from early endosomes as well [64].

The enlarged vacuoles in $PI(3,5)P_2$-defective yeast mutants cannot release water even when exposed to hyperosmotic shock [5], which suggests an inability to regulate the water content

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of the vacuole. Similarly, vacuoles in mammalian cells are likely due to defects in the regulation of osmolarity within the endomembrane system. The *Vac14*$^{Ingls/Ingls}$ and Fig4$^{-/-}$ mouse mutants exhibit extreme hydrocephalus [26,32], and the vacuoles that form in *Vac14*$^{-/-}$ or Fig4$^{-/-}$ MEF cells are not filled with lipid [22,42].

PI(3,5)P_2 regulates some ion channels

The defects in water homeostasis may be linked to defects in ion homeostasis. Indeed, overexpression of the TRPML1 calcium channel in *Vac14*$^{-/-}$ MEF cells suppresses the formation of vacuoles [61]. In *S. pombe*, mutations in a calcium permease (SPAC521.04c) rescue the enlarged vacuoles in the *fab1Δ* mutant [70]. Suppression in both cases may be due to regulation of calcium flux.

A role for phosphoinositide regulation of ion channels is better understood on the plasma membrane where multiple ions channels are activated by PI(4,5)P_2. In some cases, PI(4,5)P_2 directly interacts with the channel; in other cases, PI(4,5)P_2 recruits regulators [71]. Similarly, PI(3,5)P_2 activates ion channels on endosomes and lysosomes, including mucolipin transient receptor potential channels (TRPML1, TRPML2, TRPML3) and yeast homolog, yeast vacuolar conductance (Yvc1), and two-pore channels (TPC1, TPC2, TPC3) [61,72]. While the mechanism of PI(3,5)P_2 regulation of TPCs is not known, PI(3,5)P_2 interacts directly with the cytoplasmic N-terminus of TRPML1. PI(3,5)P_2 is also important for calcium dynamics in muscles. PI(3,5)P_2 directly activates the ryanodine receptors (RyR1, RyR2), which release calcium from the sarcoplasmic reticulum in skeletal and cardiac muscles, respectively [62,73].

PI(3,5)P_2 plays a role in the acidification of the vacuole

Vacuoles in *fab1Δ*, *vac7Δ*, and *vac14Δ* yeast are less acidified than wild-type vacuoles [8,33]. Similar phenotypes occur in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Arabidopsis thaliana* [49,66,74–76]. These acidification defects may contribute to the formation of large vacuoles; vacuolar ATPase function is required for both vacuole fission and fusion [77]. However, PI(3,5)P_2 plays additional roles in vacuole morphology. A limited increase in PI(3,5)P_2 corrects acidification of the vacuole without correcting vacuole size [1,5]. PI(3,5)P_2 effectors involved in acidification have not been identified, although the vacuolar ATPase is a likely candidate. While assembly of the vacuolar ATPase does not require PI(3,5)P_2 [5], PI(3,5)P_2 may regulate vacuolar ATPase activity.

Fab1/PIKfyve is required for multiple pathways in the endomembrane system

Membrane trafficking defects also contribute to the formation of enlarged vacuoles. In yeast, PI(3,5)P_2 and Atg18 are required for fission of the vacuole [38,78] and retrograde traffic from the vacuole to the Golgi [37,79]. These defects contribute to but do not fully account for the large vacuoles in the *fab1Δ* mutant. The vacuoles in *atg18Δ* are not as enlarged as observed in *fab1Δ* yeast [38]. Thus, defects in water and ion homeostasis, vacuole acidification, as well as defects in membrane trafficking and vacuole fission, each contribute to the enlarged vacuoles caused by low levels of PI(3,5)P_2 (Fig. 4B).

Roles for Fab1/PIKfyve in the multivesicular body pathway—Fab1/PIKfyve function has been linked to multivesicular body (MVB) formation, a protein degradation pathway. MVB formation involves the ubiquitination and capture of cargoes on the limiting membrane of late endosomes, which are then internalized via invagination and formation of intraluminal vesicles (ILV). Degradation of protein cargoes occurs when the MVB fuses with the lysosome. In *fab1Δ* yeast, fewer ILVs are formed [1]. Formation of ILVs requires several ESCRT proteins, including Vps24. While controversial [80], one study suggested

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that Vps24 binds $PI(3,5)P_2$ [81]. Thus, the partial defect in ILV formation may be due to a requirement for $PI(3,5)P_2$ in Vps24 function. In addition to a possible role in forming ILV vesicles, $PI(3,5)P_2$ may be required for sorting some protein cargoes (reviewed in [82]). How $PI(3,5)P_2$ regulates cargo sorting remains to be determined.

Loss of PIKfyve activity has also been linked to events that occur after cargo sorting. *Drosophila* Notch, Wingless and Dpp (a fly homologue of TGFβ) accumulate in the MVB and are not degraded in a *Drosophila fab1* mutant. Thus, Fab1 may also function downstream of cargo internalization [49]. Delayed epidermal growth factor receptor (EGFR) degradation due to inhibition of PIKfyve [64,83] may also be due to similar defects: either trafficking problems after sorting ligands into the MVB or loss of protease activity in lysosomes that are not properly acidified.

PIKfyve is required for protein trafficking from endosomes to the TGN—Similar to yeast [37,79], PIKfyve is required for retrograde traffic of proteins from endosomes to the trans-Golgi network (TGN). Knockdown of PIKfyve inhibits retrograde traffic of the cation-independent mannose-6-phosphate receptor (CI-MPR), sortillin (a related receptor) and furin [48]. In *Vac14*$^{-/-}$ fibroblasts [42], or following inhibition of PIKfyve [13], CI-MPR localizes to endosomes and cathepsin D, one of its ligands, is missorted [42]. Inhibition of PIKfyve also delays trafficking of the Shiga Toxin B subunit from endosomes to the TGN [13]. These defects may be due in part to misregulation of SNX1 and SNX2, retromer proteins that bind directly to $PI(3,5)P_2$. In addition, PIKfyve binds to two proteins required for retrograde trafficking: p40, a Rab9 effector, and JLP, a kinesin adaptor required for microtubule based transport from endosomes to the TGN [84,85].

Regulation of PIKfyve in response to insulin—PIKfyve is required for insulin-mediated Glut4 translocation. Insulin stimulates the glucose transporter (Glut4) to transiently translocate to the plasma membrane, which facilitates glucose uptake. Regulated Glut4 trafficking occurs in both adipocytes and muscle. Suppression of PIKfyve activity reduces insulin induced Glut4 translocation in cultured adipocytes (reviewed in [54]). PIKfyve is also required for Glut4 trafficking in animals; a muscle-specific knock-out of *PIKfyve* in mice causes a defects in Glut4 translocation and glucose uptake [86]. The precise role(s) of PIKfyve in Glut4 translocation are not known.

Insulin stimulation regulates PIKfyve activity [41,46,87], in part by Akt, which phosphorylates PIKfyve on serine 318 [87]. Moreover, EGF stimulation, which promotes EGFR internalization and degradation, also induces Akt phosphorylation of PIKfyve on serine 318 [83]. However, in cells the degree of activation of PIKfyve due to phosphorylation of serine 318 is relatively modest. Thus, there are may be additional Akt phosphorylation sites on PIKfyve, as well as additional PIKfyve activators.

One outcome of insulin activation of PIKfyve in adipocytes is an effect on mTOR, a major regulator of cell metabolism. Insulin-induced translocation of mTOR to the plasma membrane, as well as mTOR activity, requires PIKfyve [41]. The recruitment of mTOR to the plasma membrane in response to insulin may occur through direct interactions with $PI(3,5)P_2$. However, whether $PI(3,5)P_2$ is found on the plasma membrane of adipocytes is not known.

Fab1/PIKfyve is required for autophagy in metazoans—Autophagy requires Fab1/PIKfyve. Autophagy delivers cargoes to the lysosome for degradation. Suppression of Fab1/PIKfyve results in impaired clearance of autophagic organelles. In *C. elegans*, mutations in PPK-3 (Fab1) cause an increase in autophagosomes [75]. Similarly, *Drosophila fab1* mutant larvae accumulate autophagosomes and amphisomes [88]. In NIH3T3 or HEK293 cells, and

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in primary cultured hippocampal neurons, inhibition of PIKfyve with YM201636 or MF4 causes an accumulation of autophagosomes and the autophagic marker, LC3-II [13,64,89]. Similarly, the brains of mice with mutations in Fig4 have elevated levels of LC3-II and p62, another marker of autophagy [32,53,69,90]. Together, the above studies indicate that PIKfyve has multiple roles in the endomembrane system.

Roles for lysosomal PI(3,5)P_2 in the regulation of transcription—Endosomal PI(3,5)P_2 may also regulate some transcriptional pathways. Expression of pheromone responsive genes in *S. pombe* is defective in a *fab1Δ* mutant [16]. Similarly, in *S. cerevisiae* PI(3,5)P_2 modulates transcription via interaction with Tup1 and Cti6 [56]. PI(3,5)P_2 provides a site on the yeast vacuole for assembly of the Tup1/Cyc8/Cti6 transcription complex. These findings predict that PI(3,5)P_2 on lysosomes may regulate additional transcription pathways.

Fab1/PIKfyve may function at the plasma membrane—In addition to multiple functions on endosomes, a small pool of Fab1/PIKfyve may function at or near the plasma membrane. In adipocytes, PIKfyve activity may contribute to localization of mTORC1 to the plasma membrane. Furthermore, PIKfyve has been implicated in phagocytosis and pinocytosis [91,92]. Further evidence for a potential role for Fab1/PIKfyve at the plasma membrane comes from the *Physcomitrella patens* class II formins, which bind PI(3,5)P_2 [60] and require Fab1 activity for their localization at the cell cortex. PIKfyve has also been implicated in actin remodeling in mammalian cells (reviewed in [54]). In addition, when expressed heterologously in *Xenopus* oocytes, several plasma membrane localized ion channels and carrier proteins require PIKfyve activity (reviewed in [54,82,93]). Thus, while most PIKfyve is associated with endosomal membranes, PIKfyve may also have roles at the plasma membrane.

Fab1/PIKfyve, Vac14, and Fig4 IN PLANT AND ANIMAL PHYSIOLOGY

PIKfyve plays critical roles in development. Knockout of *PIKfyve* in mice results in very early lethality: *PIKfyve*$^{-/-}$ embryos did not survive past E3.5 [14] and, in an independent knock-out, embryos did not survive past E8.5 [18]. Similarly, *Drosophila fab1* and *C. elegans* (*ppk*-3) mutants display early lethality [49,75]. In *Arabidopsis thaliana* the two *Fab1* genes, *FAB1A* and *FAB1B*, play critical roles in development [66,67]}, perhaps due in part to hyposensitivity to auxin signaling [94].

Analysis of a *PIKfyve*$^{\beta geo/\beta geo}$ hypomorphic mutant mouse with partial PIKfyve activity, which dies perinatally, has revealed post-development roles of PI(3,5)P_2 and PI5P in animal physiology [6]. Similarly, *Vac14*$^{-/-}$ mutant mice, which also have less Fig4 protein [6,32], die perinatally [42]. Fig4$^{-/-}$ mice can live up to 6 weeks [22]. *Vac14*$^{Ingls/Ingls}$, a missense mutation that disrupts binding of Vac14 with PIKfyve, survives up to 3 weeks [26]. Differences in lethality may be largely due to differences in strain background. Early lethality is rescued in Fig4$^{-/-}$ mice by neuronal-specific, but not astrocyte-specific, expression of Fig4 [95]. Thus, loss of PI(3,5)P_2 and PI5P in neurons likely contributes to early lethality of the Fig4$^{-/-}$ mice and other PIKfyve-related mouse models.

Multiple tissues require PIKfyve

Vac14, Fig4 and *PIKfyve* are expressed globally. Accordingly, defects in the corresponding mouse mutants occur in multiple tissues. Hearts of the *Vac14*$^{-/-}$, Fig4$^{-/-}$ and *PIKfyve* hypomorph mutants have vacuoles [6] and in the two latter mutants, there is a spongiform-like phenotype in the spleen as well. Moreover, the lungs and kidneys of the *PIKfyve* hypomorph have a spongiform-like appearance. Conditional knock-out of *PIKfyve*

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(*PIPKIII*), in intestinal cells, causes vacuole formation and defects in membrane trafficking in the gut epithelia, which ultimately lead to early lethality [18].

Vac14, PIKfyve and Fig4 proteins are most abundant in the nervous system, which fits with findings that the nervous system is profoundly affected in the corresponding mutant animals [6,44]. Fig4$^{-/-}$, *Vac14*$^{-/-}$ and *Vac14*$^{Ingls/Ingls}$ mice display degeneration of the brain, including enlarged ventricles, increased apoptosis and severe spongiform encephalopathy; large vacuoles in the cell bodies of neurons are also observed in the peripheral nervous system [22,26,42]. The *PIKfyve* hypomorph has similar defects [6]. Consistent with the importance of PI(3,5)P_2 and PI5P in the nervous system, a mouse with a neuron-specific knock-out of *Vps34*, displays juvenile lethality and neurodegeneration, and has reduced PI3P and PI(3,5)P_2. PI5P was not measured [96].

Myelination is reduced in the central and peripheral nerves of Fig4$^{-/-}$ mice [22,95,97]. Fig4 may be particularly abundant during development of myelinating cells and dorsal root ganglia sensory neurons [98], although Fig4$^{-/-}$ controls, which would indicate whether the antigen detected by the anti-Fig4 antibody was bona fide Fig4, were missing. Interestingly, hypomyelination in Fig4$^{-/-}$ mice is rescued by neuron-specific expression of Fig4 [97]. Heterozygous Fig4$^{+/-}$ mice show no signs of neurodegeneration or increased susceptibility to trauma induced degeneration [99]. *Mtmr2*$^{-/-}$ Fig4$^{-/-}$ double mutant mice have more severe hypomyelination and neurodegeneration, which suggest that loss of PI5P contributes to these phenotypes [53].

PIKfyve in neurons

Vac14, PIKfyve and Fig4 have specialized roles at the synapse. AMPA-type glutamate receptors, which mediate fast neurotransmission in the brain, cycle between endosomes and the plasma membrane. Notably, trafficking of the AMPA receptor subunits, GluA1 and GluA2, are modulated by the PIKfyve complex. shRNA silencing of PIKfyve impairs trafficking of GFP-HA-GluA2 [100], and addition of PI(3,5)P_2 promotes trafficking of heterologously expressed GluA1 [101]. In *Vac14*$^{-/-}$ cultured hippocampal neurons, GluA1 and GluA2 are increased on the plasma membrane with a concomitant increase in postsynaptic strength [44]. Similarly, in cultured cortical neurons, internalization and degradation of the L-type voltage-gated calcium channel subunit, $Ca_V1.2$, requires PIKfyve [100].

In addition to postsynaptic defects, *Vac14*$^{-/-}$ neurons also displayed an increased probability of presynaptic vesicle fusion [44]. Similarly, PIKfyve is a negative regulator of calcium-dependent exocytosis in neurosecretory cells [102]. Together, PIKfyve and potentially PI(3,5)P_2, PI5P or both negatively regulate the excitatory response of neurons, which may explain why defects in the PIKfyve complex are linked to excitotoxic neuronal death.

Further determination of roles for PI(3,5)P_2 and PI5P signaling at the synapse will likely come from identification of proteins that binds these lipids and/or interact with the Vac14, PIKfyve, or Fig4. Potential candidates include clavesin and nitric oxide synthase (nNOS). Clavesin (clathrin vesicle-associated Sec14 protein), is expressed solely in the brain and binds PI(3,5)P_2. Knockdown of clavesin causes enlarged late-endosomes/lysosomes similar to those seen with suppression of PIKfyve activity [59]. nNOS, which functions at the synapse in the regulation of neurotransmission, binds Vac14 through a PDZ domain *in vitro* [103]; a functional interaction between Vac14 and nNOS at the synapse has not been tested.

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MUTATIONS IN GENES THAT ENCODE THE VAC14, PIKFYVE, AND FIG4 COMPLEX ASSOCIATED WITH HUMAN DISEASES

Mutations in FIG4 underlie a severe form Charcot Marie-Tooth (CMT) type 4J [22]. In CMT, progressive deterioration of nerves and/or demyelination throughout the peripheral nervous system results in reduced nerve conduction velocity and sensory sensation. These defects overlap with those observed in the Fig4$^{-/-}$ mouse. The most common genotype in CMT4J patients is FIG4 compound heterozygosity: one null allele and the other encoding the missense mutation, isoleucine 41 to threonine (I41T) [22]. The mutation retains partial function. In Fig4$^{-/-}$ mice, overexpression of a Fig4-I41T transgene significantly suppresses the early lethality [22,32]. That Fig4-I14T has a modest functional defect, yet causes peripheral neuropathy, underscores the importance of precise modulation of PI(3,5)P_2 and/or PI5P levels in the nervous system.

CMT4B1 and CMT4B2 are caused by loss-of-function mutations in MTMR2 and MTMR13 respectively (reviewed in [104]) and have clinical symptoms that overlap with those observed in CMT4J. The clinical symptoms in common between CMT4B and CMT4J may be due to either less PI5P or elevated PI3P.

A range of mutations in Fig4 were found in 7 out of 473 patients with ALS and 2 patients with PLS [105]. Mutations in Fig4 may be causative in other neurological diseases as well. Moreover, mutations in Fig4 can cause defects in additional tissues. Homozygous null mutations in Fig4 cause Yunis-Varón syndrome, a severe autosomal-recessive congenital disorder, which affects multiple tissues, including the heart, skeletal muscle, skeleton and brain [106]. The diversity of affected tissues underscores the importance of the Vac14/PIKfyve/Fig4 complex in human physiology.

To date, neither *PIKfyve* nor *Vac14* have been linked to neurological disease. Heterozygous null mutations in *PIKfyve* are associated with Francois-Mouchetee Fleck Corneal Dystrophy (CFD) [107], which results in white flecks throughout the corneal stroma of the eye that do not affect vision. Corneal flecks are thought to be enlarged vacuoles in swollen keratocytes [108]. Interestingly, Vac14 mRNA is down-regulated in a large subset of patients with chronic fatigue syndrome [109]. Based on the common molecular functions of PIKfyve, Vac14 and Fig4, it is tempting to speculate that mutations in *PIKfyve* and *Vac14* will be discovered that are linked to neurological disorders.

Conclusions

The roles and regulation of PI(3,5)P_2 parallel those of other PIP species. Notably, PI3P is a precursor for PI(3,5)P_2, which in turn is a precursor for PI5P. The interconversion between these lipids predicts that there are pathways where these lipids spatially and temporally control multi-step pathways.

Compared with PI3P, PI4P and PI(4,5)P_2, the levels of PI(3,5)P_2 are exceedingly low. The difficulty of measuring the low levels of PI(3,5)P_2 in cells, and the lack of a fluorescent probe to monitor its spatial and temporal dynamics have provided major hurdles towards elucidating the roles and regulation of PI(3,5)P_2. A more complete picture of the pathways that rely on PI(3,5)P_2 and PI5P will likely provide insights into how minor defects in the regulation of these lipids leads to profound human diseases. Recent observations that mutations in Fig4 cause defects with striking similarities to lysosomal storage disorders may also provide insight into the links between these lipids and disease [110]. Moreover, as whole exome sequencing of patients becomes feasible, more diseases linked to this pathway will likely be discovered. The severity of CMT4J, ALS and Yunis-Varon syndrome

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underscores the importance of uncovering the molecular mechanisms that regulate the Vac14/PIKfyve/Fig4 complex, as well as the discovery of new cellular pathways that are regulated by $PI(3,5)P_2$ and PI5P.

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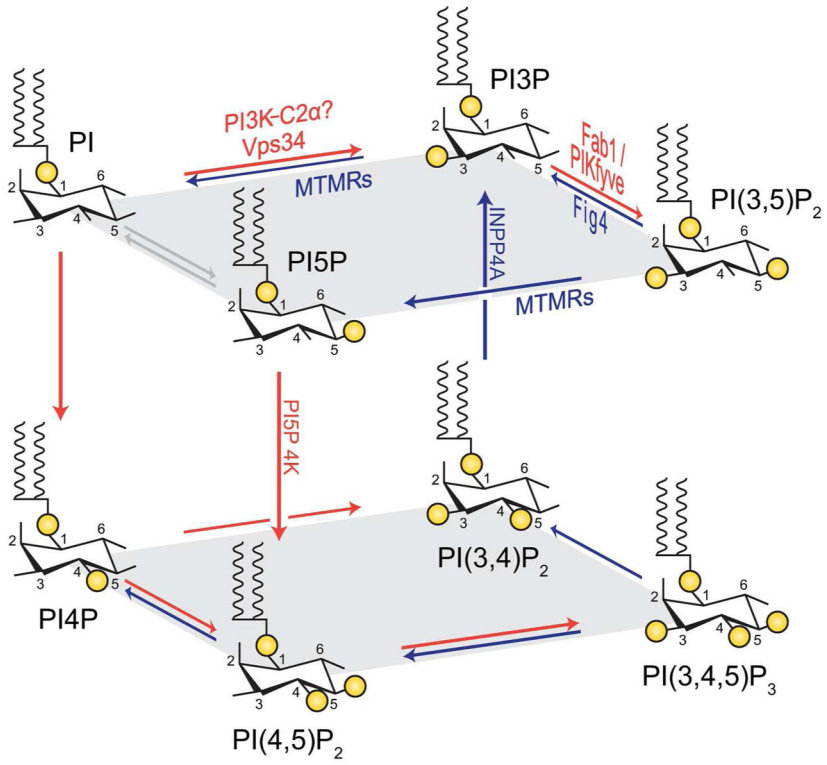

Figure 1.

Interconversion among the seven known phosphoinositide lipids occurs via action of specific lipid kinases (red arrows) and phosphatases (blue arrows). Selected kinases and phosphatases are shown. While controversial, direct conversion of PI to PI5P via PIKfyve activity may contribute to the PI5P pool (gray arrows). INPP4A phosphatase, which causes neurodegeneration in mice [118], and the type II PI5P 4-kinase [119,120], which has a role in the regulation of PI5P levels, were not discussed in this review.

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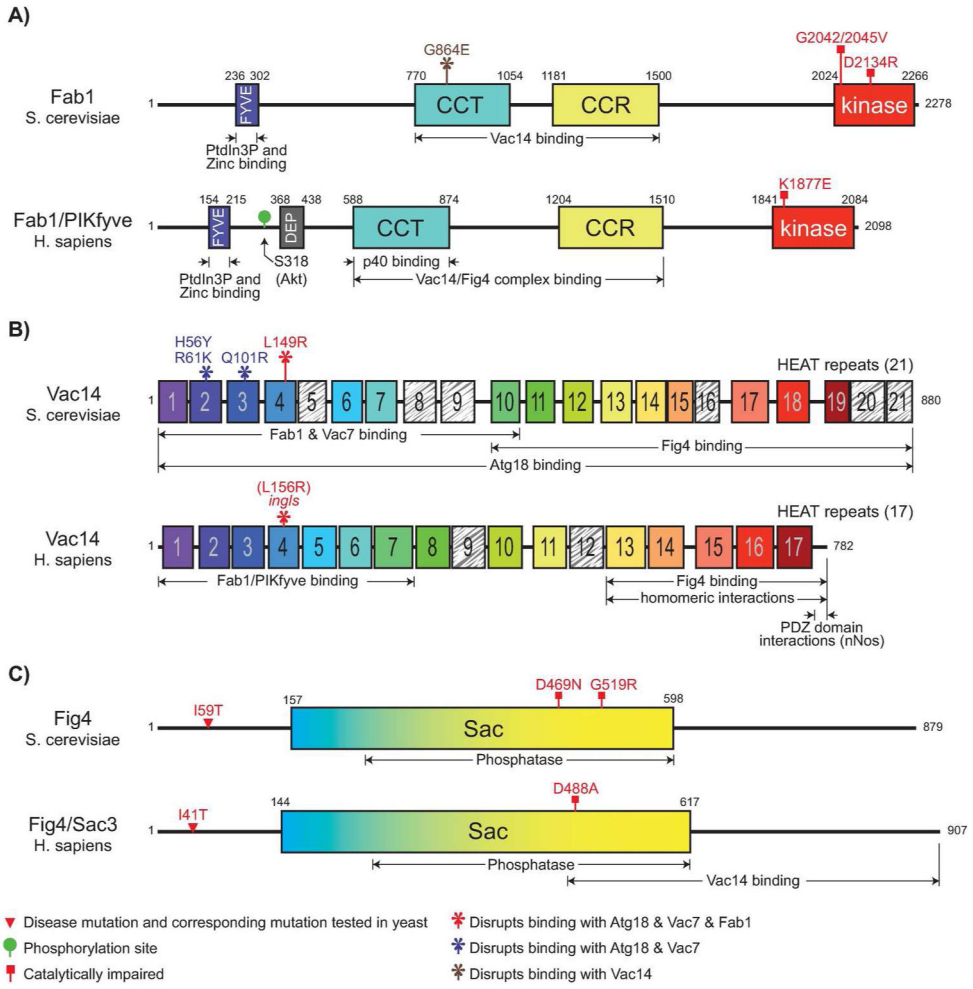

Figure 2.

Fab1/PIKfyve, Vac14 and Fig4 are conserved in most eukaryotes. Domains of *S. cerevisiae* and human Fab1/PIKfyve, Vac14 and Fig4/Sac3 are shown. **A:** Fab1 domains include FYVE (binds PI3P), DEP (unknown function; present in chordate and insect Fab1), CCT (homologous to the chaperone Cpn60/TCP-1 family; mediates interactions with Vac14), CCR (a conserved cysteine rich domain found only in Fab1/PIKfyve; part of the Vac14 binding region), kinase (catalytic site for conversion of PI3P to PI(3,5)P_2). **B:** Vac14 is composed of tandem HEAT repeats, which are rod-like helical structures that mediate protein-protein interactions. **C:** Fig4 contains a Sac domain, which is a module found in several lipid phosphatases. Note, the number of amino acids in mouse PIKfyve and human PIKfyve are not identical. The catalytically impaired mutation in mouse PIKfyve, K1831E, is indicated on the schematic of human PIKfyve, K1877E. The boundaries for FYVE, CCT, CCR, kinase, and Sac domains were identified as follows: 1) conserved in multiple sequence alignments and 2) contained unbroken secondary structure elements predicted by the program Jpred. Sequences for Fab1/PIKfyve were from the following species: *Saccharomyces cerevisiae* (budding yeast, NP_116674), *Schizosaccharomyces pombe* (fission yeast, NP_596090), *Candida albicans* (human pathogen, CAC42810), *Ashbya gossypii* (cotton pathogen, NP_985045), *Arabidopsis thaliana* (plant, NP_001078484), *Drosophila melanogaster* (fly, NP_611269), *Apis mellifera* (honey bee, XP_393666), *Anopheles gambiae* (mosquito, XP_314118), *Caenorhabditis elegans* (worm, CAA19436),

and *Homo sapiens* (human, NP_055855). The Sac domain in Fig4 was defined through alignment of the following Sac domain proteins in *S. cerevisiae*: Inp51, Inp52, Inp53, Sac1 and Fig4.

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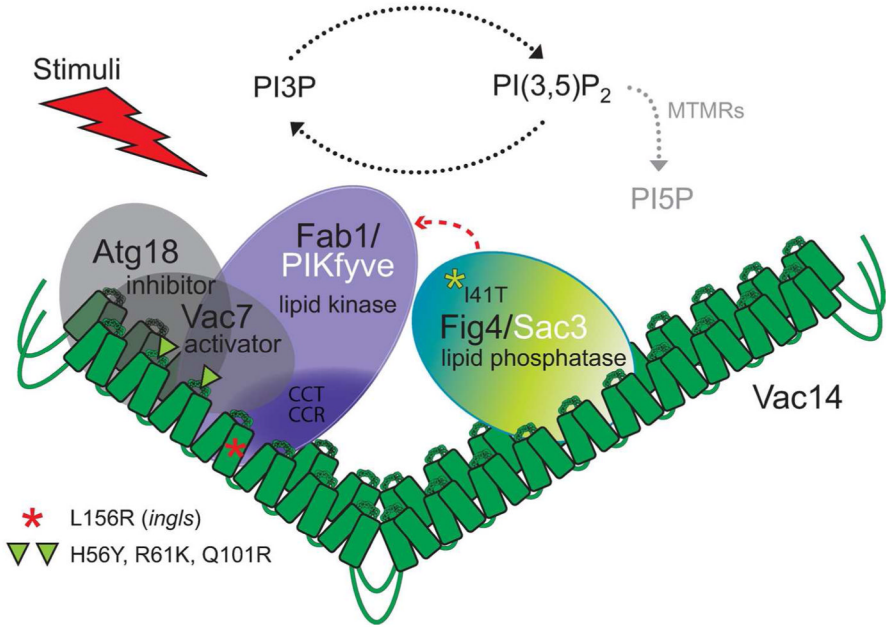

Figure 3.

Schematic of the Fab1/PIKfyve, Vac14, Fig4/Sac3 complex. Vac14 oligomerizes with itself and nucleates the complex through direct interactions with Fab1/PIKfyve and Fig4/Sac3. In yeast, Vac14 also directly interacts with Atg18 and Vac7. The yeast Vac14 point mutants, H56Y (HEAT repeat loop 2), R61K (HEAT repeat loop 2) and Q101R (HEAT repeats loop 3), each disrupt binding of Atg18 and Vac7. Thus, Atg18 and Vac7 may bind overlapping or identical sites of Vac14 [26]. The Vac14-L156R mutation, found in *ingls* mice, and corresponding mutation Vac14-L149R in yeast, disrupts Vac14 interaction with Atg18, Vac7 and Fab1. This suggests that all three proteins bind overlapping sites on Vac14. The point mutation, Fig4-I41T found in patients with CMT4J, disrupts the interaction between Fig4 and Vac14, although the major portion of human Fig4 that interacts with Vac14 resides within residues 478–907 [28]. In mammalian cells, myotubularin related proteins (MTMRs) can convert $PI(3,5)P_2$ to PI5P and may provide the majority of cellular PI5P.

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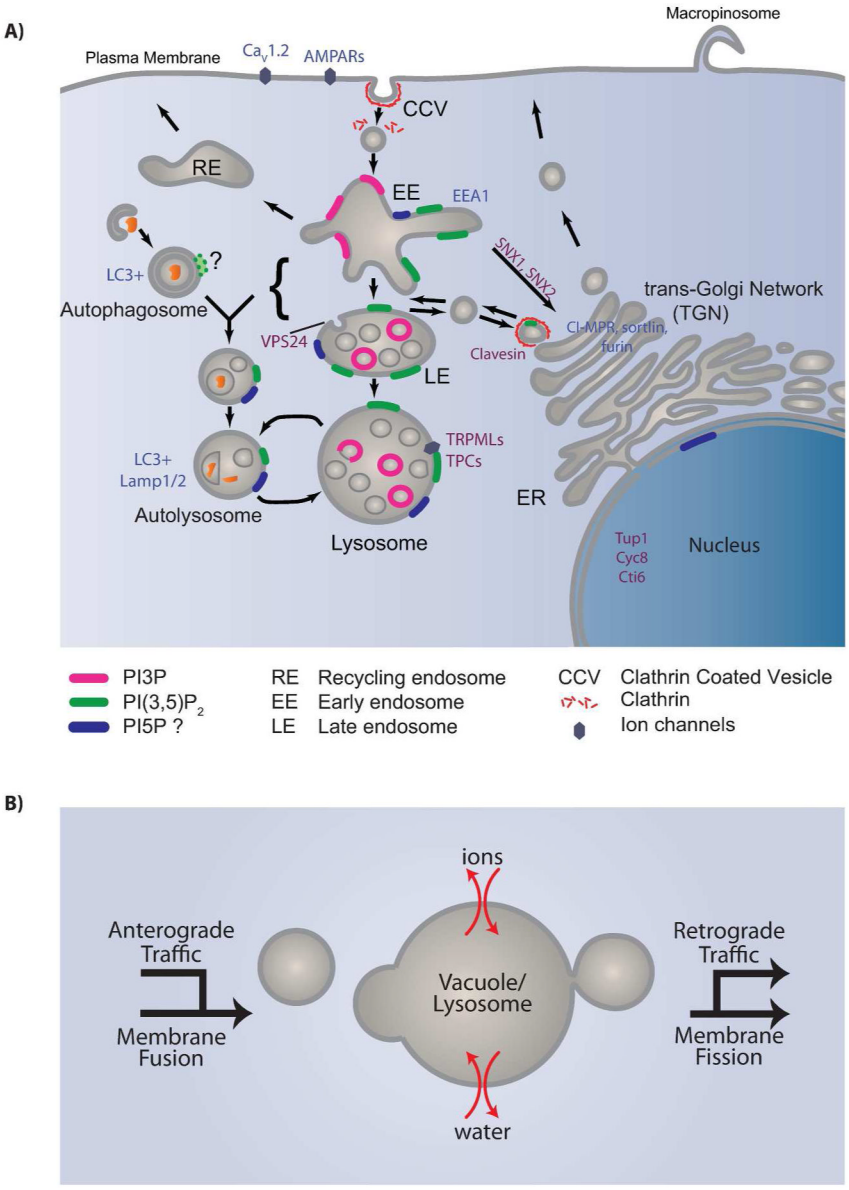

Figure 4.

A: Localization of $PI(3,5)P_2$ and PI5P inferred from the localization of PIKfyve and Vac14. $PI(3,5)P_2$ localizes on early endosomes, late endosomes and lysosomes. Localization of $PI(3,5)P_2$ on autophagosomes is less clear. PI5P may also be present at all or some of these locations. To further establish the locations of these lipids, suitable lipid probes need to be developed. $PI(3,5)P_2$ effectors and trafficking pathways affected in PIKfyve/Vac14/Fig4 deficient cells are also indicated. Purple: known $PI(3,5)P_2$ effectors. Blue: proteins affected by $PI(3,5)P_2$ and/or PI5P. **B:** The size of a yeast vacuole or mammalian lysosome is dependent on ion and water homeostasis, as well as the net sum of anterograde traffic, retrograde traffic, membrane fusion and membrane fission.

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Table 1

Pathways regulated by Fab1/PIKfyve, Vac14, and Fig4 complex

Pathways affected	Known PI(3,5)P_2 Effectors	Species and reference
PI5P biogenesis	MTMRs† [6,111]	Cell culture [6,111]
Vacuole fission	Atg18 [37]	*S. cerevisiae* [33,37]
Retrograde traffic from the vacuole	Atg18 [37]	*S. cerevisiae* [37,79]
Acidification of vacuoles or endolysosomes		*S. cerevisiae* [8],[33]; *C. elegans* [75]; *D. melanogaster* [49]; *A. thaliana* [66];
Ion channel function	TRPML1, TRPML2, TRPML3 [61]; TPC1, TPC2, TPC3 [72]; RyR1, RyR2 [62,73,112]	Cell culture and mouse [61,62,72,73,112]
Autophagy in metazoans		*C. elegans* [75]; *D. melanogaster* [88]; Cell culture [13,64]; Mouse [32,53,69,90]
Fluid phase endocytosis		Cell culture [13,42,113]; *D. melanogaster* [49]
Sorting of cargoes at the MVB	Vps24† [81]	*S. cerevisiae* (Reviewed in [82])
Traffic of cell surface receptors to lysosomes		Cell culture [13]; D. melanogaster [49]
Endosome-to-TGN traffic	SNX1, SNX2 [58]	Cell culture [13,42,48,58,85]
Glut4 translocation in response to insulin		3T3 adipocytes (Reviewed in [54])
mTORC1 translocation to the plasma membrane in response to insulin	Raptor [41]	3T3 adipocytes [41];
Cortical actin array dynamics	Formins [60]	*P. patens* [60]
GAL1 induction in the absence of GAL4 pathway	Tup1, Cti6 [56]	*S. cerevisiae* [56]
AMPA receptor and $Ca_v1.2$ trafficking		Neuron culture [44,100,101]
Exocytosis		Neuron culture [44,102]

† Potential binding of Vps24 [114] and MTMRs [115] are controversial.

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Table 2

Phenotypes of PI(3,5)P_2 deficiency in model organisms.

Phenotypes	Species and reference
Enlarged endosomes and lysosomes	*S. cerevisiae* [1,8,9,33]; *S. pombe* [16]; *C. albicans* [74]; Cell culture [6,13,22,42,48,64,68]; *C. elegans* [75]; *D. melanogaster* [49]; *A. thaliana* [67]
Sensitivity to heat and calcium	*S. cerevisiae* [8]; *S. pombe* [16]
Secretion and response to mating hormones	*S. pombe* [16]
Hyphae formation on solid medium	*C. albicans* [74]
Inviable pollen, slow root growth, curled leaves, dwarfed plants, abnormal flowers	*A. thaliana* [66,67]
Stunted growth	*P. patens* [60]
Early lethality	*C. elegans* [75]; *D. melanogaster* [49]; mouse [6,14,22,26,42]
Dilute coat color	Mouse [22,26]
Neurodegeneration	Mouse [6,22,26,42,53,90,97,116,117]
Increased pre- and post-synaptic strength	Neuron culture [44]
Myelination defects	Mouse [53,97,116,117]
Abnormalities in heart, lung, kidney, thymus, and spleen	Mouse [6,22]

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Table 3

Human Disease

Disease	Affected Gene	Reference
Charcot Marie-Tooth type 4J (CMT4J)	Fig4 I41T* Fig4 L17P*	[22,105,116]
Amyotrophic lateral sclerosis (ALS)	Mutations in Fig4	[105]
Primary lateral sclerosis (PLS)	Mutations in Fig4	[105]
Yunis–Varon syndrome	Mutations in Fig4	[106]
Charcot Marie-Tooth type 4B1	Mutations in MTMR2	Reviewed in [104]
Charcot Marie-Tooth type 4B2	Mutations in MTMR13	Reviewed in [104]
Francois-Mouchetee Fleck Corneal Dystrophy	Mutations in PIKfyve	[107,108]
Chronic Fatigue Syndrome	Down regulation of Vac14	[109]

* Patients are compound heterozygotes with a null allele of Fig4 and Fig4-I41T, or Fig4-L17P.