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REVIEW ARTICLE

# Function of the phosphatidylinositol transfer protein gene family: is phosphatidylinositol transfer the mechanism of action?

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**Abstract**

Phosphatidylinositol transfer proteins (PITPs) bind and facilitate the transport of phosphatidylinositol (PI) and phosphatidylcholine between membrane compartments. They are highly conserved proteins, are found in both unicellular and multicellular organisms, and can be present as a single domain or as part of a larger, multi-domain protein. The hallmark of PITP proteins is their ability to sequester PI in their hydrophobic pocket. Ablation or knockdown of specific isoforms *in vivo* has wide ranging effects such as defects in signal transduction via phospholipase C and phosphoinositide 3-kinase, membrane trafficking, stem cell viability, *Drosophila* phototransduction, neurite outgrowth, and cytokinesis. In this review, we identify the common mechanism underlying each of these phenotypes as the cooperation between PITP proteins and lipid kinases through the provision of PI for phosphorylation. We propose that recruitment and concentration of PITP proteins at specific membrane sites are required for PITP proteins to execute their function rather than lipid transfer.

**Keywords:** *Golgi; cytokinesis; membrane traffic; phospholipase C; phosphatidylinositol 4-phosphate; PI 3-kinase; PI 4-kinase; phosphoinositides; PITP; neurodegeneration*



## Introduction

Lipids are the major constituent of biological membranes and play fundamental regulatory roles in signal transduction and membrane traffic. Lipids can be moved between organelles either as part of vesicles budding from one membrane and fusing with another, or by the action of proteins that can bind and transport lipids. Lipid transport proteins (LTPs) specific for different hydrophobic ligands have been identified. There are several families of LTPs in the human genome that are grouped by sequence and structural similarity of their lipid-binding domain, including PITP domain proteins (five members), START domain proteins (15 members), glycolipid transfer proteins (GLTP) (two members), NSL-TP (five members), CRAL_TRIO domain proteins (~29 members), and oxysterol-binding proteins (OSBP) (12 members) (Cockcroft, 2007a). Studies of the function of these proteins reveal that LTPs integrate lipid metabolism and homeostasis with membrane traffic and cell signaling in a complex manner. Here, we specifically focus on the function of the PITP family.

The phrase "phosphatidylinositol transfer protein" (PITP) was coined to describe a biochemical activity that facilitates the transport of one phospholipid molecule, phosphatidylinositol (PI), or phosphatidylcholine (PC), between membrane compartments *in vitro*. The first mammalian PITP protein was purified in 1974 from brain cytosol and the isolated protein was found to be present as two species that could be distinguished on the basis of their isoelectric point. The two species were demonstrated to be due to the presence of either PI or PC bound to the protein and the two species could be interconverted when incubated with appropriate lipid vesicles (Helmkamp et al., 1974; Van Paridon et al., 1987). PITP

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was subsequently cloned in 1989 (Dickeson et al., 1989). The first PITP was a soluble protein, 271 amino acid (aa) residues in length with a calculated molecular weight of 32 kDa. Since these initial observations, the number of PITP proteins has expanded and structural studies provide the first details regarding the architecture of the PITP domain. The crystal structures of PITPs bound to their phospholipid cargo as well as their apo-form devoid of cargo have provided a molecular understanding of how PITPs can bind and transfer lipids. The biological actions of PITP proteins have been started to be unraveled through the use of model organisms including mice, flies, and zebra fish, as well as cultured cell lines. The cellular function of these proteins is increasingly being linked to cell signaling and membrane trafficking. In this review, we discuss the PITP family-examining members both in mammals and in other organisms including flies and fish to appraise the scope and biological function of these PITPs whose hallmark is PI binding. The reader is directed to several excellent reviews that provide alternative proposals on PITP/RdgB function (Wirtz, 1991, 1997; Hsuan and Cockcroft; 2001; Allen-Baume et al., 2002; Lev, 2004. Phillips et al., 2006b; Bankaitis et al., 2007; Cockcroft, 2007b. Cockcroft and Carvou, 2007; Trivedi and Padinjat, 2007).

## Structure of PITP domain and molecular mechanism of docking to membranes

In humans, proteins containing the PITP domain are encoded by five genes; two genes give rise to splice variants increasing the total number to seven (Figure 1). Based on phylogenetic analysis, the proteins are grouped as Class I PITPs, which contain PITPα and PITPβ, and Class II PITPs which contain the RdgB proteins (Allen-Baume et al., 2002). Two of the genes code for single domain proteins (Class I: PITPα and PITPβ) whose crystal structures have been solved. They reveal a hydrophobic pocket that can accommodate a single phospholipid molecule, either PI or PC (Figure 2). PITPα and PITPβ share 77% sequence

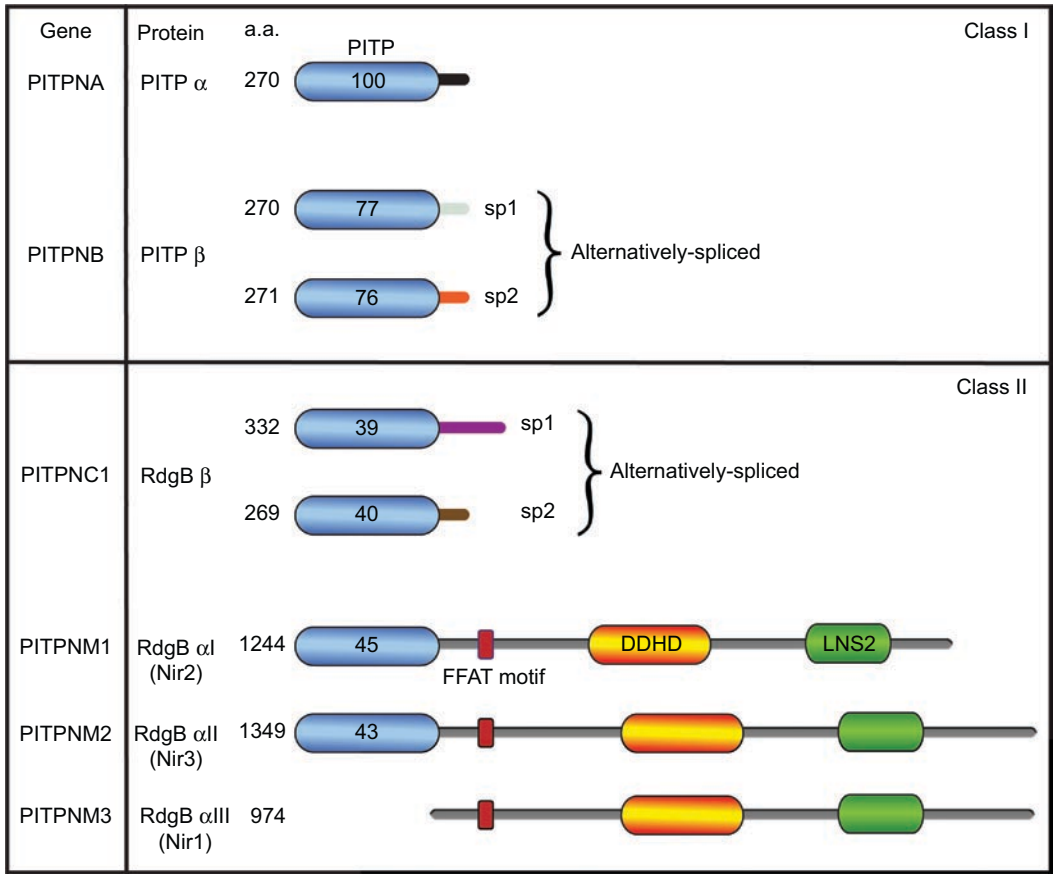


**Figure 1.** Structural organization of the human PITP family. Human PITP family members are arranged into two classes. Alternative nomenclature is indicated in parentheses next to each protein. The length of each protein in amino acids (aa) is given to the left of the PITP domain; the number inside the blue PITP domain indicates the percentage sequence identity of that domain in comparison with PITPα. Splice variants that have different C-terminal tails are indicated by different colors. Individual domains are color-coded: blue, PITP domain; red, FFAT motif; orange–yellow, DDHD domain; green, LNS2 domain.

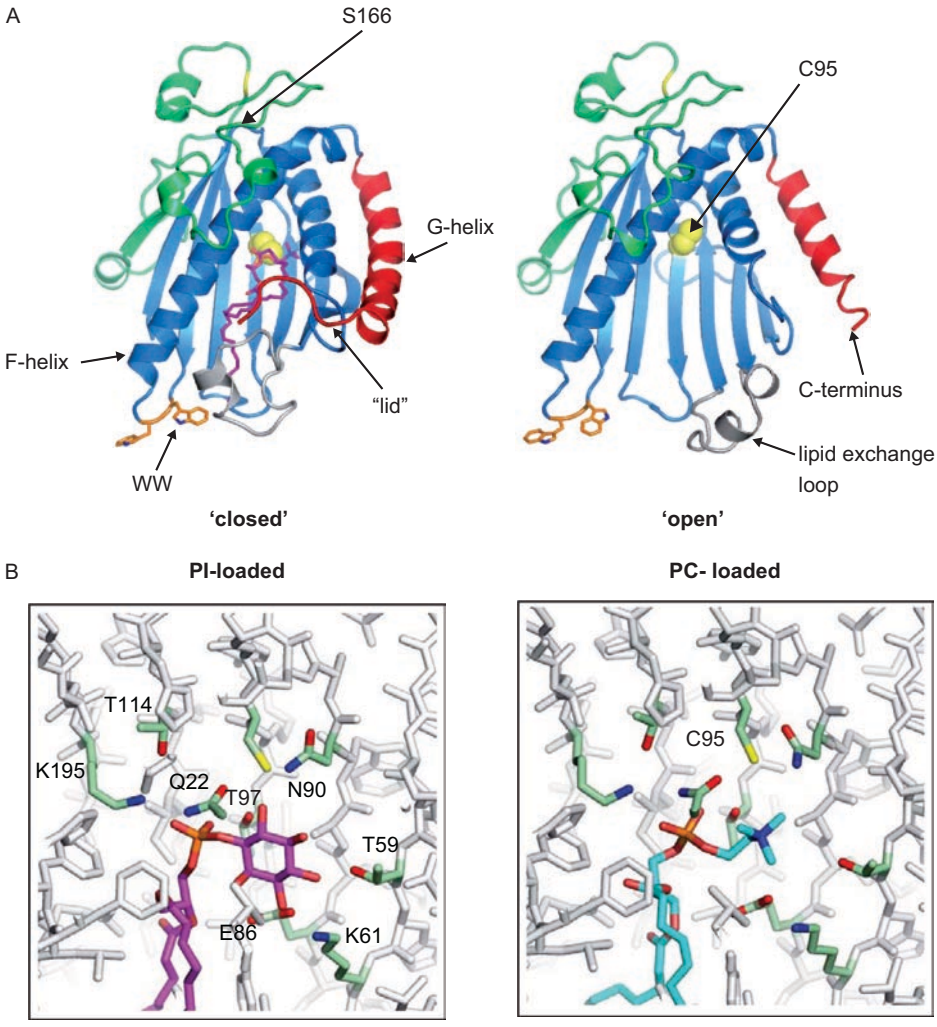


**Figure 2.** Open and closed forms of PITPα and the lipid-binding site in the cavity. (A) Comparison of the lipid-loaded "closed" conformation with the lipid-free "open" form of PITPα. The lipid-binding core residues are colored in blue, the G-helix and the extended 11 amino acids at the C-terminus that form the lid are colored in red, the regulatory loop is colored in green, and the lipid-exchange loop is colored in gray. In the lipid-free "open" form, the lipid-exchange loop and the G-helix have moved, and the C-terminal region is disordered. The diagrams were generated using PYMOL software with PDB files 1t27 and 1kcm. (B) A stick model showing PI (magenta carbon atoms) (left panel) and PC (blue carbon atoms) (right panel) loaded in the lipid-binding pocket. The four residues that make contact with the phosphate moiety of the phospholipid, Q22, T97, T114, and K195 are labeled, as are the four residues that make contact with the inositol head group, K61, N90, T59, and E86. The location of C95 (green carbon atoms and yellow sulfur atom) is also shown.

identity, although the three other members (Class II RdgB family) are more divergent and share ~40% sequence identity (Figure 1).

The lipid-binding cavity consists of eight β-strands that form a large concave sheet flanked by two long α-helices (Figure 2A). This cavity is closed by a "lid" composed of a C-terminal α-helix (the G-helix) and an 11-aa extension. The phospholipid is buried within the protein with the polar head group interacting with aa residues located at the far end of the cavity away from the lid. The phospholipid has access to the membrane interface only when the lid is displaced. The sn-1 and sn-2 fatty acyl chains of the ligand occupy distinct sites with the methyl ends being close to the opening of the lipid-binding cavity. The protein is thus "closed" for transport through the aqueous compartments and has to "open" for lipid exchange.

The apo-structure devoid of ligand provides a glimpse of what may occur at the membrane interface (Schouten et al., 2002). In the lipid-free structure, the G-helix is dislodged (Figure 2A) such that the lipid-binding cavity is now facing the membrane interface. In addition, a loop containing helix B dubbed the "lipid-exchange loop" has swung out of position. Hydrophobic residues are exposed that could allow the protein to remain membrane-associated (Wirtz et al., 2006). Nonetheless, when cells are disrupted, PITP proteins rarely remain associated with membranes suggesting that the lipid-free form of PITP is a very transient species within cells.

To capture PITPs in the lipid-free form, cells can be treated with thiol-modifying reagents such as *N*-ethyl maleimide (NEM). NEM covalently links to Cys95 of Class I PITPs, which lies deep in the lipid-binding pocket (Figure 2B) preventing lipid binding due to steric hindrance (Shadan et al., 2008). NEM only has access to the PITP interior when it is in the process of exchanging its lipid cargo at the membrane: NEM literally "freezes" the protein in the act of lipid exchange. Treatment of cells with NEM traps a population of PITPα and PITPβ to membranes. In addition, the residual cytosolic population of PITPs has lost its transfer activity. Since NEM is unable to inhibit the PITP transfer activity if the proteins are treated in aqueous solution, it follows that the PITP proteins have been modified during a cycle of lipid exchange at the membrane and have subsequently been released into the cytosol. The inactive PITP proteins in the cytosol have been found to be dimerized, which can be rationalized if the modified proteins are unable to bind lipid and are trapped in the "open" conformation. In the "open" conformation, hydrophobic residues are exposed and therefore the proteins likely dimerized end-to-end to shield these residues (Tilley et al., 2004; Shadan et al., 2008). This is reminiscent of treatment of PITPα with subtilisin, a proteolytic enzyme that cleaves 24 aa residues at the C-terminus. Not only is the protein inactive for lipid transfer, but it is also now present as a dimer (Prosser et al., 1997).

Treatment of cells with NEM reveals that Class I PITP proteins are constantly interacting with the membrane interface to exchange their lipid cargo in cells, and it is remarkable how frequently they do this. For PITPβ, nearly all the molecules undergo a round of lipid exchange within a 2-min period. For PITPα, a small population of PITPα molecules does not experience interaction with the membrane by 10 min. Thus, we concluded that PITPα and PITPβ show differences in their propensity to interact with membranes.

How does the conformational change in PITPs take place when the protein approaches the membrane interface? Two tryptophan residues (WW203/204) are located at the tip of the loop that faces the membrane interface (Figure 2A), and mutation of these two residues to alanine leads to loss of membrane docking and lipid transfer (Tilley et al., 2004; Shadan et al., 2008). Two different *in vitro* assays have been used to assess PI transfer: one assay uses permeabilized HL60 cells as the donor membrane, whereas the second uses rat liver microsomes. Both use synthetic liposomes as the acceptor compartment. Using either assay PI transfer is considerably diminished for the WW mutant. In a third *in vitro* assay where support for phospholipase C activation is monitored (this assay is a measure of PI phosphorylation), the WW/AA mutants are unable to restore G-protein-stimulated phospholipase C signaling. Finally, in two *in vivo* assays, the WW/AA mutant is dysfunctional. First, when PITPα and PITPβ WW/AA mutants are expressed in cells, they are unable to be trapped onto membranes upon NEM treatment (Shadan et al., 2008). Second, the WW mutant of rat PITPβ is unable to rescue the defects in COPI-mediated retrograde traffic from the Golgi to the endoplasmic reticulum (ER) observed in PITPβ knockdown HeLa cells. Reexpression of wild-type PITPβ is able to reinstate Golgi–ER retrograde traffic (Carvou et al., 2010).

All these results combined clearly suggest that the two tryptophan residues are essential for PITP function and we proposed that they insert into the membranes. These residues are located at the end of helix F, which is adjacent to helix G (see Figure 2A). Insertion of the tryptophan residues into the membrane could indirectly perturb the helix G and consequently the C-terminal 11-aa residues. One or two tryptophan residues are conserved at these positions in nearly all members of the PITP family. Tryptophan side chains are known to have preference for membrane interactions due to their aromatic ring and molecular shape, and many membrane-active proteins use tryptophan residues for this purpose (Yau et al., 1998; Killian and von Heijne, 2000).

Two other studies have examined the importance of these two tryptophan residues and have reached a different conclusion: the WW mutant was as effective as wild-type PITP for rescue. In both these studies, the mutants were tested in a heterologous reconstitution system. In one study, the mutant was tested for its ability to rescue secretory function and PI(4)P levels in the temperature-sensitive mutant of Sec14p ($Sec14^{ts}$) in *Saccharomyces cerevisiae* (Phillips et al., 2006a). In yeast, mammalian PITPα and PITPβ have been found to rescue the $Sec14^{ts}$ mutant despite being a member of the CRAL_TRIO family of lipid transfer proteins with no sequence or structural similarity to PITPs. What Sec14p does share with mammalian PITPs is the ability to facilitate PI/PC transfer. In the second study, rat PITP was used to rescue zebrafish phenotypes (Ile et al., 2010). Injection of morpholinos into zebrafish embryos to knockdown PITPα causes early developmental arrest followed by inviability (Ile et al., 2010). Mammalian PITPα, but not PITPβ, rescues this phenotype. Additionally, the WW PITPα mutant was also examined and at 24 h postfertilization (hpf), only 7% of the embryos developed (8/108) compared with 26% (27/108) with the wild-type PITPα (values computed based on the data tabulated in Figure 9C in Ile et al., 2010). The authors concluded that the "WW mutant rescues the early developmental arrest of PITPα morphants with efficiencies similar to those recorded for ectopic PITPα expression." However, we would suggest that these data could be interpreted as the WW mutant is not as effective as the wild-type protein in effecting a rescue in zebrafish embryos.

## Binding of PI by PITP requires several conserved residues in the lipid-binding pocket

PITPα and PITPβ have dual specificity for ligand binding: they can bind either PI or PC (Figure 2B). PITPα has a 16-fold higher affinity for binding to PI than it does to PC (de Brouwer et al., 2002). PC accounts for ~40% of the cellular phospholipids compared with PI, which is 5–7% in most mammalian cells. Thus the higher affinity compensates for the amount of the individual lipid. In general, when PITPα has been purified from tissue, ~50% of the protein is loaded with PI and 50% with PC (Helmkamp et al., 1974; Thomas et al., 1993). PITPβ from rat liver cytosol, when subjected to separation by native isoelectric focusing and subsequently analyzed after western blotting with PITPβ antibodies, was also present as two species in roughly equal amounts, PI-loaded and PC-loaded (Morgan et al., 2006). The common feature shared by PI and PC is the glycerol backbone, the acyl chains, and the phosphate moiety. Comparison of the structures of PITPα complexed with either PC or PI reveals that both lipids occupy the same shape in the lipid-binding cavity—the backbones of both structures are able to be superimposed. In fact, the only difference is that the inositol headgroup makes more hydrogen bonds with specific aa residues than the choline moiety (Yoder et al., 2001; Tilley et al., 2004; Vordtriede et al., 2005).

Residues that are common in the recognition of both PI and PC are those making contact with the phosphate moiety of each lipid, namely Gln22 and Thr114, which form hydrogen bonds with the phosphate moiety via a water molecule and Thr97 and Lys195, which form direct hydrogen bonds to each of oxygen of the phosphate moiety (Figure 2B). Not surprisingly, these residues are conserved in the majority of the PITP sequences available.

Thr59 and Glu86 are part of the lipid headgroup-binding cavity and both interact with the headgroup of PI and PC, but in distinct ways. Thus mutation of these residues has effects on both PI and PC binding and transfer. More importantly, the effect of the mutation is dependent on which aa has been substituted. For example, mutation of Glu86 to Ala leads to a decrease in PI binding/transfer with no effects on PC binding/transfer. However, mutation to Gln causes a reduction in both PI and PC binding/transfer (Tilley et al., 2004).

Thr59 is an interesting residue and has been studied in great detail; not only is it part of the lipid headgroup-binding cavity, but it is also a consensus phosphorylation site for protein kinase C, and has been reported to be phosphorylated in PITPα in a minor capacity (Morgan et al., 2004). Thr59 would not normally be accessible for phosphorylation in the crystal structure of either PI- or PC-loaded PITPα. Mutation of this residue in PITPα to A, E, or S has different effects on lipid binding and transfer. Mutation of T59 to E, which is also a phospho-mimetic, causes complete loss of PI binding and transfer, although PC binding is unaffected. In contrast, mutation to alanine, which is also phospho-defective, diminishes both PI and PC transfer. Threonine and serine are chemically very similar and thus the hydrogen bonds between PITPα and the inositol head group could potentially still form. Nonetheless, mutation of Thr59 to Ser causes a significant decrease (70%) in PI binding compared with wild-type PITPα with increases in PC binding. However, PI transfer activity is only marginally reduced, although PC transfer activity is enhanced (Tilley et al., 2004). With this mutant, binding and transfer do not strictly mirror each other suggesting that these assays monitor different aspects of the protein's capability, although both assays are conducted in a similar manner. Both the binding and transfer assays make use of permeabilized HL60 cells where the endogenous PITPs have been removed. To measure lipid transfer, HL60 cells, prelabeled to near equilibrium with [$^3$H]inositol or [$^3$H]choline to measure PI or PC transfer, respectively, are exposed to exogenously added recombinant PITP proteins and liposomes. Lipid transfer from the cells to liposomes is monitored. To measure lipid binding, HL60 are grown in [$^{14}$C]acetate to label the cellular lipids to equilibrium and His-tagged PITP proteins are exposed to the permeabilized cells for lipid exchange to take place. The PITP proteins are recaptured and the lipid bound is identified by thin layer chromatography or by mass spectrometry (Ségui et al., 2002; Hunt et al., 2004; Tilley et al., 2004). PITPα and PITPβ only bind to PI and PC and to no other lipid in any significant amounts. PI and PC are a heterogeneous population of molecular species that can be distinguished by their acyl chains at the sn-1 and sn-2 position. PITPα has a defined acyl chain preference when presented with a total population of cellular lipids. The lipid bound by PITPα is enriched in species possessing shorter acyl chains with a preference order: 16:1>16:0>18:1>18:0 >20:4 (Hunt et al., 2004).

A separate study has also examined the mutation of threonine 59 in PITPα (Alb et al., 1995). This residue was mutated to A, S, E, V, D, N, and Q, and it was reported that all these mutants were unable to transfer PI, and only T59A retained 50% of its transfer ability. Threonine 59 is also conserved in *Drosophila* RdgBα and has been mutated (Milligan et al., 1997). In this case, T59A had no transfer activity, whereas T59E was shown to be wild-type, contrary to that observed for PITPα. These two studies preceded our knowledge of the PITPα structures and with this hindsight these results are surprising and should be reexamined. We

have recently reassessed the *Drosophila* RdgBα mutants and observed that T59E does not bind or transfer PI, although PC transfer is unaffected exactly as observed for PITPα. On the other hand, T59A retains partial binding and transfer for both PI and PC, similar to that observed for PITPα (Cockcroft, Garner, and Raghu, unpublished).

### *Residues important for PI binding*

Are there any residues that only affect PI binding and transfer with no affect on PC binding/transfer? Residues Lys61 and Asn90 are unique in that they only make specific contacts with the inositol ring. Mutations made in Lys61 and Asn90 to prevent formation of hydrogen bonds between the inositol ring of PI and PITPα or PITPβ confirm that these two residues are essential for only PI binding and transfer (Tilley et al., 2004; Carvou et al., 2010). PC binding and transfer is unaffected. In our opinion, these mutants are the most appropriate to use when examining the individual requirements for PI and PC transfer in biological functions.

### *Residues important for PC binding*

Mutants that affect PC transfer are harder to predict from the crystal structures. Cysteine95 is in the binding pocket and is in close proximity to the headgroup-binding site for both PI and PC (Figure 2). However, mutation of this residue to Thr or to Ala does not affect PI transfer but eliminates PC transfer (Carvou et al., 2010). Cys95 is conserved in Class I PITPα and PITPβ from all species but is not conserved in the Class II RdgB family. It is replaced with threonine in the majority of RdgBα and RdgBβ proteins from all species. Another locus that has been identified that plays a modest role in affecting PC transfer is Phe225. When the large aromatic residue is present at this locus, PC transfer activity is robust. When occupied by the small aliphatic residue, leucine, as found in rodent PITPβ, PC transfer is lower (Vordtriede et al., 2005). In human PITPα and PITPβ, this residue is phenylalanine. In comparison, in RdgBα this residue in an alanine and in RdgBβ it is a glycine. Thus from this analysis we predicted that the RdgB family have low PC transfer activity in comparison with the Class I PITPs. Not only is Cys95 replaced with Thr, but also Phe225 is replaced with a small aliphatic residue.

In summary, there are a number of residues that are essential for the lipid binding in the hydrophobic cavity, which can be grouped into three categories: (1) residues essential for binding the phosphate moiety (Q22, T97, T114, and K195), (2) residues essential for binding inositol (T59, N90, E86, and K61), and finally (3) residues important for PC binding (C95 and F225).

## Domain architecture and phylogenetic distribution of PITPs

PITPα and PITPβ consist of a single domain (IP_trans, PFAM: PF020121). However, the PITP domain can also be found in combination with other domains including the DDHD domain (PF02862), PH domain (PF00169), LNS2 domain (PF08235), oxysterol-binding domain (PF01237), and DUF547 domain (PF04784). Representative members of PITP proteins with these domains are shown in Figure 3. DUF547, LNS2, and DDHD domains are conserved in many proteins but are currently of unknown function. The PITP domain is occasionally broken by the presence of inserted residues. Despite this, these domains exhibit up to ~30% identity to PITPα.

PITP orthologs are broadly distributed across eukaryotic phyla. Proteins with a PITP domain have been found in both multicellular and unicellular organisms. Of the five eukaryotic supergroups—Excavates, Rhizaria, Unikonts, Chromalveolates, and Plantae (Embley and Martin, 2006; Koonin, 2010)—proteins with a PITP domain are present in at least four of the five supergroups (Figure 3C). Rhizaria is the only group in which PITP proteins have not been reported but there are no complete sequenced genomes as yet. In the Unikont group, which contains both animal and unicellular organisms, PITPs are present not only in Metazoans but also in Amoebozoa (e.g. *Dictyostelium*) and Fungi (e.g. microsporidia, the parasitic fungus, *Encephalitozoon cuniculi*). In the Plantae supergroup, which also contains both multicellular and unicellular organisms, PITP proteins are found in red algae, rice, castor bean, grape, and sorghum. PITP proteins are not found in the well-studied model organism *Arabidopsis thaliana*, however. The two other groups contain the free-living unicellular organisms. PITPs are present in *Giardia* (Diplomonad) and *Trichomonas vaginalis* (Parabasalids), part of the Excavate Group, and in *Plasmodium, Toxoplasmosis*, and *Theilera* (Apicomplexa) and in *Phytophthera ramorom* (Oomycetes), part of the chromalveolates group (Cockcroft and Carvou, 2007) (Figure 3C). Of note is the absence of this domain in the extensively studied yeasts, *S. cerevisiae* and *Schizosaccharomyces pombe*. The presence of PITPs in virtually all the supergroups indicates that PITP is an ancient gene and that its absence in many organisms such as yeast and *Arabidopsis* could be due to gene loss during evolution.

Conservation of the residues required for binding the phosphate moiety and inositol head group of PI is high amongst PITP domains from all species. An exception is the sea urchin PITPs. There are three sea urchin PITPs, all lacking Q22 and T115 (for binding the phosphate moiety) and T59 and E86 (for inositol and choline binding). Plant PITPs, in particular, castor bean, lack many of the

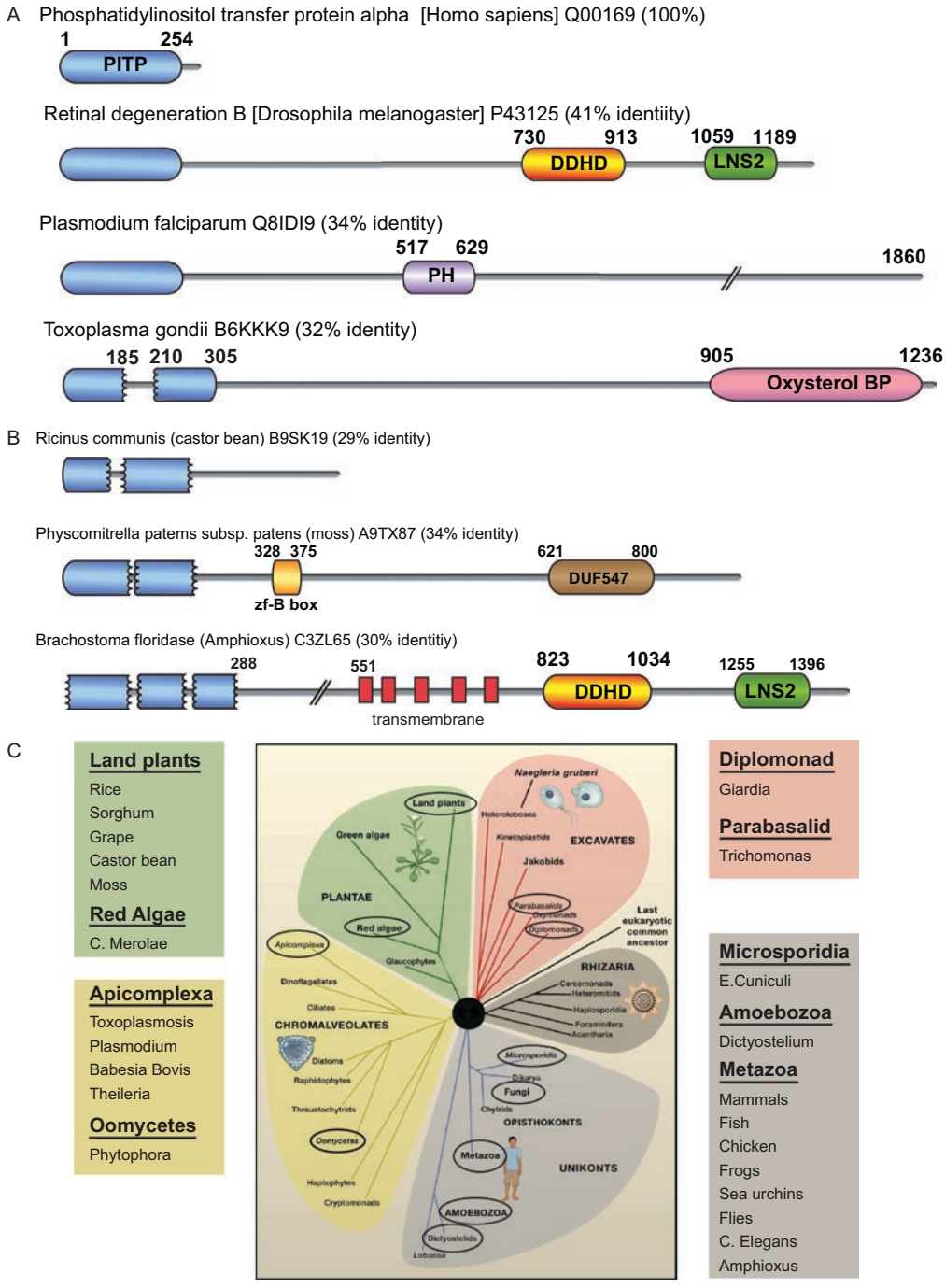


**Figure 3.** Representative proteins with a unique domain architecture in which the PITP domain is present. The PITP domain is present as a single domain protein and this represents the largest subset. The PITP domain is ~254 amino acids. DDHD domain (PF02862; IPR004177) is 180 residues long and contains four conserved residues that may form a metal-binding site. The domain is named after these four residues. This pattern of conservation of metal-binding residues is often seen in phosphoesterase domains and is also seen in a family of probable phospholipases. LNS2 domain (Lipin/Ned1/Smp2) is of unknown function found in phosphatidate phosphohydrolase (lipins) and many other proteins (PF08235; IPR013209). PH domain (PF00169; IPR001849) is 100 amino acid residues and can bind to phosphoinositides. Oxysterol_BP (PF01237) is a domain of 400 amino acids that can bind sterols. B-Box zinc finger (INTERPRO entry IPR000315) is 40 residues in length and is found in transcription factors, ribonucleoproteins and proto-oncoproteins. DUF547 domain is of unknown function found in >400 proteins (PF04784 and IPR006869). Residues that make contact with the phosphate moiety of either lipid, namely Q22, T97, T114, and K195, are conserved in all the sequences. (A) PITP family members where the amino acids (T59, K61, E86, and N90) required for binding the inositol head group are conserved. (B) Proteins with a PITP domain where key residues required for inositol binding are not conserved (PF, PFAM entry; http://www.sanger.ac.uk/Pfam; IPR, interpro entry). (C) Identification of PITP in four of the five supergroups. Organisms containing PITPs are circled in the general outline of eukaryotic evolution. (Diagram modified from Koonin, 2010.)

key residues for both phosphate and inositol binding. Residues for PC binding are less conserved in all species. In Figure 3, the PITPs have been divided into [A] and [B], where [A] are PITPs containing all the residues necessary for phosphate and inositol binding, and [B] are proteins that have high sequence identity to PITPs are annotated as PITPs in the databases, but lack residues either for binding the phosphate moiety or for inositol binding. This latter category includes amphioxus, the modern survivor of an ancient chordate lineage with a fossil record dating back to the Cambrian period. In addition to the putative protein depicted in Figure 3B, amphioxus also contains a minimal domain class I PITP (C3Z486), 269 aa residues long and 62% identical to human PITPα and 64% identical to PITPβ, and contains all of the residues required for PI and PC binding. It is conceivable that PITP domains lacking the residues necessary for binding the phosphate moiety and/or the inositol head group of PI have evolved to bind other hydrophobic ligands at the expense of PI binding. Alternatively, evolution may have operated in the opposite direction, where the PITP domains lacking in consensus residues for PI binding evolved to bind PI when phosphoinositides were increasingly being used for cell signaling, membrane traffic, and other important functions.

### *Regulation of lipid transfer by Ser166 phosphorylation*

Serine residue 166 is conserved in the majority of PITP sequences analyzed and lies in the regulatory loop (Figure 2A). Ser166 is localized in a small pocket between the 165–172 loop and the rest of the protein and is not solvent-accessible in both the PI- and PC-loaded structures of PITPα or PITPβ, as well as in the apo-structure of PITPα. Nonetheless, Ser166 lies in a consensus sequence for phosphorylation by protein kinase C and is phosphorylated upon stimulation of HL60 cells with either FMetLeuPhe or PMA (Morgan et al., 2004). In addition, PITPα can also be phosphorylated *in vitro* by protein kinase C (Van Tiel et al., 2000b; Morgan et al., 2004). Finally, analysis of rat brain cytosol indicates the presence of phosphorylated PITPα at Ser166 (Morgan et al., 2004). When PITPα is phosphorylated at Ser166, it is unable to exchange its bound PI for PC, although bound PC can still be exchanged for PI.

In cells, phosphorylation of PITPα is likely to occur at the membrane and the membrane could facilitate structural changes. There are precedents for such changes in proteins that are active at the membrane (Heitz and Van Mau, 2002). For the phosphorylation site to become accessible, structural changes may go further than seen in the apo-structure, converting PITPα into a "molten globule" that allows partial unfolding of the protein. The steroidogenic acute regulatory protein (StAR), a lipid transfer protein with a preference for cholesterol, is reported to undergo such a transition when it binds to membranes (Bose et al., 1999, 2000). Another protein similar to StAR is Bet v1; when this protein binds to membranes, it undergoes a major structural rearrangement as well as affecting membrane structure (Mogensen et al., 2007). One possibility is that PITPα can dock onto the membrane in two different orientations, one where the regulatory loop faces the membrane and the other where the tryptophan residues face the membrane. Under conditions where docking is favorable in the orientation where the regulatory loop faces the membrane, the protein would have to undergo extensive changes including relaxation of the 165–172 loop such that the Ser166 is available for phosphorylation. Ancillary proteins may even facilitate this process. Thus to permit phosphorylation at Ser166, a distinct structural form is postulated. Mutation of Thr59 to Ala makes PITPα a much better substrate for protein kinase C and shifts the equilibrium to this form, which can be resolved on native PAGE. The elution profile observed by size exclusion chromatography of phosphorylated PITPα is distinct from non-phosphorylated PITPα, demonstrating that phosphorylated PITPα is structurally distinct from the non-phosphorylated form (Morgan et al., 2004).

## Sec14: a yeast PI-binding protein domain unrelated to PITP

In *S. cerevisiae* and other yeasts, a biochemical activity similar to mammalian PITP was identified that facilitated both PI and PC transfer and was attributed to a protein of similar size to PITPα. This protein is Sec14p, which interestingly bears no sequence or structural homology to mammalian PITPs. Despite this, the product of the *SEC14* gene is able to rescue phospholipase C signaling in mammalian cells washed of cytosol (Cunningham et al., 1996). Likewise, mammalian PITPs can rescue the temperature-sensitive *SEC14* allele, but not the null mutant. Sec14p is an essential protein and mutations lead to a complex phenotype in yeast: secretory traffic to and from the *trans*-Golgi is disrupted and growth is impaired. Extensive changes in lipid metabolism occur including reduction in PI(4)P levels at the Golgi, an increase in the rate of PC synthesis, increased turnover of PC via the PC-phospholipase D (Spo14), and decreased PC turnover by the PC-phospholipase B (Nte) (Howe and McMaster, 2006; Curwin et al., 2009; LeBlanc and McMaster, 2010). Regulation of PC and phosphoinositide metabolism by Sec14p is thought to provide the appropriate balance of lipids for membrane traffic from the *trans*-Golgi. However, the precise mechanism of how Sec14p regulates membrane traffic is unclear.

The yeast Sec14p is a minimal SEC14 domain-containing protein. The mammalian homolog is

the CRAL_TRIO domain, named after two proteins containing it. Many proteins in the human proteome contain the CRAL_TRIO domain, either alone or in combination with other domains (Saito et al., 2007). The minimal domain proteins bind hydrophobic ligands such as tocopherols (vitamin E), squalene, and 11-*cis*-retinol/11-*cis*-retinaldehyde. However, ligands for many CRAL_TRIO domains have not been identified so the possibility that mammalian cells contain a Sec14 family member that binds and transfers PI and PC similar to PITPα and PITPβ remains a formal possibility. *Dictyostelium discoideum* contains four PITPs related to mammalian PITP as well as Sec14p; two of the four Dd-PITPs as well as Dd-Sec14p can transfer PI and are able to stimulate PI(4,5)$P_2$ synthesis for phospholipase C signaling (Swigart et al., 2000). Along with the difference in sequence and structure between PITP and Sec14p, the significant difference between the two protein domains is the orientation of the bound phospholipid: in PITPs the head group of the lipid lies deep inside the hydrophobic pocket, whilst in Sec14p the head group lies at the opening of the cavity. Therefore, caution is necessary when parallels are drawn between these two families.

## PITP function in model organisms and cultured cell lines

The emerging pattern regarding the function of PITPs is one of the complexity where the phenotypes associated with loss of different members of the PITP family vary depending on the organism (Figure 4). In contrast to the specific requirement for RdgBα in flies in phototransduction for example, RdgBαI, which can rescue the fly phenotype, is embryonic lethal in mice (Lu et al., 2001). PITPα is required for zebrafish development, although mice are born in the absence of PITPα but die shortly after birth (Alb et al., 2003; Ile et al., 2010). An underlying theme, however, is witnessed at the molecular level.

As discussed above, proteins with a PITP domain bind PI. PI, although quantitatively a minor component of cell membranes, is the most versatile of phospholipids. Three of its hydroxyls can be phosphorylated by lipid kinases either individually or in combination giving rise to seven phosphorylated derivatives. These phosphorylations are understood to take place *in situ*, in the organelle membrane in which they are required. PI is exclusively synthesized at the ER and since PI is an insoluble amphiphilic lipid, mechanisms are required to facilitate its transfer from the ER to the target organelle membrane across the aqueous cytosol. PITPs are ideal for this job; lipid transfer proteins that can move phosphorylated PI have not been found. In this way, the PITP family of proteins works together with PI kinases to perform their biological function (Figure 5).

PI kinases can be subdivided into the 3-kinase and the 4-kinase families, and PITPs are able to potentiate the *in vitro* activity of both families. Each phosphorylated derivative of PI can bind to specific protein modules within effector proteins at target membranes, including PH domains, FYVE domains, ENTH domains, and regions of basic patches found in many cytoskeletal proteins. Phosphorylated derivatives of PI are present in many cellular organelles. Phosphoinositides play essential roles in cell signaling, membrane trafficking, dynamics of the actin cytoskeleton, modulation of ion channels, endocytosis, and a myriad of other functions. Since PI is the ultimate source of seven phosphoinositides, and these lipids have many functions in cells, it appears that PITPs may participate in many processes dependent on phosphoinositides. The possibility that some PITPs have tissue-specific functions is highly probable, thus in one cell type a PITP may participate in cell signaling and the same PITP in a different type of cell may function in membrane trafficking.

The C-terminal region of PITPs is likely to be responsible for the specific function of different PITPs isoforms. The PITP domain itself is always located at the N-terminus of the protein but the C-terminus has almost no similarity between isoforms (Figure 1). For PITPβ and RdgBβ, where the transcript is differentially spliced into two expressed splice variants, the main difference also lies in the C-terminal residues. This suggests that the specific function of the PITPs is derived from the C-terminal region interacting with different proteins or lipids. Below we examine the individual members of the PITP family and identified the pathways in which individual PITP proteins participate in mammalian biology, and then move on to *Drosophila*, zebrafish, and lastly the flatworm *Planaria* to examine common threads in PITP function.

## PITP function in mammals

### *PITPα*

PITPα was the first PITP protein to be isolated and its activity recognized as lipid transfer. Early studies using reconstitution assays in permeabilized cell systems to identify cytosolic factors required for exocytosis and phospholipase C signaling used brain cytosol as the starting material. PITPα was purified as the reconstituting factor able to restore $Ca^{2+}$-dependent exocytosis and the stimulation of phospholipase C signaling through G-protein-coupled receptors (Hay and Martin, 1993; Thomas et al., 1993). In both experiments, PITPα worked together with the lipid kinases to generate PI(4,5)$P_2$, which was the lipid required for exocytosis and was the substrate for phospholipase C activity. Subsequent

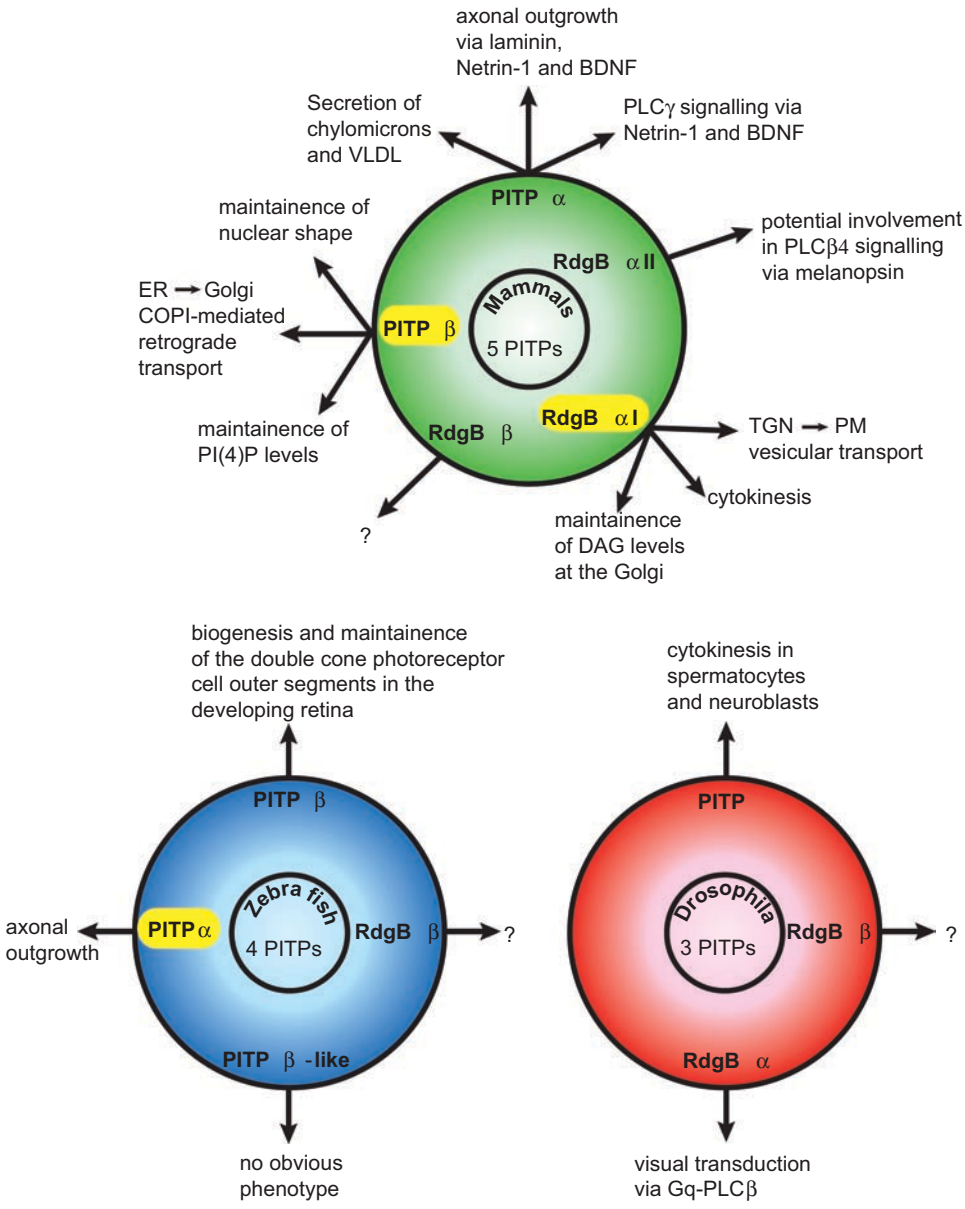


**Figure 4.** PITP function in model organisms and in cultured cells. The function of the individual PITPs in the different organisms is indicated. The PITPs that are highlighted in yellow are those that when absent cause embryonic lethality.

studies have mainly been carried out in permeabilized cell preparations or using purified lipid kinases such as type III PI3K and have yielded a singular theme: PITPα is able to enhance the activity of the lipid kinases, both 4-kinases and 3-kinases (Cunningham et al., 1995; Kauffmann-Zeh et al., 1995; Fensome et al., 1996; Kular et al., 1997; Panaretou et al., 1997). Furthermore, different cell types make use of PITPα in a specific manner.

### PITPα in neuronal function

The first indication of the biological function of PITPα came from the identification of the mutation responsible for the mouse *vibrator* phenotype (Weimar et al., 1982; Hamilton et al., 1997). *Vibrator* is an autosomal recessive mutation that occurred spontaneously in the DBA/2J strain of mice. It is a retroposon insertion in an intron of the PITPα gene, *PITPNA*. The insertion creates a hypomorphic *PITPNA* allele with reduced mRNA expression. Protein expression is reduced by ~80%. Homozygous *vibrator* mice have severe action tremor and progressive neurodegeneration in the brain and spinal cord, and they die by postnatal day 30. Degenerative changes in neurons are observed in the brain stem, cerebellum, spinal cord, and dorsal root ganglia. Affected neurons display progressive intracellular vacuolation suggestive of defective ER.

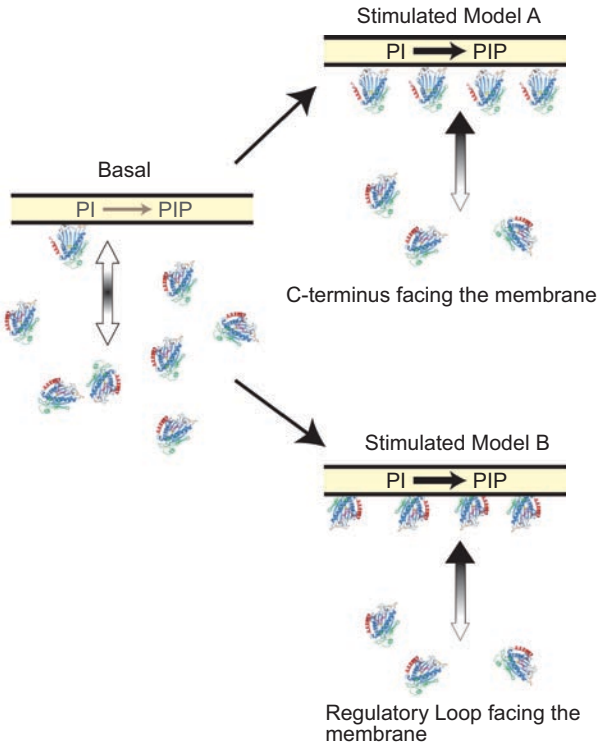


**Figure 5.** Model of PITP function. Under basal conditions, Class I PITPs sample the membrane continuously and do not impact substantially on PI phosphorylation. Upon stimulation, PITP molecules are recruited by binding to target proteins such as the EGF receptor or the netrin-1 receptor, DCC. Accumulation of PITP increases the resident time at the membrane permitting the phosphorylation of PI at localized sites. Two models are presented: Model A—the C-terminus faces the membrane and the PI molecule would not be able to be phosphorylated although loaded onto PITP. Model B—the regulatory loop of PITP faces the membrane interface and the close proximity to the membrane induces a molten globular state. Under these conditions, the inositol ring is accessible for phosphorylation without the lipid released from the hydrophobic cavity. Serine 166, which is located in the regulatory loop, not normally available for phosphorylation in the soluble structures of PITPα, PITPβ, or the apo-form, is now also accessible for phosphorylation. Phosphorylation at this residue has a regulatory function by inhibiting PI exchange but not PC exchange.

Cerebellar dysfunction is observed in the *vibrator* as well as in the knockout mouse. The cerebellum is primarily involved in coordination of movements and ataxia is observed in these mice. Histochemical analysis of the adult mouse cerebellum shows restricted PITPα expression in the molecular layer and punctate immunoreactivity in the granule cell layer with low signals detected in the Purkinje cell layer and the white matter tract. The staining observed in the cerebellum suggests an axonal function for PITPα. Similarly, in the adult hippocampus PITPα is strongly expressed in the CA3 cells as well as in the stratum radiatum (Cosker et al., 2008).

During neuronal maturation of hippocampal neurons *in vitro*, PITPα levels increase. During the first few days of development, PITPα is specifically found in the axonal processes including their growth cones. In mature neurons, PITPα immunoreactivity colocalizes with the presynaptic marker, synaptophysin. Although PITPα is a soluble protein and localizes throughout the cytosol and nucleus when expressed in cultured cells (De Vries et al., 1996; Larijani et al., 2003), the localization of PITPα in neurons is discrete. This localization suggests that PITPα may be retained in the axons by interacting with other components.

Clues to the cellular function came from studies where PITPα levels were either increased or decreased in neuronal preparations. For the establishment of the neuronal network during development, neurons extend axons over considerable distances to make contact with their targets. The extremity of the axon (the growth cone) grows toward its target guided by receptor-directed ligands that ensure the correct trajectory. One guidance cue is netrin-1. Netrin-1 is released by floor plate cells in developing spinal cord, stimulating the growth and orientation of commissural neurons toward floor plate cells (Serafini et al., 1996). Netrins constitute a family of highly conserved secreted proteins that are structurally related to laminins. Laminins are the structural components of basement membranes of tissues and can bind to receptors such as integrins initiating signaling cascades (Yurchenco and Wadsworth, 2004; Cirulli and Yebra, 2007).

Previous studies indicated that growth cone guidance by NGF, BDNF, netrin-1, or myelin-associated glycoprotein (MAG) required coactivation of phospholipase Cγ and PI 3-kinase signaling pathways (Ming et al., 1999). Two studies have examined the role of PITPα in isolated neurons (Xie et al., 2005; Cosker et al., 2008). Using different strategies, both studies conclude that PITPα is required for signaling through $PI(4,5)P_2$ during neuronal development. In one study, PITPα was required for phospholipase C signaling and in the second study it was required for PI 3-kinase signaling. Both PI 3-kinase and phospholipase C use $PI(4,5)P_2$ as substrate and this accords with previous studies that PITPα can support both phospholipase C and PI 3-kinase signaling (Thomas et al., 1993; Kular et al., 1997).

The receptor for netrin-1 is DCC (deleted in colorectal cancer) and its C-terminus recruits PITPα following activation with netrin-1 (Xie et al., 2005). This interaction was first identified in a yeast two-hybrid system where the C-terminal domain (P3) of netrin-1 was used as bait to identify interacting partners. This domain is conserved in neogenin, a homolog of DCC, and this also binds to PITPα via the PITPα C-terminus. PITPβ, whose C-terminus is different, does not interact with DCC. Unlike NGF, EGF, and BDNF, which are receptor tyrosine kinases and therefore can recruit and activate PLCγ via its SH2 domains, DCC does not harbor tyrosine kinase activity. Regardless,

netrin-1 does stimulate phospholipase Cγ and cortical neurons prepared from *vibrator* mice that have reduced PITPα are refractory to netrin-1 stimulation (Xie et al., 2006). Previous studies with the EGF receptor had shown that PITPα is found in a complex with PLCγ and a Type II PI 4-kinase (Kauffmann-Zeh et al., 1995) suggesting that following activation with an agonist, some receptors can recruit PITPα, PLCγ, and a PI 4-kinase into a complex to generate the second messengers, diacylglycerol (DG) and inositol trisphosphate (Cockcroft, 1998).

The interaction between DCC and PITPα implies that PITPα is important for axon guidance. Netrin-1 provides a migrational cue in the developing nervous system and attracts the spinal commissural axons to cross the midline. Using the chick embryo as a model system, commissural axons expressing a dominant-negative PITPα mutant (Hara et al., 1997) were unable to cross to the other side (Xie et al., 2005). When morpholinos to ablate PITPα were injected into zebrafish embryos, a loss of spinal cord was observed together with defects in motor-axon outgrowth (Xie et al., 2005). In a separate study, injection of morpholinos also caused early developmental arrest followed by inviability of the embryo (Ile et al., 2010). Mammalian PITPα, but not PITPβ, was able to rescue the phenotype. The mutant T59D, defective in PI binding and transfer, was unable to effect a rescue.

In addition to netrin-1, laminin also stimulates axonal outgrowth, and hippocampal neurons plated on a laminin substrate make much longer axons compared with controls (Lein et al., 1992). It was found that overexpression of wild-type PITPα enhances axonal outgrowth provided the neurons are plated on laminin substrate (Cosker et al., 2008). Importantly, this enhancement of axonal outgrowth requires the PI-binding function of PITPα; the PITPα K61A mutant, which is unable to bind or transfer PI does not support axonal outgrowth. The signaling pathway that PITPα is required for in this system is the PI 3-kinase pathway as inhibitors of PI 3-kinase reversed the effects of PITPα overexpression.

**PITPα requirement in intestinal and liver function**

Although PITPα levels are merely reduced in the *vibrator* mouse, mice that are totally devoid of PITPα show additional phenotypes (Alb et al., 2003). These mice die earlier (40% mortality at 48 h) and those that survive for longer are smaller in size and severely ataxic. The mutant animals exhibit low body fat and the enterocytes and hepatocytes of these mice accumulate lipid. Moreover, the animals are hypoglycemic and the pancreatic islet number is greatly reduced. Mice are also deficient in plasma triacylglycerol and vitamin E (α-tocopherol). Reexpression of PITPα specifically in the small intestine corrects the intestinal disorder and the hypoglycemia, but does not rescue the neonatal lethality in these animals (Alb et al., 2007). These results confirm that the neurodegenerative phenotype is a cell-autonomous defect and not a consequence of intestinal steatosis and hypoglycemia.

The accumulation of fat in enterocytes and liver observed in the PITPα-null mice suggests that the major problem is the inability to assemble or secrete chylomicrons from the enterocytes and very low-density lipoproteins (VLDL) from the hepatocytes. Following ingestion of fat, enterocytes produce the triglyceride-rich chylomicrons. The intestine also synthesizes VLDL but its assembly occurs constitutively, whereas chylomicron assembly is a characteristic property of the enterocytes during the postprandial state. Chylomicrons also transport fat-soluble vitamins such as vitamin E into the blood. Each chylomicron particle has one apo B-48 and is packaged in the lumen of the ER. Chylomicron assembly is also deficient in the clinical syndrome of abetalipoproteinemia and chylomicron retention disease. Abetalipoproteinemia is due to mutations in the microsomal triacylglycerol transfer protein (*mttp*) gene and is characterized by the virtual absence of apoB-containing lipoproteins in the plasma. MTTP protein is located at the site of apoB translocation and facilitates concerted transfer of lipids and folding of apoB as it exits the ribosome and enters the ER lumen. Patients suffer from neurological disorders, visual impairment, and exhibit acanthocytosis where the red cells are spiculated (Blasiole et al., 2007). Chylomicron retention disease and Anderson's disease, both inherited recessive disorders, are characterized by chronic diarrhea and failure to thrive. Both diseases are caused by a specific defect in the secretion of intestinal lipoproteins; secretion of lipoproteins by the liver is not affected. Patients with chylomicron retention disease and Anderson's disease selectively retain chylomicron-like particles within membrane-bound compartments. Sar1B mutations are responsible for both diseases (Jones et al., 2003; Shoulders et al., 2004).

How does PITPα participate in the apo48-containing chylomicron secretion? These lipoproteins vastly exceed the size of canonical ER-to-Golgi transport vesicles, which are only 60–70 nm in diameter (Williams, 2008). In the PITPα-null enterocytes, the lipid accumulates in the ER suggesting that passage of these particles to the Golgi is disrupted. One possibility is that PITPα together with the ER-localized PI 4-kinase makes PI(4)P required for COPII/SAR1b-coated vesicles. Assembly of COPII-coated vesicles when reconstituted *in vitro* with purified coat proteins and chemically defined liposomes requires PI(4)P or PI(4,5)$P_2$ for the binding of the three coat assembly proteins (Matsuoka et al., 1998). Moreover, Sar1 has been reported to activate a type II PI 4-kinase at the ER and the PI(4)P assisted in Sar1-induced COPII nucleation at ER exit sites (Blumental-Perry et al., 2006). Since the ER is the site of PI synthesis and therefore the richest source of PI, PI delivery would not be a factor here. However, if

we assume that PITPα works together with a lipid kinase, then PITPα might facilitate PI(4)P production specifically at the ER exit sites. Future studies are clearly necessary to examine the requirement for PITPα in chylomicron secretion in the enterocyte and VLDL secretion in the liver. PITPα might provide a novel point of control for dietary lipid absorption. Overconsumption of lipid-rich diets together with a sedentary lifestyle is a major health issue worldwide, and disabling PITPα could be a way to limit absorption of dietary fat.

Although the exact mechanism of action of PITPα in intestinal and liver function is not yet clear, it is clear that the ability of PITPα to bind and transfer PI is important. PITPα knockout mice were engineered to express the PITPα mutant, T59D. As described earlier, Thr59 is important for PI binding and substitution with glutamic acid (T59E) or aspartic acid (T59D) causes loss of PI binding and transfer, although PC binding and transfer remains intact (Alb et al., 1995; Tilley et al., 2004). These mice exhibited the same phenotypes as the mice that had their PITPα gene ablated. In conclusion, PI binding/transfer is the essential biochemical activity of PITPα in animals.

**PITPα and aging**

The molecular and cellular basis for aging is an important area of research as humans live longer. Age-associated diseases have increased in the modern era and create a major economic burden on society. PITPα is enriched in the brain as discussed earlier, and neuronal function is understood to decline with age. Studies in aging mice and in mice where Parkinson's disease is induced have observed decreased PITPα levels (Chalimoniuk et al., 2006). In contrast, supplementation of the diet with vitamin C has been shown to increase PITPα levels in humans (Griffiths et al., 2009).

**PITPα as a signature gene for human retinal pigment epithelium**

In a recent study, signature genes of the human retinal pigment epithelium (RPE), the polarized cell layer critical for photoreceptor function and survival, were assessed for their association with age-related macular degeneration (AMD). PITPα has been identified as a signature gene of the RPE, and this study revealed that *PITPNA*, the gene encoding PITPα, together with *TIMP3*, *GRAMD3*, and *CHRNA* signature genes, have potential roles in AMD pathogenesis (Strunnikova et al., 2010). The RPE is the source and target of many retinal degenerative diseases and defects in RPE function can affect the integrity and viability of the neighboring cells, primarily photoreceptors. Numerous studies have focused on regenerating or replacing damaged RPE using embryonic stem (ES) cells or from induced pluripotent stem (iPS) cells. Several human ES cells can be induced to develop RPE phenotypes. There is a potential for PITPα to be used as a biomarker for an RPE phenotype as monoclonal antibodies specific for PITPα are widely available. Further studies will be required to understand the precise involvement of PITPα in this system and the importance of PI binding and transfer.

**PITPα and the nucleus**

PITPα is found in the nucleus (De Vries et al., 1996). PITPα is not totally inactivated by NEM treatment even after 10 min, suggesting that the nuclear pool of PITPα may not be closely associated with membranous structures. The function of the nuclear pool of PITPα is not known but it is increasingly clear that phosphoinositides play important role in the nucleus (Irvine, 2003) and therefore PITPα could be well-positioned for a specific function in this organelle.

**PITPα and cell proliferation**

The function of PITPα has been explored in NIH3T3 cells, where stable cell lines overexpressing PITPα were examined. In these cell lines, PITPα levels show a 2–3-fold increase accompanied by increased rates in proliferation. Additionally, these cells have increased levels of lyso-PI suggesting the activation of a phospholipase $A_2$ (Snoek et al., 1999). It was suggested that PITPα is responsible for the production of a mitogenic factor that is derived from arachidonic acid. Although the identity of this factor has not yet been clarified, it has been suggested that the factor is an eicosanoid and that it protects cells from apoptosis and regulates growth (Schenning et al., 2004; Bunte et al., 2006). Identification of this eicosanoid and the phospholipase $A_2$ responsible for the production of arachidonic acid is an outstanding question that remains to be explored.

***PITP*β**

PITPβ was discovered in a screen looking for gene products able to rescue the temperature-sensitive *SEC14* mutant in *S. cerevisiae* (Tanaka and Hosaka, 1994). In these mutant cells, secretion from the Golgi to the plasma membrane is defective and rat brain cDNA was used to identify genes that could rescue this phenotype. The gene that rescued this defect encoded a protein that was similar in sequence to PITPα and was thus called PITPβ.

*PITPNB*, the gene encoding PITPβ, can be alternatively spliced. PITPβ splice variant 1 (the original PITPβ identified) is a product of the first 11 exons of the gene, whereas splice variant 2 is a product of the first 10 exons followed by the 12th exon, exon 11 being skipped. Consequently, the C-terminal region of these two splice variants differs by 17 aa residues. To distinguish between the two splice variants, we referred to these two proteins as PITPβ-sp1 and PITPβ-sp2 (Morgan et al., 2006). As the C-terminal region forms the lid that closes the hydrophobic cavity

and has to dislodge to allow the opening of the cavity for lipid exchange, the general prediction is that this region provides the specificity for interactions with particular membranes and possibly other proteins.

Analysis of the expression of the two splice variants indicates that both are expressed in cultured cells as well as in cells obtained from animal tissues. The lipid-binding properties of both splice variants of PITPβ have been characterized and like PITPα, both splice variants bind and transfer PI and PC. The C-terminus of PITPβ-sp1 contains a serine residue (Ser262) that is constitutively phosphorylated by protein kinase C (Van Tiel et al., 2002; Morgan et al., 2006). This phosphorylation has no effect on the transfer activity of PITPβ-sp1 *in vitro*. Both splice variants of PITPβ localize to the Golgi, the ER, and the nuclear envelope determined using an antibody that recognizes both splice forms (Morgan et al., 2006; Carvou et al., 2010). This localization is at odds with another study that reported that PITPβ localizes exclusively at the *trans*-Golgi network (TGN) (Phillips et al., 2006a). It is therefore plausible that the two splice variants may be differentially distributed and have unique functions.

**PITPβ is required for retrograde traffic from the Golgi to the ER**

Deletion of the PITPβ gene is embryonic lethal indicating an essential function of PITPβ in mammals (Alb et al., 2002). The first clues concerning the cellular role of PITPβ come from studies where both splice variants of PITPβ are depleted by RNAi in HeLa cells (Carvou et al., 2010). Two morphological changes are immediately apparent when the control cells are compared with the knockdown cells. First, the shape of the nucleus is distorted and second, the Golgi has a compacted morphology. Moreover, the Golgi is resistant to the actions of brefeldin A. Brefeldin A disrupts the ARF GTPase cycle causing the Golgi to tubulate and the tubules merge with the ER. Examination of membrane transport identifies a specific defect in retrograde traffic from the Golgi to the ER mediated by COPI-coated vesicles. The KDEL receptor retrieves escaped ER proteins and is an important cargo of COPI-coated vesicles. The KDEL receptor cycles between the Golgi and the ER but is arrested at the Golgi compartment in PITPβ-knockdown cells. Likewise, ER–Golgi intermediate compartment (ERGIC)-53 that cycles between the ERGIC and the ER via COPI-coated vesicles is also affected in PITPβ-knockdown cells. ERGIC-53 is likewise arrested at the ERGIC.

The retrograde transport defect observed in PITPβ-knockdown cells can be rescued when wild-type PITPβ is reexpressed (Carvou et al., 2010). However, expression of mutants deficient in PI or PC transfer is unable to rescue the retrograde transport defect. In addition, a PITPβ mutant (WW/AA) that is unable to dock on membranes is unable to rescue the phenotype. These results clearly indicate that the biochemical characteristics of PITPβ studied *in vitro* are the essential activities that are required *in vivo*.

**PITPβ maintains a pool of PI(4)P**

In PITPβ-knockdown cells, a ~40% drop in PI(4)P is observed indicating that a potential function of PITPβ is to provide PI for phosphorylation by a PI 4-kinase (PI4K). Two sequence-unrelated PI4K families, each containing two members, have been characterized: Type II (α and β) and Type III (α and β) (Balla and Balla, 2006). Type IIIα localizes at the nuclear envelope and perinuclear ER, although Type IIIβ localizes to the Golgi. Type II enzymes also localize to the Golgi, as well as TGN and endosomes (Balla and Balla, 2006). Although several studies have suggested that PI(4)P is restricted to the TGN (D'Angelo et al., 2008), the widespread location of PI4K enzymes would suggest otherwise (Smith and Wells, 1983. 1984; Cockcroft et al., 1985). Establishing the location of PI(4)P in cells is technically challenging and PH domains that bind PI(4)P (e.g. FAPP1 and OSBP) have been used extensively (Levine and Munro, 1998. 2002; Lenoir et al., 2010). More recently, PI(4)P antibodies have also been used (Hammond et al., 2009). Both methods detect PI(4)P at the TGN but do not necessarily identify all the different pools (see Balla et al., 2009; Balla and Várnai, 2009 for a critical evaluation). In PITPβ-knockdown cells, the TGN pool of PI(4)P does not appear to be sufficiently disrupted as TGN-to-plasma membrane transport, sphingomyelin and glycosphingolipid synthesis are not affected (Carvou et al., 2010). These activities depend on TGN PI(4)P synthesized by PI4KIIα and PI4KIIIβ (Tóth et al., 2006; D'Angelo et al., 2007).

What is the function of PI(4)P in COP1 vesicle traffic? COP1 vesicles are formed on *cis*-Golgi membranes and require ARF1; activation of ARF1 is accomplished by an ARF-GEF, which exchanges the GDP bound to ARF1 with GTP. The large ARF-GEF, GBF1 localizes at the *cis*-Golgi and interacts with the γ-COP subunit of COP1 to recruit the COP1 complex to the membranes independently of its interactions with ARF1 (Deng et al., 2009). PI4KIIIα-generated PI(4)P is required for recruitment of GBF1 (Dumaresq-Doiron et al., 2010). In the PITPβ-knockdown cells, ARF1 and COPI are concentrated in the compacted Golgi. Since PI(4)P is also depleted in PITPβ-knockdown cells, one possibility is that the exchange factor GBF1 is prevented from being recruited and therefore ARF1 activation cannot occur (Dumaresq-Doiron et al., 2010). If recruitment of ARF1.GDP, COP1, and GBF1 are independent events, and if PI(4)P is required to allow the complex to form, this could explain why PITPβ can lead to the blockade in COP1 vesicle formation and hence arrest of retrograde traffic.

An alternative possibility pertains to the knowledge that the phenotype observed in PITPβ-knockdown

cells is reminiscent of cells treated with latrunculin that causes both Golgi compaction and defects in Golgi–ER retrograde transport (Egea et al., 2006), and distortions in nuclear shape (Münter et al., 2006). Latrunculin affects the actin cytoskeleton suggesting that PITPβ via the production of PI(4)P somehow regulates the dynamics of the actin cytoskeleton. Golgi compaction, defects in retrograde transport, and changes in nuclear shape are also observed when Nesprin-2 is mutated. Nesprin1/2 are large ~800 kDa proteins that belong to the spectrin superfamily. They interact with actin filaments via their N-terminus, although the C-terminal KASH domain projects into the nuclear envelope lumen and binds the C-terminal luminal domain of SUN1 projecting from the inner nuclear membrane (Worman and Gundersen, 2006). The possibility that PITPβ regulates the activity of nesprins is truly exciting and warrants further investigation.

**PITPβ and sphingomyelin metabolism**

In a previous review, we have discussed whether PITPβ can bind and transfer sphingomyelin (Cockcroft and Carvou, 2007). In brief, PITPβ-sp1 has been shown to transfer sphingomyelin in addition to PI and PC. Depending on the assay used to measure transfer, some studies show robust sphingomyelin transfer, although other studies do not (De Vries et al., 1995; Ségui et al., 2002; Phillips et al., 2006a). PITPβ function has also been examined in NIH3T3 cells in stable cell lines that overexpress over 10-fold excess of PITPβ. When treated with exogenous sphingomyelinase, these cells show a more rapid replenishment of plasma membrane sphingomyelin compared with wild-type cells (Van Tiel et al., 2000a). Based on this observation, it has been suggested that PITPβ stimulates sphingomyelin transport to the plasma membrane. However, similar experiments performed in COS-7 cells where PITPβ was expressed transiently do not show any changes in sphingomyelin synthesis when treated with sphingomyelinase (Ségui et al., 2002). In a recent study, where PITPβ levels were reduced by RNAi, sphingomyelin levels were not altered (Carvou et al., 2010) suggesting if PITPβ does influence sphingomyelin metabolism, it may do so under specific conditions.

***RdgBβ***

RdgBβ was the last of the three soluble mammalian PITPs to be identified after PITPα and PITPβ: very little is known about this protein. RdgBβ was originally cloned in 1999 as a PITP of 332 aa residues (38 kDa) from a human infant brain cDNA library in a screen for novel human homologs of Dm-RdgBα (Fullwood et al., 1999). A subsequent study reported that RdgBβ is encoded by the *PITPNC1* gene and has two splice variants that differ from one another exclusively at their C-terminal tail (Takano et al., 2003). The coding region for PITPNC1 is made up of 10 exons in total: the short splice variant, sp2 (268 aa) is translated from an mRNA transcribed from exons 1–9; the long splice variant, sp1 (332 aa) is translated from an mRNA transcribed from exons 1–8 and 10.

Northern analysis suggests that RdgBβ-sp1 is ubiquitously expressed with particularly strong expression in the heart, muscle, kidney, liver, and peripheral blood leukocytes (Fullwood et al., 1999). The presence of RdgBβ-sp1 has been reported in adult mouse heart as well as in the brain at stages E15, P0, and P49 by RT-PCR. RdgBβ-sp2 was also observed in the heart and brain, as well as in the mouse P49 liver, kidney, and testes (Takano et al., 2003). Immunofluorescence microscopy of HEK-293 cells expressing FLAG-RdgBβ-sp1 shows diffuse staining throughout the cytoplasm. PC12 cells expressing RdgBβ-sp1-GFP or RdgBβ-sp2-GFP confirmed the sp1 localization to the cytoplasm, and observed that sp2 was present in the nucleus as well as in the cytoplasm (Takano et al., 2003).

Unlike Class I PITPs, which are present in both unicellular and multicellular organisms, RdgBβ appears to be restricted to metazoans; it is present in mammals, fish, frogs, chickens, and flies (Cockcroft and Carvou, 2007). Human RdgBβ is 43% identical and 62% similar to human PITPα in the PITP domain. The RdgBβ proteins contain all the key residues that have been identified in other PITPs essential for the binding of the inositol head group of PI (Tilley et al., 2004). Examination of its PI transfer activity indicates that like PITPα and β, the recombinant protein is active *in vitro* (Fullwood et al., 1999). A recent study examined the expression of genes when human dermal fibroblasts were stimulated to proliferate with serum, and other growth factors including EGF, FGF, and PDGF, indicative of their normal role in wound healing. Increased expression of the *PITPNC1* transcripts was observed as was increased expression of 14-3-3γ, a regulatory factor in the PI 3-kinase pathway. Since PITPs are known to enhance the activity of PI 3-kinases, it is possible that RdgBβ could play a specific role in the proliferative response in dermal fibroblasts (Gu and Iyer, 2006).

Several phosphoproteome screens indicate that RdgBβ-sp1 is phosphorylated *in vivo*. In the developing mouse brain, Ser274 located at the C-terminus is constitutively phosphorylated (Ballif et al., 2004). The same residue was also found to be phosphorylated in HeLa cells during entry into mitosis (Dephoure et al., 2008). Additionally, in human ES cells, RdgBβ was observed to be phosphorylated, but this time at Ser299 (Brill et al., 2009).

What is the significance of these two phosphorylations in RdgBβ-sp1? Examination of the sequence surrounding the two serine phosphorylation sites in the C-terminal extension of RdgBβ-sp1 suggests that this region could

bind 14-3-3 proteins. The distance between the two phosphorylated serines is sufficient for 14-3-3 proteins to bind in tandem. 14-3-3 are homodimeric proteins that adopt a horseshoe-shaped structure that binds to two phosphoserine/threonine residues (Liu et al., 1995; Xiao et al., 1995). These residues reside at the C-terminus of RdgBβ-sp1, which is the region most likely to form the lid that regulates the entry or exit of the lipid from the hydrophobic cavity. Binding of 14-3-3 to the C-terminus would have major consequences for the function of RdgBβ-sp1. Essentially, the C-terminus would be immobilized and therefore would not be able to exchange its lipid cargo. Our prediction is that phosphorylation keeps RdgBβ-sp1 in an inactive state and dephosphorylation would be required for RdgBβ-sp1 to function as a transfer protein, at least *in vitro*. In a recent study, we have identified that RdgBβ-sp1 is maintained as a complex with 14-3-3 proteins *in vivo* and this interaction controls the RdgBβ-sp1 activity *in vitro* (Garner et al., 2011). The complex is unable to mediate PI transfer.

The RdgBβ proteins are present as two splice variants in mammals and it is only the splice variant with the long C-terminal extension that is bound to 14-3-3 proteins. In flies, zebrafish, sea anemone, and *Ciona intestinalis*, the C-terminus is not conserved. They do not contain 14-3-3 consensus binding sites. However, the 14-3-3-binding site is conserved in mouse, human and chimp, and frog (*Xenopus tropicalis*). In the frog, there are two RdgBβ proteins of 329 and 333 in length, which are not splice variants and only one of them has the appropriate residues for binding 14-3-3 proteins. Zebra fish RdgBβ does contain an equivalent serine residue at position 274 and this residue is also phosphorylated (Lemeer et al., 2008).

The biological function of RdgBβ is unknown. In a screen looking for genes that affect embryonic muscle pattern formation in *Drosophila*, RdgBβ was identified together with 66 other genes (Staudt et al., 2005). Further studies are required to examine the function of RdgBβ in intact organisms and in cultured cell lines.

### *RdgBαI and RdgBαII*

The founding member of the RdgB family is Dm-RdgBα (*Drosophila* retinal degeneration B [*rdgB*]) that was identified as an essential component of visual transduction and maintenance of the ultrastructure of retinal sensory neurons (photoreceptors) in flies (Vihtelic et al., 1993). Subsequently, mammalian RdgBαI was cloned as a mouse homolog of Dm-RdgBα that could rescue the mutant fly phenotype (Chang et al., 1997). Rescue of both retinal degeneration as well as the abnormal electroretinogram (ERG) was facilitated. A second mammalian homolog, RdgBαII that possesses 56% identity to RdgBαI was subsequently identified (Lu et al., 1999). Transgenic expression of this homolog in RdgBα null mutant flies suppressed retinal degeneration but did not restore the electrophysiological light responses. Unlike RdgBαI, which is ubiquitously expressed, RdgBαII is only expressed in the retina and dentate gyrus in the mouse (Lu et al., 1999).

What is the function of the two RdgBα proteins in mammals? Here, we will restrict our discussion to RdgBαI and examine the potential function of RdgBαII in the section where Dm-RdgBα is described. Mice knockouts for both RdgBα genes have been carried out, and although mice ablated for the RdgBαII gene appear completely normal, deletion of RdgBαI is embryonic lethal (Lu et al., 2001). Lethality provides no clues to its function except that it is required during development. The use of RNAi to reduce RdgBαI expression in cultured cells provides important clues to its role in mammalian cells.

RdgBα proteins are large membrane-associated 160 kDa proteins with an N-terminal PITP domain. When Dm-RdgBα was cloned, it was proposed to be an integral membrane protein consisting of six transmembrane domains (Vihtelic et al., 1993). This assumption was based on the hydrophobicity profile of the six segments. Subsequent biochemical analysis of both Dm-RdgBα and human RdgBαI indicate that these proteins associate with membranes via protein–protein interactions (Lu et al., 1999; Litvak et al., 2002a).

RdgBα proteins were also independently identified as binding partners for protein tyrosine kinase PYK2 and were designated Nir proteins (PYK2 N-terminal domain-interacting receptors). All three Nir proteins (Nir1, Nir2, and Nir3—Nir3 will be discussed briefly in the next section) bound to the N-terminal domain of PYK2 via a conserved motif of ~300 aa residues located at the C-terminus (Lev et al., 1999). We will use the RdgB nomenclature throughout (see Figure 1 for alternative nomenclatures). Aikawa et al. (1999) also cloned RdgBαI (and named it PITPnm) and found that the N-terminal 378 residues formed a complex with type III PI 4-kinase. This construct contained both the PITP domain as well as the FFAT motif. This motif is also present in other lipid transfer proteins including members of the OSBP family and the StART family member, CERT (Loewen et al., 2003). The FFAT motif binds to VAP proteins, which are ER-localized integral membrane proteins. Localization studies of the full-length RdgBαI protein also indicate that the protein localizes to the ER and the Golgi (Aikawa et al., 1999; Litvak et al., 2002a). In contrast, expression of the PITP domain (1–257 aa) of RdgBαI shows cytosolic staining (Litvak et al., 2002a) suggesting that the FFAT motif is sufficient to target the protein to membranes.

#### RdgBαI and the CDP-choline pathway

What does RdgBαI protein do at the Golgi? This has been examined in HeLa cells where the protein was

depleted using RNAi (Litvak et al., 2005). These cells were found to have disorganized Golgi with swollen cisternae. Functionally, protein export from the TGN to the plasma membrane was arrested in the knockdown cells and could be rescued when the full-length protein was reexpressed. However, rescue could also be achieved by the PITP domain alone. They also reported that DG levels in the Golgi apparatus were reduced, whilst PC levels had increased. The interpretation of this data was as follows: RdgBαI inhibits the CDP-choline pathway of PC synthesis at the TGN to maintain a pool of DG that is the critical lipid for Golgi secretory function (Litvak et al., 2005). This model of RdgBαI function is similar to that proposed originally for Sec14p in yeast (Phillips et al., 2006b). Interestingly, levels of PI(4)P at the Golgi were unaffected, measured using a reporter construct of OSBP-PH that binds to PI(4)P. Several outstanding questions need to be addressed to validate the model, for example, how does RdgBαI inhibit the CDP-choline pathway? Is the lipid-binding/transfer properties of the PITP domain required, and if so, is it the PC or the PI binding/transfer that is required or both? Although it is predicted that the PITP domain of RdgBαI will bind and transfer PI because it contains the four signature residues (K61, N90, E86, and T59) that are essential for inositol binding in the hydrophobic pocket, it is not apparent that the binding pocket will accommodate choline. In PITPα and PITPβ, Cys95, a residue that is in the binding pocket, is required for PC binding and transfer, but not for PI binding (Carvou et al., 2010). Mutation of cysteine95 to either alanine or threonine leads to loss of PC transfer. In the RdgBαI protein, this residue is a threonine. The characterization of the lipid-binding properties is needed—does RdgBαI bind PC and if does, to what extent? Importantly, is PI binding essential for RdgBαI function? This question can be easily answered using mutants such as T59E and K61A to address this crucial issue.

**RdgBαI and lipid droplets**

RdgBαI carrying the T59E mutation has been examined and has been found to be targeted to lipid droplets in HeLa cells. The PITP domain of RdgBαI with the T59E mutation is also localized to the lipid droplets. The T59E mutation is predicted to lead to loss of PI binding based on the structure function analysis of PITPα (Tilley et al., 2004). Studies of the PITP domain of Dm-RdgBα, however, suggest that this mutation does not affect PI transfer (Milligan et al., 1997). Clearly, this needs to be resolved. The wild-type protein as well as the solitary PITP domain also translocated to lipid droplets when cells were incubated with oleic acid, which induces lipid droplet accumulation. Oleic acid-treated cells enhanced threonine phosphorylation on the RdgBαI protein but not of the RdgBαI-T59A mutant. Finally, RdgBαI-T59A was unable to translocate to lipid droplets. From these studies, it would appear that induction of lipid droplets leads to the phosphorylation of threonine 59, a residue that resides in the heart of the lipid-binding pocket and this phosphorylation causes the translocation of RdgBαI to lipid droplets. Based on the structure of PITPα, this would require the PITP domain to go through a large conformational change to allow a protein kinase to get access to T59. Although RdgBαI translocates to the lipid droplets, it is not clear whether the protein is required for their formation.

**Does RdgBαI maintain PI(4)P levels?**

Although no effects on PI(4)P levels were observed in RdgBαI-knockdown cells, in cells depleted of VAP proteins and treated with 25-hydroxycholesterol, PI(4)P levels were greatly reduced (Peretti et al., 2008). In addition to RdgBαI, CERT and OSBP also contain the FFAT motif that binds to VAP proteins. CERT and OSBP both have a PH domain that binds to PI(4)P. CERT transfers ceramide and OSBP binds 25-hydroxycholesterol, which causes its translocation to the Golgi and enhances CERT-mediated ceramide transport (Perry and Ridgway, 2006). In VAP-knockdown cells treated with 25-hydroxycholesterol, CERT, OSBP, and RdgBαI become cytosolic, leading to the reduction not only in PI(4)P, but also in DG and sphingomyelin (SM) at the Golgi. Defects in membrane traffic out of the Golgi are also observed. Overexpression of RdgBαI restores all these defects including PI(4)P levels, DG levels, targeting of OSBP and CERT to the Golgi and membrane traffic. The full-length protein is much more effective than expression of the solitary PITP domain of RdgBαI, suggesting that the other domains are required for correct targeting.

RdgBαI appears to have potent effects on PI(4)P levels but only when the cells are treated with 25-hydroxycholesterol and VAP proteins are knocked down (Peretti et al., 2008). When RdgBαI is knocked down, only DG levels are affected. The PITP domain of RdgBαI is presumed to bind both PI and PC and these data could be rationalized if both forms had separate effects. Under normal conditions, it is the ability of RdgBαI to inhibit the CDP-choline pathway that keeps DG levels high at the Golgi. An alternative pathway to keep DG levels high is to allow synthesis of sphingomyelin. SM synthase uses ceramide and PC to make sphingomyelin and DG. The lack of change in PI(4)P levels in the RdgBαI-knockdown cells could be explained if PI(4)P is sequestered at the Golgi by binding to CERT and OSBP and therefore does not get degraded. In the VAP-knockdown cells, CERT and OSBP are delocalized from the Golgi and therefore the PI(4)P is accessible to phosphatases and hence can turnover. It cannot be replaced in the absence of RdgBαI.

A plausible mechanism of action of RdgBαI at the Golgi can be suggested based on the published data: under normal conditions, RdgBαI transfers PI to the

Golgi, which gets phosphorylated to PI(4)P. RdgBαI also exerts an inhibitory effect on the synthesis of PC via the CDP-choline pathway, which has a sparing effect on DG. PI(4)P allows CERT to increase ceramide levels which, along with PC, is consumed by SM synthase to produce DG and SM. This leads to a drop in PC levels and increased DG levels required for transport out of the TGN. The drop in the PC levels causes RdgBαI to be released from the membranes. PI(4)P levels drop due to turnover and CERT and OSBP depart from the membranes. PC levels rise again because the inhibition on the CDP-choline pathway is removed, and an increase in PC levels allows RdgBαI to bind and release PI to the membranes. What this cycle suggests is that binding of RdgBαI to the Golgi is in part determined by the lipid composition, and increased PC levels are conducive to RdgBαI binding. This model could be tested by targeted depletion of the CDP-choline pathway from the TGN.

**RdgBαI and cell division**

RdgBαI localizes to the Golgi apparatus in interphase cells but is recruited to the cleavage furrow and midbody during cytokinesis, where it colocalizes with the small GTPase RhoA (Litvak et al., 2002b). This relocalization is regulated by protein kinases including Cdk1 and Plk1. Cdk1 phosphorylates RdgBαI at Ser382 and this facilitates its dissociation from the Golgi. The phosphorylated protein relocalizes to the cleavage furrow and midbody during cytokinesis and is then required for docking of Plk1, essential for completion of cytokinesis (Litvak et al., 2004). Plk1 binds to RdgBαI by binding of its Polo Box domain to the Cdk1 phosphorylation sites on RdgBαI. It is thought that the RdgBαI-Plk1 interaction is required for cleavage furrow ingression.

In summary, RdgBαI has multiple functions in cells. Not only is it important for Golgi secretory function and lipid droplet formation, but it also has important functions during cell division. These functions appear to be specific for mammalian cells, as knockdown of the single *Drosophila* RdgBα has a major impact on phototransduction alone.

***RdgBαIII***

In mammals, there is a third RdgBα protein, RdgBαIII (also known as PITPNM3 or Nir1), which is 974 aa residues long and lacks the PITP domain (Lev et al., 1999; Lev, 2004) (Figure 1). Mutations in this gene are the rare cause of autosomal dominant cone dystrophy in humans (Köhn et al., 2007, 2010). Two mutations have been identified, Gln626His which lies in the region that has been previously shown to interact with the tyrosine kinase PYK2 and Gln342Pro. It is not clear how mutations of these residues affect the function of the protein in the visual transduction system in humans.

## PITP function in Drosophila

As detailed above, mammals have five genes encoding proteins that contain a PITP domain; in flies, the situation is simplified. In *Drosophila*, there are three PITP proteins that are representative members of Class I and Class IIa and b PITP family in mammals: Dm-PITP (corresponding to mammalian PITPα/β), Dm-RdgBα (corresponding to mammalian RdgBαI/II), and Dm-RdgBβ (corresponding to mammalian RdgBβ) (Figure 4). Genetic analysis has revealed that these PITP proteins have specific functions in the fly. It is becoming clear that different organisms make distinct use of their PITP proteins, but in all cases the PITP proteins cooperate with lipid kinases to facilitate the synthesis of a phosphorylated derivative of PI. Dm-RdgBα is essential for phototransduction, whilst Dm-PITP is required for cytokinesis. In the fly, the visual transduction cascade is mediated by phospholipase C-catalyzed hydrolysis of $PI(4,5)P_2$ (Hardie and Raghu, 2001). A mammalian counterpart is not present as the visual transduction in mammals depends on guanylate cyclase/cyclic GMP.

***Dm-RdgBα***

*rdgB* was one of the first *Drosophila* retinal degeneration mutants identified (Hotta and Benzer, 1969) and was characterized by multiple phenotypes. Visual transduction in the fly is well-established (Hardie et al., 2001; Wang and Montell, 2007). Detection of light by the receptor rhodopsin leads to the activation of phospholipase Cβ (NorpA) via the heterotrimeric G-protein, Gq. Hydrolysis of $PI(4,5)P_2$ initiates the signaling cascade downstream to cause activation of Trp channels, the principal source of calcium influx into the *Drosophila* photoreceptors (Katz and Minke, 2009; Raghu and Hardie, 2009). How does RdgBα fit into the visual transduction pathway in the fly? Examination of the phenotypes provides some clues to its function.

The phototransduction defects are evidenced by abnormal termination of the light response and profound loss of the ERG amplitude shortly after initial light exposure. The mutation also causes a light-enhanced retinal degeneration. Lack of RdgBα function leads to derangement of the cell ultrastructure. The photoreceptor is a giant polarized cell whose apical domain forms the specialized light-sensing organelle, the rhabdomere. The rhabdomere consists of the closely packed photoreceptive microvilli and in the *rdgB* mutant, this rhabdomere membrane undergoes vesiculation and is internalized and lost resulting in reduction in the size of the apical membrane.

Dm-RdgBα localizes in the region immediately beneath the light-sensing rhabdomeric membrane, the subrhabdomeric cisternae (SRC), as well as in the

adjacent plasma membrane. The microvilli are devoid of Dm-RdgBα (Vihtelic et al., 1993; Suzuki and Hirosawa, 1994; Yoon et al., 1996). This subrhabdomeric region is continuous with the main ER system of the photoreceptor where PI synthesis occurs. Thus the close proximity of the SRC to the rhabdomeres could suggest that membrane contact sites between the rhabdomere and the SRC are regions of lipid transfer. The gap between the SRC and the photoreceptor membrane is ~10 nm, sufficiently narrow for the direct transfer of PI between these opposing membranes by the PITP domain of RdgBα, back and forth in accordance with the concentration gradient of PI (Figure 6A). PI is the precursor for the synthesis of $PI(4,5)P_2$, the substrate for phospholipase C (Figure 6A). The consumption of this lipid during phototransduction is rapid and intense and enzymes involved in the resynthesis of PI all lead to retinal degeneration. In principle, RdgBα is appropriately localized to replenish the plasma membrane pool of PI and lipid kinases in this compartment would regenerate the substrate (Figure 6A). Given that the PITP domain alone is sufficient to rescue the phenotypes of the *rdgB* mutant, this would imply that PI transfer is the essential function of Dm-RdgBα (Milligan et al., 1997).

The mutant Dm-RdgBα-T59E is unable to rescue the retinal degeneration phenotype yet partially rescues the light response. This residue is conserved in all PITP proteins (>130 sequences) and participates in binding to the inositol ring of PI. Mutation to glutamine abrogates PI binding and transfer in PITPα. It has been reported that the T59E mutant of the PITP domain of Dm-RdgBα is not compromised for PI transfer. An independent analysis of this mutant could not confirm this result (Cockcroft, Li, and Garner, unpublished). The T59E mutant of Dm-RdgBα is unable to transfer PI. Thus, we concluded that the ability to bind/transfer PI is critical for RdgBα function in flies.

Since $PI(4,5)P_2$ is the critical lipid required for cell signaling, and the activity of the phospholipase C results in consumption of $PI(4,5)P_2$, replenishment of PI to the rhabdomere offers an attractive mechanism for Dm-RdgBα function. Additional evidence that Dm-RdgBα functions within the context of G-protein-coupled $PI(4,5)P_2$ hydrolysis comes from genetic analysis of proteins that work within the PI cycle. The retinal degeneration phenotype of Dm-RdgBα is light-dependent in that it can be both protected by rearing flies in the dark and accelerated by rearing flies in a light/dark cycle. Such protection is observed in mutants that have reduced levels of the rhodopsin receptor or have been reared in vitamin A-deficient medium that reduces the levels of functional rhodopsin receptors (Stark and Sapp, 1987), and in *norpA* mutants that lack functional phospholipase Cβ (Paetkau et al., 1999). In contrast, retinal degeneration is accelerated in flies that express the constitutively active Gq. Retinas of double mutants of DGq1 with mutant *rdgB* degenerated even in the dark. DGq1 stimulation of *rdgB* retinal degeneration in the dark was NorpA-dependent (Lee et al., 1994). PI synthase (dPIS) is required for a key step during $PI(4,5)P_2$ regeneration, the production of PI. Overexpression of dPIS suppresses the retinal degeneration resulting from two other mutations affecting $PI(4,5)P_2$ cycling, *rdgB* (retinal degeneration B) and *cds* (CDP-DG synthase) (Wang and Montell, 2006).

Together these data provide compelling genetic evidence to support the idea that the *rdgB* mutant phenotype requires ongoing $PI(4,5)P_2$ hydrolysis. This conclusion is supported by another study that used an electrophysiological biosensor, the $PI(4,5)P_2$-sensitive Kir channel. The channel was targeted to the microvillar membranes. Depletion of $PI(4,5)P_2$ and its recovery during phototransduction was measured indirectly by recording the activity of the Kir channel. Comparison of wild-type and *rdgB* mutant flies showed that microvillar $PI(4,5)P_2$ levels in wild-type flies had recovered by ~30 sec after stimulation yet in *rdgB* mutant flies a recovery of no more than 50% wild-type levels was observed after several minutes (Hardie et al., 2001). This study provides the most direct evidence supporting a role for RdgBα in $PI(4,5)P_2$ resynthesis during cell signaling.

An additional role for phosphoinositides is in the recycling of arrestin. Arrestin trafficking is impaired in *rdgB* and *cds* mutants (Lee et al., 2003). The C-terminal domain of arrestin binds to $PI(3,4,5)P_3$ *in vitro*, and mutation of this site delays arrestin shuttling and results in defects in the termination of the light response, which is normally accelerated by prior exposure to light. Disruption of the arrestin/$PI(3,4,5)P_3$ interaction also suppresses retinal degeneration caused by excessive endocytosis of rhodopsin/arrestin complexes. These findings indicate that light-dependent trafficking of arrestin is regulated by direct interaction with phosphoinositides and is required for light adaptation. Since phospholipase C activity is required for activation of *Drosophila* phototransduction, these data point to a dual role of phosphoinositides in phototransduction (Lee et al., 2003). Both functions depend on RdgBα.

Although the function of the single *Drosophila* RdgBα in phototransduction has been characterized, mammals do not share the same signal transduction cascade for phototransduction. However, in recent studies, the intrinsically photosensitive retinal ganglion cells (pRGCs) have been shown to respond to light. The photopigment is melanopsin that transduces its signals via a G-protein coupled to phospholipase Cβ4 (Graham et al., 2008; Bailes and Lucas, 2010; Moldrup et al., 2010). These cells modulate behavioral responses to light including synchronization of the circadian clock to light/dark cycles, regulation of pupil size, sleep propensity, and pineal

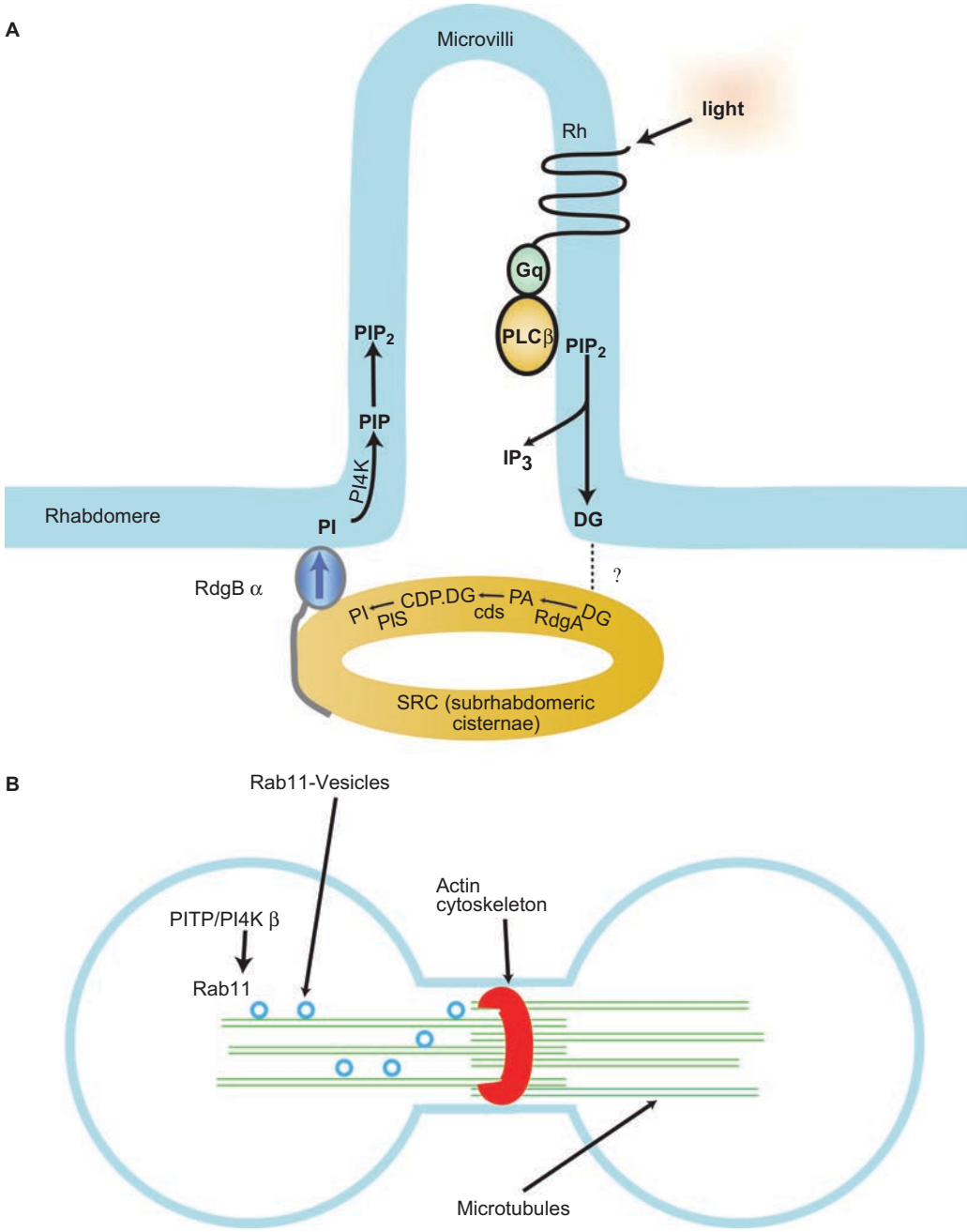


**Figure 6.** The function of PITP proteins in phototransduction and cytokinesis in *Drosophila*. (A) *Drosophila* photoreceptors are polarized cells where the apical domain (the rhabdomere) composed of microvilli is the site of phototransduction. Light-activated rhodopsin (Rh) initiates hydrolysis of PI(4,5)$P_2$ by phospholipase Cβ via the G-protein, Gq. The enzymatic machinery for recycling DG to PI resides in the subrhabdomeric cisternae (SRC), which is in close proximity to the rhabdomere. DG kinase (*rdgA*), CDP-DG synthase (cds), and PI synthase (PIS) successively transform the DG back to PI. RdgBα is localized to the SRC and could transfer PI to the PI 4-kinase (PI4K) residing in the rhabdomere. It is not known how DG is translocated from the rhabdomere to the SRC. (B) Cytokinesis in *Drosophila* spermatocytes is dependent on PITP (*gio*), PI 4-kinaseβ (*fwd*), and Rab11. Genetic analysis indicates that PITP and PI 4-kinaseβ lie upstream to Rab11. Rab11 regulates membrane addition to the advancing cleavage furrow.

melatonin production. Thus, this signaling cascade is similar to phototransduction in flies and potentially mammalian RdgBα might work in this pathway. RdgBαII expression is restricted to the retina and the dentate gyrus and could potentially function in the melanopsin signal transduction pathway in a manner similar to flies. The availability of the knockout mice for RdgBαII, which show no gross phenotype could now be studied for defects in light-sensitive behavioral responses such as regulation of pupil size (Lu et al., 2001).

***Dm-PITP***

Cytokinesis is the process that separates two daughter cells at the end of cell division. In animal cells, cytokinesis is mediated by an actomyosin ring that forms during late anaphase. This actomyosin ring assembles just beneath the equatorial cortex and constricts while remaining anchored to the plasma membrane, thus mediating furrow ingression (Figure 6B). Addition of new membrane is required for furrow ingression. At the end of cytokinesis, the actomyosin ring disassembles and the addition of new membrane seals the remaining intracellular bridge, leading to the final separation (abscission) of the daughter cells. Defects in meiotic cytokinesis in spermatocytes can be easily recognized, and mutations in *gio* and *fwd* were 2 of 19 genes identified, which were required for meiotic cytokinesis (Giansanti et al., 2001). *Gio* and *fwd* encode for a Dm-PITP and Dm-PI 4-kinaseβ, respectively, and were two out of eight mutants specifically required for regulation of the actomyosin ring constriction and cleavage furrow ingression (Gatt and Glover, 2006; Giansanti et al., 2006). Fwd was originally identified as a requirement for cytokinesis in spermatocytes (Brill et al., 2000). The same cytokinesis defect was observed in *gio* mutant neuroblasts in the brain. Mutations in Gio results in the abnormal localization of Golgi-derived vesicles at the cell equator; in wild-type spermatocytes, these vesicles lie at the poles and are excluded from the central region of the cells. Failure of fusion of these Golgi-derived vesicles with the invaginating furrow appears to be responsible for the defects in cytokinesis. The same vesicle phenotype was observed in *fwd* mutants. The acroblast is a structure that forms at the end of the second meiotic division through the aggregation and fusion of Golgi vesicles. In spermatids of *gio* mutants, acroblast assembly fails to occur and the vesicles are dispersed within the cytosol. These results indicate that membrane traffic of vesicles is essential for actin remodeling during animal cytokinesis, and PITP and a PI 4-kinaseβ are essential for this process.

In *Drosophila* spermatocytes, Rab11 is also required for cleavage furrow ingression. Rab11 regulates membrane addition to the advancing cleavage furrow (Giansanti et al., 2007; Polevoy et al., 2009). The phenotypes observed with *gio*, *fwd*, and *rab11* mutants are virtually identical and these defects include incomplete constriction of the actin rings, central spindle disorganization, and accumulation of Golgi-derived vesicles at the cell equator. Most interestingly, Rab11 recruitment depends on Gio and Fwd and mutant Rab11 is found to be associated with the Golgi-derived vesicles described above. Rab11 is not required for Gio localization, whereas Gio and Fwd are essential for Rab11 localization. Finally, Rab11 is also required for acroblast formation. This analysis indicates that a PITP and PI 4-kinaseβ are upstream to Rab11 (Figure 6B). In mammalian cells, an interaction between Rab11 and PI 4-kinaseβ is also observed and in this case, PI 4-kinaseβ is also required for Rab11 localization at the Golgi (de Graaf et al., 2004). Whether a PITP is required for Rab11 localization is not known in mammalian cells. Both PITPβ and RdgBαI are potential candidates as both localize to the cleavage furrow during cytokinesis (Litvak et al., 2004. Cockcroft and Carvou, 2007).

## PITP function in zebra fish

Zebrafish (*Danio rerio*) is increasingly becoming a popular model organism to study biological phenomenon. As a vertebrate, it resembles mammals in its development and gene knockdown by morpholino oligonucleotides is easily accomplished. Zebrafish harbor two copies of many genes owing to a genome-wide duplication event that took place after the bony fishes diverged from the common ancestor with humans some 350 million years ago. Examination of genes for PITP proteins in zebrafish indicate that there are three Class I PITPs rather than the two Class I PITPs found in mammals (Tilley et al., 2004; Cockcroft and Carvou, 2007; Ile et al., 2010). Of the three Class I PITPs, one is a homolog of PITPα (*PITPNA*) and two are homologs of PITPβ, namely zf-PITPβ (*PITPNB*) and zf-PITPβ-like (*PITPNBL*). Of the Class II PITPs, a homolog of RdgBβ is also present. The zf-RdgBβ is shorter than mammalian RdgBβ (289 aa compared with 332 aa) with the major difference being in the C-terminus. In addition, there is a RdgBα homolog lacking its PITP domain (Elagin et al., 2000), much like the mammalian RdgBαIII (Nir1) discussed above (see also Figure 1). Zf-RdgBα possesses the FFAT motif followed by the DDHD domain and LNS2 domain.

Class I PITPs from zebra fish possess transfer activity similar to mammalian PITPs and are able to rescue the growth and secretory defects associated with the temperature-sensitive *SEC14* mutant in yeast, similar to that observed for mammalian Class I PITPα and PITPβ (Ile et al., 2010). Mammalian Class I PITPs stimulate PI(4)P production when reconstituted in mammalian cells as well as in a yeast reconstitution assay. Zebra fish PITPs also reconstitute PI(4)P synthesis and this is dependent on a PITP that is able to bind PI (Ile et al., 2010). Thus, the biochemical properties of zebra fish PITPs are indistinguishable from mammalian PITPs.

The role of zf-PITPα in zebra fish embryos has been investigated using antisense morpholino oligonucleotides to knockdown expression. Microinjection of PITPα morpholinos results in developmental defects that are grossly similar to morpholino knockdown of the netrin receptor neogenin (Mawdsley et al., 2004; Xie et al., 2005). A loss of spinal cord neurons is observed and, when present, defects in motor-axon outgrowth. The similarity in phenotypes observed in both neogenin and

PITPα morphant embryos supports the view of PITPα as a downstream mediator of neogenin signaling in zebra fish embryos similar to that observed in mammalian cells (Xie et al., 2005).

Zebrafish PITPβ is also present as two variants (PITPβ-sp1 and sp2) and are expressed ubiquitously throughout the fish embryo during development. Expression is observed at 6 and 24 h postfertilization, at 6 days postfertilization, and in adult fish. PITPβ expression in the eye is particularly high and is restricted to the double cone photoreceptor cells. Double cone photoreceptor cells are comprised of one long and one short cone cell fused along most of its length and when morpholinos are injected, defects are found in the outer segment of the cells. No other developmental defects are found. Of note is the loss of retinal arrestin 3-like (Arr3l) protein in morpholino-treated embryos. When morpholinos against Arr3l are used, no obvious phenotypes are observed and the staining of zf-PITPβ is unaffected. The impact of PITPβ deficiency is overcome at later times and the double cone photoreceptor cells subsequently develop normally. Morpholinos against PITPβ-like proteins when injected causes no significant changes in development. Whether the two PITPβ proteins compensate for each other and therefore need to be knocked down simultaneously would be interesting to explore.

## Planaria: PITP and stem cell proliferation

Although we have focused on phenotypes in mice, *Drosophila* and zebra fish as examples, mention should be made of the identification of a PITP required for regeneration in the model system, *Planaria*. This organism is used to study regeneration following wounding where a stem cell population begins to proliferate and subsequently differentiate to replace the wounded structures. PITP is required for stem cell proliferation (Reddien et al., 2005). This observation accords with PITPβ that appears to be essential for stem cell proliferation as mouse stem cells ablated of PITPβ are inviable (Alb et al., 2002).

## Conclusions

### *What is the underlying mechanism for PITP function?*

We provide a snapshot of the functions known currently of the different PITPs in the three organisms discussed above (Figure 4). Distinct phenotypes are observed in mammals and flies when specific PITP proteins are knocked down either in genetic models or in cultured cell lines. The picture that emerges is that PITPs operate in many biological situations including neurite path finding and outgrowth, membrane vesicle traffic, cell signaling, and cytokinesis.

The fundamental biochemical property of all the PITPs tested to date is the ability to bind PI, and therefore the simplest way to rationalize these observations is that PITPs permit the maintenance of specific pools of phosphorylated PI (the possibility that PI itself is bioactive should not be excluded). Phosphorylated PIs play diverse roles in cell biology (Di Paolo and De Camilli, 2006; Lemmon, 2008; Kutateladze, 2010), which could explain why the effects of PITP knockdowns are not always predictable. Also, since several PITPs are expressed in the same cell, it is likely that there is some level of redundancy. From the summary shown in Figure 4, two observations can be made: first, knockdown of a single PITP can give rise to a number of cell-specific defects and second, PITP orthologs give different phenotypes depending on the organism. The real challenge will be to identify the different networks that PITPs engage in and examine their requirement on a case-by-case basis.

### *PITPs—are they really lipid transporters that function by PI delivery?*

The most challenging question is whether PITP proteins simply function as lipid transporters or by some undefined mechanism? The minimal domain Class I PITP proteins, PITPα and PITPβ are certainly capable of lipid transfer and intuitively, it provides a logical explanation for their mode of action. However, arguments that militate against PITPs functioning as a simple delivery system can be put forward: in intact cells, both PITPα and PITPβ are constantly sampling the membranes and undergoing a change in conformation from the closed soluble form to the open membrane-associated form (Shadan et al., 2008). PITPs dock on the surface where the WW residues reside facing the membrane and the regulatory region away from the membrane interface (Figure 2). This is the basal activity under resting conditions. The results with the studies using NEM to trap the PITPs onto membranes support this interpretation.

When cells are stimulated, PITPs are immobilized at the membrane by binding to a membrane-associated component such as the receptor (Kauffmann-Zeh et al., 1995; Larijani et al., 2003; Xie et al., 2005). In the case of PITPα, stimulation with either EGF or netrin-1 causes recruitment and greater interaction with PI at the membranes. In the case of PITPβ, which is already associated with Golgi, ER, and nuclear envelope membranes, the interaction of the membrane-associated pool with PI is enhanced following stimulation (Larijani et al., 2003). We would argue that the local increase in concentration at specific sites is the mechanism that PITPs use in executing their function (Figure 5). Immobilized PITPs at the membrane by definition do not deliver lipids from one

membrane compartment to another, but we propose that they participate in a metabolic reaction. During stimulation, PITPα can get phosphorylated in its regulatory loop and this affects the activity of the protein. We propose that PITPs can also dock on the membrane in the opposite orientation following stimulation. We further propose that at the membrane the protein undergoes a greater change in structure than that observed in the apo-structure. This interpretation is supported by the knowledge that PITPα does get phosphorylated at Ser166 by protein kinase C *in vivo* following stimulation despite being inaccessible in the apo-structure (Morgan et al., 2004).

What does the PITP do at these targeted sites? One possibility is that the recruitment of PITP to the membrane affects membrane structure. Alternatively, PITPs make PI accessible to the lipid kinases for phosphorylation. Prior to the knowledge of the PITP crystal structures, it had been proposed that PI bound to PITPα could be presented to the lipid kinases for phosphorylation and subsequently to phospholipase C (Cunningham et al., 1995; Cockcroft, 1998). The general concept was that the lipid kinases could not readily access membrane PI in the bilayer and that PITPs could extract it and present it to the lipid kinases for phosphorylation. This model is not tenable if it is assumed that PITP can only dock on the membrane with the C-terminus facing the membrane. In this case, the inositol head group would not be accessible to the lipid kinases for phosphorylation. However, if PITPs undergo a more drastic structural change such that the regulatory loop is sufficiently disordered and PITPs can dock in the opposite orientation then the inositol head group could be accessible to the lipid kinases. It has been repeatedly observed that PITPs affect lipid kinase activity. PITPα and β can enhance the activity of PI 3-kinase and PI 4-kinase *in vitro* and when added to permeabilized cells (Fensome et al., 1996; Kular et al., 1997, 2002; Panaretou et al., 1997; Way et al., 2000). Although there is a lack of specificity in these *in vitro* situations, we would argue that other mechanisms bring a specific PITP in close proximity to the membrane such as cell surface receptors that interact with the C-terminus of the PITPs. At the membrane, the protein could undergo major structural rearrangements that allow PI bound to PITP to get phosphorylated. Designing experiments to test this hypothesis *in vivo* is the next challenge but the ability of PITPs to bind PI is an essential activity required for PITPs to function. Mutations that cause loss of PI binding are inactive in all the various assays tested including expression from a transgene in mice (Alb et al., 2007).

Our studies have revealed that under basal conditions, both PITPs sample membranes continuously and that upon stimulation the resident time at the membrane is increased. We proposed that when PITPs engage in a complex with other proteins, they become functionally active (Figure 5). We suggested that the increase in resident time is required for PITPs to undergo a structural change that allows access to the inositol head group for phosphorylation to take place. Here, we suggested that "function" should be taken to mean the ability of a PITP protein to cooperate with a lipid kinase to generate site-specific phosphorylated PI. Genetic evidence from *Drosophila* show that the single Class I PITP functions together with PI 4-kinaseβ (Giansanti et al., 2007), whilst studies in *Caenorhabditis elegans* show that a Class I PITP (CE: 24759) functions together with Vps34, a Class III PI 3-kinase (Lee et al., 2008).

Can this concept include the RdgBα proteins where the PITP domain is part of a multi-domain protein? The PITP domain is always at the N-terminus of the proteins and the C-terminus forms the lid in PITPα and PITPβ. Under resting conditions, it is the dislodgement of the lid that is proposed for release of lipid. In the RdgBα proteins, the C-terminus is very long and contains a number of additional domains. Thus, its ability to dislodge the entire C-terminus may be limited. However, if the lipid kinases can get access via the regulatory loop as proposed for the single domain PITPs, the concept can be easily accommodated for both the single domain proteins as well as the proteins that contain a PITP domain. One prediction is that when the PITP domain is part of a multi-domain protein, these proteins will not exhibit basal lipid transfer as seen for Class I PITPs.

For the RdgBα proteins, this model would work equally well if the PITP domain of the RdgB proteins was responsible for providing PI to the lipid kinases in an opposing membrane. In the *Drosophila* photoreceptors, RdgBα is associated with the subrhabdomeric region that is part of the ER but the lipid kinases are present on the opposing plasma membrane. During stimulation by light, engagement of the PITP domain of RdgBα by the proteins of the opposing membrane would stimulate the regeneration of the substrate for phospholipase C. In mammalian cells, RdgBαI is reported to be localized to the Golgi and ER. ER localization is due to the FFAT motif that binds to integral VAP proteins. ER-localized RdgBαI could potentially regulate Golgi-localized PI kinases when the two membranes are in close proximity.

In Figure 5, we provided two alternatives modes of PITP action following stimulation. Under resting conditions, PITPs are constantly sampling membranes and dock with the C-terminus facing the membrane. Under these conditions, PI kinase activity is minimal. Following stimulation, PITPs accumulate on the membranes and could be oriented as under resting conditions (Model A) or alternatively, PITPs could dock in a different orientation with the regulatory loop facing the membrane (Model B). In this orientation, PITPs undergo a more dramatic structural change that allows the lipid kinases to have access to the inositol head group, although the lipid is still localized on the PITP protein.

### *Are PITPs lipid sensors that regulate lipid homeostasis?*

An alternative model for PITP function is that they are lipid sensors. Such a model has been previously presented for Sec14p (LeBlanc and McMaster, 2010). For the lipid sensor model to work, PITP would need to "sense" the lipid composition of specific membranes and be able to convey this information to downstream effectors. To date, it is not clear how PITP proteins can sense the lipid environment and thus regulate lipid homeostasis. Overexpression of PITPα or PITPβ does not affect lipid homeostasis and neither are there any differences in the rates of PI or PC turnover when PITP proteins are depleted using RNAi (Ségui et al., 2002; Carvou et al., 2010). There is no experimental evidence available to suggest that PITPs could function in this manner.

### *Are there separate functions for PI- and PC-loaded PITP proteins and are both activities essential for function?*

When a PITP molecule is bound to either PI or PC, can the cellular environment such as the membranes sense the cargo lipid? Taking the case of PITPα, the structures of the PI- and PC-loaded PITPs are superficially similar; however, the major difference between the two is the charge of the protein. This charge difference is the basis for the separation of the PI-loaded and the PC-loaded protein by isoelectric focusing or by anion-exchange chromatography. PITPα loaded with PI is more negatively charged and associates with lipid vesicles more strongly compared with PC-loaded PITPα. Intracellular membranes have different charges (Yeung et al., 2006; Yeung et al., 2008) as well as different lipid compositions (van Meer et al., 2008). Thus, it is an intriguing possibility that PITPs are recruited at specific membrane domains and the charge of the PITP proteins can also influence the outcome. Phosphorylation of proteins also affects charge and the splice form, PITPβ-sp1 is constitutively phosphorylated at Ser262 (Van Tiel et al., 2002; Morgan et al., 2006). Although mutation of this residue has no impact on transfer activity when assessed *in vitro*, the possibility that this phosphorylation has functional consequences remains to be analyzed.

To assess whether both the PI and PC binding/transfer activity of PITP proteins is required *in vivo*, mutants deficient in PI and PC binding/transfer were examined for their ability to rescue COP1-mediated retrograde transport in PITPβ knockdown cells (Carvou et al., 2010). These mutants were unable to rescue the retrograde transport phenotype. This is intriguing as mutants defective in PC binding/transfer should in principle still promote PI(4)P synthesis. Thus, it would appear that PITPβ does more than just support PI(4)P synthesis; PC-loaded PITPβ may regulate other activities that are required for the protein to execute its function at the Golgi. Whether both activities need to reside in the same protein was not assessed and coexpression of the two mutants would be able to address this question in the future. Whether all the biological activities of PITP depends on both PI and PC binding/transfer will need to be assessed not only for the individual PITPs but also for the different biological functions that PITPs can support. A single PITP such as PITPα is required for phospholipase C signaling, axonal outgrowth, as well as chylomicron assembly. Whether PC binding is essential for all these activities remains to be explored.

## Perspectives

Since the first discovery of PITPα as a protein that can transfer PI *in vitro*, this activity has influenced our thinking of how PITPs could function *in vivo*. Thus, it is counter intuitive to suggest that the transfer activity is not the key to PITP function but that the ability to bind PI and to be recruited to the membrane is the essential activity. Knowledge of the PITP structure at the membrane surface under conditions when PITPs are functionally "active" would greatly assist in our understanding—in particular, knowledge of the potential orientations of the PITP molecule at the membrane interface would provide a valuable insight into its actions.

Future studies should be directed at tracking single molecules of PITPs loaded with their specific lipid cargoes in living cells and to follow their itinerary as they go about their business. We favor a model where the single domain PITPs constantly undergo lipid exchange as part of their basal activity—this would not consume any energy as lipid transfer *in vitro* is not dependent on ATP. Following stimulation of the cells, PITPs are recruited and accumulate at the membrane and in some way are permissive for PI phosphorylation. We present two potential models and further experiments will be required to distinguish between them. PITPs are fascinating proteins and many questions remain. Because of their involvement in multiple pathways, an understanding of their basic function will give clues as to how these proteins participate in diverse biologies including neural and intestinal function, cytokinesis, and stem cell viability.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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