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Synthesis and Function of Membrane Phosphoinositides in Budding Yeast, *Saccharomyces cerevisiae*

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Abstract

It is now well appreciated that derivatives of phosphatidylinositol (PtdIns) are key regulators of many cellular processes in eukaryotes. Of particular interest are phosphoinositides (mono- and polyphosphorylated adducts to the inositol ring in PtdIns), which are located at the cytoplasmic face of cellular membranes. Phosphoinositides serve both a structural and a signaling role via their recruitment of proteins that contain phosphoinositide-binding domains. Phosphoinositides also have a role as precursors of several types of second messengers for certain intracellular signaling pathways. Realization of the importance of phosphoinositides has brought increased attention to characterization of the enzymes that regulate their synthesis, interconversion, and turnover. Here we review the current state of our knowledge about the properties and regulation of the ATP-dependent lipid kinases responsible for synthesis of phosphoinositides and also the additional temporal and spatial controls exerted by the phosphatases and a phospholipase that act on phosphoinositides in yeast.

1. Introduction

Phosphoglycerides comprise a major class of the phospholipids in biological membranes. These phospholipids consist of a glycerol backbone to which are esterified two fatty acids (at the 1- and 2-hydroxyls) and an inorganic phosphate (to the 3-hydroxyl, following standard nomenclature). The phosphate moiety can (except in the case of phosphatidic acid, PtdOH) be esterified, in turn, to one of a variety of specific substituents (ethanolamine, PtdEth; choline, PtdCho; serine, PtdSer; and so forth). The phosphate and its substituent are referred to as the head group. The head group of phosphatidylinositol (PtdIns) is unique among phosphoglycerides in the sense that three of the hydroxyl groups of the six-membered inositol ring are subject to reversible covalent modification by phosphorylation (Figure 1). Thus, PtdIns is a substrate for a number of different lipid kinases [1–3] and phosphatases [3–7], and can yield an ensemble of different, biologically active derivatives, collectively termed phosphoinositides (PIPs). Moreover, several PIPs are substrates for phospholipases [8–10], thereby generating a number of products that serve as second messenger molecules with biological functions on their own. PIPs also serve as specific membrane-anchored determinants for the recruitment of a wide range of proteins, which can interact with these lipids via a conserved set of modular binding motifs [11–15]. Therefore, PIPs are ideally suited to serve as key regulators of fundamental biological processes in all eukaryotic cells.

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In the 1940s, inositol was found as a natural constituent of brain phospholipids [16,17], although the exact chemical structure of these lipids remained unknown for some time until subsequent studies addressed this question in more detail [18–22]. In 1953, Mabel and Lowell Hokin demonstrated that certain phospholipids, especially PtdIns, are rapidly metabolized by cells in response to external stimuli, thereby founding the new discipline of lipid signaling [23,24]. Thereafter, and for the past six decades, PtdIns and its derivatives have been the focus of intense scientific study, owing largely to the recognition that these might not be solely structural components of the lipid bilayer, but also key regulators of a variety of different cellular processes.

The field received substantial new impetus with the discovery in the early 1980s that PtdIns $[4,5]P_2$, which had been suggested to be produced by successive phosphorylation of PtdIns [18,25,26], might serve as the major substrate of phospholipase C isoforms (PLC) (Figure 2A). The archetypical PtdIns$[4,5]P_2$-initiated signal transduction pathway [27] involves hydrolytic degradation of PtdIns$[4,5]P_2$ by a receptor-stimulated phospholipase C (PLC) in response to external stimuli [28–32], thereby generating two second messengers: 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins$[1,4,5]P_3$). In animal cells, DAG activates certain isoforms of protein kinase C (PKC) [33–35], ultimately leading to changes in gene expression, whereas Ins$[1,4,5]P_3$ (IP3) binds to a group of intracellular receptors that interact with calcium channels, causing the release of Ca^{2+} from intracellular stores [33,36–39], triggering Ca^{2+}-dependent responses. Moreover, important physiological roles for more highly phosphorylated, water-soluble inositol-phosphates derived from IP3 are emerging [600,601].

Furthermore, over the last decade and a half, evidence emerged demonstrating functions for PIPs in a range of cellular processes, beyond the use of PtdIns$[4,5]P_2$ as a precursor to second messengers. A role for PtdIns and its derivatives in vesicle-mediated membrane trafficking first arose from genetic studies in the yeast *Saccharomyces cerevisiae*. It was found that the gene corresponding to one of the classical secretion-defective mutants isolated by Schekman and his colleagues, *sec14* [40], encoded a PtdIns transfer protein (PITP) that is essential for yeast Golgi function [41]. It was then discovered by Emr and coworkers that a gene product, Vps34, necessary for protein transport between the Golgi apparatus and the vacuole (the yeast equivalent to the mammalian lysosome) [42] encoded a PtdIns 3-kinase [43]. Likewise, a PtdIns 4-kinase, Pik1, was implicated in generation of exocytic vesicles from the Golgi by work from this laboratory [208] and others [210]. Further studies revealed that PIPs exert their function in vesicular trafficking, at least in part, via their ability to recruit various proteins to the cytoplasmic face of intracellular membranes. This membrane interaction is achieved by the docking of certain classes of modular domains present in these proteins, which recognize the head group of a cognate PIP [44–47]. Over the past several years, an ever-growing number of such PIP-specific binding modules have been discovered [11,14,15,48–52]. Perhaps the best-studied class of these PIP-binding motifs is the pleckstrin homology (PH) domain [53–58], which serves to anchor proteins to membranes by mediating protein-lipid and, in some cases, protein-protein interactions [59,60].

In this article, we provide an overview of the various PIPs that have been identified as physiologically relevant to the biology of *S. cerevisiae*. We also review what is currently known about the enzymes responsible for synthesis, interconversion, and breakdown of these PIPs. Budding yeast has proven to be an organism in which previously unsuspected roles for PIPs have been uncovered and analyzed incisively. For example, roles for PtdIns[3]P in endosomal and vacuolar membrane trafficking, for PtdIns[4]P in secretion, and for PtdIns$[4,5]P_2$ in actin cytoskeleton remodeling were demonstrated for the first time in this organism. Use of yeast remains at the forefront of research on the physiological functions of PIPs.

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2. Phosphoinositide kinases

The majority of PtdIns kinases have been conserved during evolution. Members of each class of these enzymes can be found in organisms as diverse as unicellular yeasts, filamentous fungi, worms, insects, vertebrates, and plants, suggesting important roles in cellular processes in all eukaryotic organisms. Historically, nearly all of these PtdIns kinase isoforms had only been described as enzymatic activities that could be separated chromatographically. Hence, they were classified originally according to their distinctive biochemical properties, such as whether they could be stimulated by certain detergents or inhibited by adenosine, resulting in categorization of PtdIns kinases into classes I, II, and III (Table 1) [61–63]. Later on, these kinases were further classified into three subgroups, according to the products they generated or their substrate specificity: PtdIns 3-kinases, PtdIns 4-kinases, and PtdInsP (PIP)-kinases [64–66]. The first enzymes to be described in more detail were the so-called Type II kinases [67–69], a group of PtdIns 4-kinases originally purified from a number of mammalian sources [61,63,70–74].

Studies in the yeast *S. cerevisiae*, an organism that had been shown in the early 1970s to possess PtdIns kinase activity [75], lead to isolation of the first genes identified in any organism that encode PtdIns kinases. In 1993, *VPS34* was found to encode a PtdIns 3-kinase [43]; and, in the same year, *PIK1* was shown to encode a PtdIns 4-kinase of Type IIIβ [76]. To date, PtdIns[3]P [78], PtdIns[4]P [79], PtdIns[3,5]P_2 [80], and PtdIns[4,5]P_2 [79] have been detected in *S. cerevisisae* (Figure 2A). PtdIns[3,4,5]P_3 has been detected in fission yeast (*Schizosaccharomyces pombe*), but not in *S. cerevisiae* [81–83].

In the following sections, we review the general biochemical properties of the members of the different PtdIns kinase classes that are present in *S. cerevisiae* cells and provide an overview of our current understanding of their physiological functions.

2.1 PtdIns 3-kinases

The first PtdIns 3-kinase ever cloned was *VPS34* from *S. cerevisiae* [43] during the course of characterizing the genes corresponding to mutations that had been identified earlier as causing defects in vacuolar protein sorting (Vps) [84–87]. However, the fact that *VPS34* encodes a PtdIns 3-kinase arose only after it was realized that its COOH-terminal half shares significant homology with the catalytic subunit (p110) of mammalian Type I PtdIns 3-kinase [88]. In addition to its conserved COOH-terminal catalytic domain, Vps34 contains two other motifs (Figure 3A) that share a high degree of homology with other PtdIns kinases, namely a so-called lipid kinase unique (LKU) domain of unknown function and a C2 domain, a motif that has been implicated in protein-lipid interactions [13,15,48, 48a] and more recently in binding to phosphotyrosine-containing sites in proteins [48b]. It was shown that *vps34*Δ cells lack PtdIns 3-kinase activity [42], which had been detected before in wild-type yeast [78], demonstrating that Vps34 is a functional PtdIns 3-kinase *in vivo* and the sole source for producing PtdIns[3]P in *S. cerevisiae*. Moreover, it was found that Vps34 phosphorylates only PtdIns at the D3 position of the inositol ring, and will not use PtdIns[4]P or PtdIns[4,5]P_2 as substrates [89], unlike most of the then known (Type I) mammalian PtdIns 3-kinases [90]. Subsequently, PtdIns 3-kinase isoforms highly related to and with the same restricted substrate selectivity as Vps34 also were found in animal cells [91a]. Vps34 also differed (Table 1) from the mammalian Type I enzymes in being relatively insensitive, even in *in vitro*, to two inhibitors, wortmannin and LY294002, widely used at the nM level to block mammalian Type I PtdIns 3-kinases both *in vivo* and *in vitro* [89,91,92].

Early studies on *vps34* mutants, utilizing both light and electron microscopy, revealed morphologically intact vacuoles (the yeast vacuole is functionally equivalent to the mammalian lysosome), which receive protein traffic from both the secretory and endocytic pathways, but

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also a number of different abnormal membranous structures in the cytoplasm of these cells [93]. Cells harboring a *vps34*Δ (null) mutation [43] are viable under normal conditions and display phenotypes that are very similar to those of the original *vps34* mutants, including an intact secretory pathway [86,94], which shares its machinery in the early stages with the vacuolar protein sorting (Vps) pathway [95,96]. Proteins following the Vps pathway are sorted away from secretory cargo and plasma membrane proteins only at a late stage of the Golgi apparatus, and, hence, receive all the modifications that take place in the ER and the early Golgi, such as proteolytic processing and glycosylation. This routing is best exemplified by the soluble vacuolar hydrolase, carboxypeptidase Y (CPY, also called proteinase C), product of the *PRC1* gene [96,97]. CPY is first synthesized as an inactive precursor, prepro-CPY, that is released into the ER lumen (Figure 4). Here, CPY undergoes proteolytic removal of its NH_2-terminal hydrophobic signal peptide and receives another posttranslational modification in the form of an NH_2-linked core oligosaccharide. The resulting intermediate form, p1CPY, is then transported to the Golgi apparatus, where further oligosaccharide modifications take place, yielding another intermediate species, p2CPY. Under normal physiological conditions, p2CPY is then sorted away from proteins destined for the cell surface at a late compartment of the Golgi apparatus and shunted instead to the vacuole. Upon delivery of p2CPY to the vacuole, it is processed further by another resident vacuolar protease to generate active mature CPY. Defects in the sorting step in the late Golgi apparatus lead to missorting and delivery of p2CPY to the cell surface. Such non-physiological secretion of CPY was exploited by a number of groups as a means to isolate mutants, like *vps34*, defective in vacuolar protein sorting [84, 86,87,94,98]. Taken together, these early studies suggested a role for the *VPS34* gene product in the process of sorting proteins to the vacuole, rather than in biogenesis of this organelle itself.

In a strategy designed to identify novel factors defective in endocytosis, Munn et al. provided genetic evidence that sorting of soluble hydrolases from the Golgi apparatus to the vacuole was connected to the process of endocytosis in *S. cerevisiae* [99], in good agreement with other findings [100–102]. To do so, they exploited the properties of *vat2*Δ cells. *VAT2* encodes one of the multiple subunits of the membrane-associated vacuolar H^+-ATPase [103–105] and cells lacking Vat2 inefficiently assemble the holoenzyme on vacuolar membranes and, as a consequence, are unable to acidify the vacuole efficaciously [106,107]. The *vat2*Δ cells are able to grow at pH 5.5, but not at pH 7. It was proposed that *vat2*Δ cells survive at pH 5.5 because fluid phase-endocytosis is sufficient to acidify endocytic compartments and, subsequently, the vacuole [107,108]. On this basis, they screened for mutations that prevented growth of *vat2*Δ cells even at pH 5.5 in an attempt to find novel mutations that cripple fluid-phase endocytosis. However, several of the mutations identified this way turned out to affect genes known to play roles in vacuolar protein sorting rather than endocytosis *per se*, including *VPS34*. These results suggested the existence of a prevacuolar endosomal compartment (PVC) (Figure 4), common to both the endocytic and vacuolar protein sorting pathways [99,102].

Comparison of different *vps* mutants revealed that *vps34* and *vps15* mutants have remarkably similar phenotypes [43,93,94,109], suggesting that these gene products might act together at the same step in vacuolar protein sorting. Additional work demonstrated that *VPS15* encodes a serine/threonine-protein kinase (Figure 3A) that is stably associated with membranous compartments via an NH_2-terminal myristoyl moiety [109,110]. Mutations of conserved residues within its kinase domain result in the missorting and secretion of soluble (lumenal) vacuolar hydrolases, like CPY [42,110]. Furthermore, Vps15 and Vps34 physically associate with each other in hetero-oligomeric complexes (Figure 5) and this interaction is required for stable membrane recruitment of Vps34 [111]. Interaction between Vps34 and Vps15 requires a short 28 residue-element near the COOH-terminus of Vps34 and two regions of Vps15, the NH_2-terminal protein kinase domain and the three HEAT repeat motifs in the center of the protein (Figure 3A) [112]. Interaction with Vps15 stimulates the enzymatic activity of Vps34

(>10-fold) [113]. Physiological significance of this interaction is supported by the finding that kinase-domain mutants of Vps15 result in vacuolar protein sorting defects that can be suppressed by overexpression of Vps34 [111]. In addition, the PtdIns 3-kinase activity of Vps34 is essential for proper targeting of certain (but not all) soluble vacuolar proteins to this organelle, whereas it appears not to be required for its interaction with Vps15 [113].

Work from quite a number of different groups classified the known set of more than 40 *VPS* genes into six categories, according to their mutant phenotypes: classes A – F [93,100]. Based on this scheme, both *VPS15* and *VPS34* are categorized as class D genes, which typically display only moderately aberrant vacuoles, temperature-sensitive growth, missorting of vacuolar proteins, and defects in vacuole segregation during mitosis [114,115].

Intracellular PtdIns[3]P levels rapidly decrease upon inactivation of Vps34 [113]. However, it was unclear what other factors are responsible for maintaining or modulating the cellular levels of this lipid. To address this issue, Wurmser et al. investigated PtdIns[3]P level in yeast cells carrying null mutations of the genes encoding the vacuolar t-SNARE, Vam3, and the Rab-like GTPase, Ypt7 [116], which had both been ascribed important roles in endosome-to-vacuole trafficking in other studies [117,118]. Deletion of either gene led to a markedly increased level of PtdIns[3]P (~ 5-fold), when normalized to PtdIns[4]P content. By contrast, cells harboring a temperature-conditional *vam3* mutant displayed a milder and opposite effect (a ~ 2-fold decrease in PtdIns[3]P) at the restrictive temperature. None of these alterations had any effect on the enzymatic activity of Vps34, suggesting that, normally, *VAM3* and *YPT7* somehow contribute to PtdIns[3]P turnover and that endosome-to-vacuole transport may be required for this process [116]. In this regard, initial studies suggested that hydrolysis of PtdIns[3]P to PtdIns occurs upon invagination of PtdIns[3]P-containing vesicles into the vacuolar lumen upon formation of so-called multivesicular bodies (MVBs) and is dependent on vacuolar hydrolases [116,120], similar to what had been reported for plant cells [119]. However, it has been shown more recently that the myotubularin-related phosphatase, Ymr1, and two synaptojanin-like phosphatases, Inp52/Sjl2 and Inp53/Sjl3, are directly responsible for conversion of PtdIns[3]P back to PtdIns [457] (see 3.2 and 3.3 below). How the action of these enzymes is coupled to the process of MVB formation is not yet clear [455].

An obvious question is what proteins act as effectors of the vacuolar sorting pathway by binding directly to the PtdIns[3]P generated by Vps34. An important clue was provided by the finding that a mammalian early endosome antigen (EEA1) co-purifies with PtdIns[3]P-containing liposomes and that its localization to endosomal compartments is blocked by wortmannin [125]. A Cys-rich motif in EEA1 with unique sequence features reminiscent of Zn^{2+}-binding RING domains was also found in two yeast proteins, Vps27 and Vac1/Pep7/Vps19, known to play roles in vacuolar protein sorting [126–129] and in Fab1, a protein found to be important for vacuolar membrane homeostasis and now known to be a PtdIns[3]P 5-kinase [130] (see 2.3.1 below). For these reasons, attention focused on the proteins containing this motif, which was dubbed the FYVE domain (for Fab1, Ydr313c/Pib1, Vps27 and EEA1) [121,122]. GFP-fusions of FYVE domains from a number of proteins, including mammalian EEA1 and yeast Vps27, Vac1 and Pib1 localized mainly to cytoplasmic puncta [120,123]. These structures were identified as prevacuolar endosomes (PVC) by colocalization with the lipophilic dye, FM4-64, which is transported from the plasma membrane to the vacuole via the endocytic pathway [131]. This subcellular localization pattern was lost when a single residue required for Zn^{2+}-coordination by the FYVE domain was mutated and when GFP-fusions of FYVE domain proteins were expressed in *vps34* cells [120,123]. Other biochemical and biophysical evidence confirms that the FYVE domain serves as a specific PtdIns[3]P-binding element [123,124]. For example, bacterially-expressed FYVE domains lacking any yeast-specific modification interact uniquely with PtdIns[3]P-containing liposomes, and not with vesicles containing any other PIPs [123]. Thus, the FYVE domain is a modular PtdIns[3]P-binding element and

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proteins that contain it are downstream effectors of this PIP [51]. Although the FYVE domain and its interaction with PtdIns[3]P may be necessary for the function of these Vps factors, it may not be sufficient. In this regard, it has been suggested that Vac1 may require multivalent tethering for its function in the Vps pathway and thus may integrate different input signals. To permit its association with the endosomal t-SNARE, Pep12/Vps6, Vac1 seems to require interaction with the GTP-bound form of the Rab GTPase, Vps21, as well as the Vps34-dependent presence of PtdIns[3]P at endosomal membranes [127].

To complicate matters, subsequent analysis has shown that Vps34 and Vps15 form several different multi-subunit complexes [132]. These complexes contain distinct subunits that interact with the Vps34-Vps15 heterodimer and function in separate membrane trafficking processes (Figure 5). One complex is composed of Vps34-Vps15, Vps30, and Apg14 (Complex I) and functions in macroautophagy [133], an evolutionary conserved mechanism for non-selective degradation and recycling of long-lived proteins and organelles, which is induced in *S. cerevisiae* by nitrogen or carbon starvation or the drug, rapamycin (Figure 6) [134–140]. Upon induction of this process, cytoplasm and organelles are enclosed into a double membrane structure, termed the autophagosome, which eventually fuses with the vacuolar membrane, thereby releasing the inner membrane and enclosed contents into the vacuolar lumen, where degradative enzymes reside [134]. In this manner, amino acids and nucleotides are recycled and, hence, cells are able to survive during conditions of prolonged nutrient deprivation and, in diploids, can muster the resources to induce the sporulation process. This same complex is also thought to play a role in the Cvt (cytoplasm-to-vacuole targeting) pathway (Figure 6) [132,135,138,140], a specific version of autophagy that functions constitutively even under nutrient-rich conditions, in contrast to starvation-induced macroautophagy [141,142]. It has been suggested that this autophagy-specific Vps34-Vps15 complex localizes to [143] and controls the organization of the pre-autophagosomal structure (PAS), presumably the site of autophagosome and Cvt vesicle formation adjacent to, but morphologically different from, the vacuole, perhaps by sorting/selecting cargo during vesicle budding [132,144].

The second and more abundant Vps34-Vps15 complex (Complex II) contains Vps30, but also Vps38 (Figure 5), and plays a role in classical CPY sorting from the Golgi apparatus to the endosome/vacuole [132,133,145]. The different protein-protein associations mediated by Vps30 provide an explanation for how the cell can achieve specificity in the light of the earlier findings that Vps34, Vps15, and Vps30 all function both in the Vps pathway and in autophagy [43,94,133]. It has been demonstrated that Complexes I and II function independently and that the Complex I-specific subunit, Apg14, is essential for recruitment to the PAS, whereas Vps38 is found mainly at endosomal structures [146]. Proper subcellular localization of Apg14 depends on two coiled-coil regions in its NH_2-terminal half, which mediate its interaction with both Vps34 and Vps30, and are necessary and sufficient for its function in autophagy [146]. However, Vps34 and Vps15 may contribute to other physiological processes in addition to the Vps pathway and autophagy, based on the fact that *vps34*Δ and *vps15*Δ mutations have additional phenotypes not displayed by *vps30*Δ mutants [94,100,132].

Indeed, using a genetic selection to isolate mutations in potentially novel PtdIns[3]P effectors, Wurmser et al. identified the *EFT1* gene [147]. *ETF1* encodes a type II integral membrane protein that functions in the Cvt pathway, but not in macroautophagy or in the Vps pathway [147]. Eft1 is able to bind PtdIns[3]P, but surprisingly it lacks a recognizable PtdIns[3]P-binding motif, and its interaction with PtdIns[3]P is mediated by a short stretch of basic residues in the cytoplasmic region of the protein [147]. The fact that Etf1 localization to prevacuolar compartments is almost completely lost in *vps34*Δ cells argues that PtdIns[3]P binding does play a role in targeting of Eft1 to specific membranes [147].

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In this regard, two additional PtdIns[3]P effectors in yeast have been identified, namely Cvt13 and Cvt20, which display specificity for pexophagy, yet another version of autophagy that exclusively targets peroxisomes and shares many, but not all, of the same components of its molecular machinery with macroautophagy [148–150] and the Cvt pathway [151]. Both Cvt13 and Cvt20 possess PX (phagocytic cell oxidase, or phox, homology) domains, a PIP-binding motif that, for all of the yeast proteins that contain it, functions as a specific PtdIns[3]P-binding module [52], in contrast to the PX domains in animal cells, some of which can bind to other PIPs [52a]. Significance of this domain for recruitment of Cvt13 and Cvt20 to punctate perivacuolar structures, identical to the PAS, was demonstrated, first, by the fact that mutant proteins harboring alterations of diagnostic PX domain residues are non-functional and, second, by the fact that GFP-fusions to each protein mislocalize to the cytoplasm in *vps34*Δ cells [151,152].

Other PX domain-containing factors known to be effectors of PtdIns[3]P include: Vam7 [153], a vacuolar t-SNARE [154]; and, Vps5 and Vps17 [155], components of the so-called retromer complex, which is thought to play a role in the retrieval of cargo from the PVC to the late Golgi apparatus [145,156,157]. All three proteins require intact PX domains and Vps34-mediated PtdIns[3]P generation for proper subcellular localization to target membranes and for vacuolar transport of CPY [153,155].

More recently, it was discovered that Atg18, Atg21, and Hsv2/Ygr223c form a novel family of PtdIns[3]P-dependent downstream effectors [158]. GFP-fusions of all three proteins localize to the vacuole and perivacuolar structures in wild-type cells, but are found exclusively in the cytoplasm in cells lacking functional Vps34 [158]. Only Atg18 function is essential for all forms of autophagy, whereas Atg21 appears to function only in the Cvt pathway and not in induced macroautophagy [158]. It has recently been suggested that Hsv2/Ygr223c is a PtdIns[3,5]P_2-binding protein and, hence, it might play a role in the regulation of processes downstream of the Fab1-dependent conversion of PtdIns[3]P to PtdIns[3,5]P_2 [159].

Additional insight has been obtained about how PtdIns[3]P contributes to the molecular mechanisms that underlie the Vps pathway. PtdIns[3]P produced by Vps34-Vps15 plays an important role in the generation of multivesicular bodies (MVBs) [160]. MVBs are structures formed via the invagination of the limiting membrane of late endosomal compartments, thereby forming new intralumenal vesicles that are subsequently delivered to the vacuolar lumen by heterotypic membrane fusion (see Figure 11) [161,162]. Specifically, it has been shown that Vps34-dependent generation of PtdIns[3]P at the endosome leads to recruitment of Vps27 (via its FYFE domain), which, in turn, serves as a membrane-docking site for the ESCRT-I complex [163]. The ESCRT-I complex consists of Vps proteins of the E class, namely Vps23, Vps28 and Vps37 [160]. This complex was shown earlier to be important for proper cargo sorting during an early step of the MVB pathway [160], whereas other complexes (ESCRT-II and ESCRT-III) containing other class E Vps proteins only function at later stages [164,165]. Therefore, it seems that the MVB pathway is initiated by controlled Vps34-dependent production of PtdIns[3]P at specific sites on the endosome.

Homologs of Vps34 have been identified in other yeasts, including *Candida albicans* [166], *Hansenula polymorpha* [167] and *Schizosaccharomyces pombe* [168,169]. All these enzymes possess PtdIns 3-kinase activity and have similar physiological functions, including roles in vacuolar protein sorting [167,169–172]. It is perhaps not surprising that PtdIns[3]P-dependent vesicular transport is required for *C. albicans* pathogenesis; *C. albicans* cells harboring a null allele or expressing a catalytically-inactive CaVps34 are avirulent in a mouse model for systemic candidiasis [170,173].

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In summary, it is now clear that several different vesicle-mediated trafficking processes that impinge on vacuolar protein transport in *S. cerevisiae*, namely the Vps, Cvt, MVB and macroautophagy pathways, require Vps34-mediated generation of PtdIns[3]P. In the Vps pathway, proteins first enter the secretory pathway by translocation from the cytoplasm to the ER and are then transported by a series of vesicular trafficking events to the late Golgi compartment [96]. Here, cargo destined for the vacuole is diverted away from those proteins destined for secretion via delivery to a late endosomal compartment [95], also called the PVC (prevacuolar compartment), similar to the mechanism by which animal cell proteins are transported to the lysosome [174]. The Vps34-Vps15 heterodimer, together with Vps30, forms two complexes of different composition. One, which contains Apg14, functions in macroautophagy and the Cvt pathway and localizes at the vacuolar membrane and at the perivacuolar pre-autophagosomal structure (PAS) [158]. The other, which contains Vps38, plays a role in the Vps pathway and is thought to be targeted to cisternae of the late Golgi apparatus [132], as well as to late endosomal structures and vacuolar membranes [146]. In this regard, it has been suggested that Vps34-dependent PtdIns[3]P generation functions in cargo selection and not in vesicle biogenesis *per se* [175]. At least in the Cvt and macroautophagy pathways, however, Vps34 function seems necessary for an early step in vesicle nucleation/ formation at the PAS. Thus, at the very least the PtdIns[3]P generated by Vps34 (together with its various regulatory subunits) is involved in regulating the size and type of vesicles formed, which must be dictated by the nature of the downstream PtdIns[3}P-binding effectors specifically recruited to each membrane.

How can Vps34 be involved in the regulation of so many different membrane trafficking processes? At least in the case of the biosynthetic Vps and Cvt pathways [142,176] and the catabolic starvation-induced macroautophagy pathway [141,142,177,178], the latter is generally not active together in the same cell with the former. Under nutrient-rich conditions, Vps34 and its associated factors are employed by the constitutive Vps and Cvt pathways, whereas it is only under severe nutrient starvation conditions that macroautophagy comes into play. Utilization of certain common components in these processes [141,142,179,180], including Vps34, provides an economic use of these factors.

2.2 PtdIns 4-kinases

In 1968, Lester and Steiner detected the deacylation products of and therefore deduced the presence of both PtdInsP and PtdInsP$_2$ in cells of baker's yeast [79]. By the late 1980s, PIPs corresponding to PtdIns[3]P, PtdIns[4]P, and PtdIns[4,5]P$_2$ were identified in this yeast, indicating that *S. cerevisiae* must possess PtdIns 4-kinase and PtdIns[4]P 5-kinase activities, in addition to PtdIns 3-kinase activity [78,181,182]. At about the same time, it became apparent that PIPs were rapidly metabolized in response to certain extracellular stimuli, such as readdition of glucose or ergosterol to starved yeast cells [183,184]. It was even reported that introduction of an anti-PtdIns[4,5]P$_2$ mAb into yeast cells by electroporation blocked growth [185]. The fact that PtdIns[4,5]P$_2$ is essential for yeast cell growth is correct, but is now based on much more solid and believable evidence (see below). Hence, at the time, it was at least conceivable that all of the major PIP-dependent pathways then known in animal cells, as well as the enzymes involved in these processes, including the archetypal generation and subsequent hydrolytic breakdown of PtdIns[4,5]P$_2$, also take place in *S. cerevisiae*.

2.2.1 Type II PtdIns 4-kinases—Type II PtdIns 4-kinases were among the first PtdIns kinases to be detected and defined biochemically in greater detail because they form the most abundant subgroup of these enzymes in most animal cells and tissues [61,63,70]. Therefore, this membrane-bound kinase activity had long been believed to be the sole source for PtdIns [4]P, the precursor for PtdIns[4,5]P$_2$, which, in turn, is the substrate for phosphoinositide-specific phospholipase C (PLC) [27,29]. Indeed, a membrane-associated type II-like PtdIns 4-

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kinase was partially purified from yeast cell extracts and characterized biochemically [181, 182,186] before the gene for any PtdIns kinase had been cloned from any organism. This enzyme had an apparent M_r of 45–55 kDa and was stimulated by Triton X-100 [181,182, 186], two hallmarks of type II enzymes (Table 1) [187]. Other characteristics of class II PtdIns kinases include inhibition by Ca^{2+} ions [72,74], by mAb 4C5G [188], and by low concentrations of adenosine (< 150 μM), as well as a relatively low sensitivity to inhibition by wortmannin [70–72,74], which strongly inhibits mammalian type I PI 3-kinases [189,190].

It turns out that Type II PtdIns 4-kinases possess a catalytic domain unrelated to that found in type I PtdIns 3-kinases and Type III PtdIns 4-kinases [191,192], which explains, in part, why it was difficult, at first, to identify the corresponding *S. cerevisiae* gene even after genes encoding PtdIns 3-kinase and PtdIns 4-kinase activities were isolated from this yeast. Moreover, it was reported that in yeast cells carrying temperature-sensitive mutations in the genes encoding both of the then known PtdIns 4-kinase isoforms (Pik1 and Stt4; see 2.2.2), nearly all (> 90%) of the PtdIns[4]P was lost within a hr after shift to restrictive temperature [193], suggesting that no other PtdIns 4-kinase might be present.

Finally, commencing in 2001 (nearly a decade after sequences for Type I and Type III PtdIns kinases became available), several groups isolated the genes encoding Type II PtdIns 4-kinases from various mammalian sources [191,192] and Drosophila [194]. The corresponding kinases behave like integral membrane proteins and display all the hallmarks of typical Type II enzymes. Furthermore, these enzymes are the founding members of a novel family of lipid kinases that have been highly conserved during evolution, but lacks, as mentioned, sequence homology to Type I and Type III enzymes [191,192,194]. Database searches revealed a single putative family member in *S. cerevisiae* [195,196], *LSB6* (Las17-binding) (Table 1 and Figure 3B). The product of this gene was originally identified in a two-hybrid screen in which Las17, the yeast ortholog of human Wiskott-Aldrich syndrome protein (WASp), a known promoter of actin filament polymerization, was used as the bait [197]. Lsb6 is a functional lipid kinase because cells overexpressing *LSB6* have increased PtdIns[4]P levels and this elevation does not require the functions of Pik1 and/or Stt4 (see 2.2.2), and, conversely, lysates prepared from an *lsb6*Δ mutant lack the PtdIns 4-kinase activity with the characteristic properties of the Type II class that can be detected in wild-type cells [195].

The enzyme encoded by *LSB6* is not essential for normal vegetative growth of *S. cerevisiae* [195,196]. Lsb6 behaves like an integral membrane protein and appears to localize to both the plasma membrane and the vacuolar membrane, and the purified detergent-solubilized protein has enzymatic properties diagnostic of Type II enzymes [195,196]. Several of the mammalian type II PtdIns 4-kinases are S-palmitoylated *in vivo* and this modification is necessary for their membrane association [191,194]. Lsb6 possesses a COOH-terminal Cys and eight other Cys resides (several of which are conserved in its mammalian counterparts); so, it is conceivable that membrane association of Lsb6 may also require its S-palmitoylation. Indeed, a recently developed algorithm to identify S-acylation sites in proteins with an accuracy of over 80% [198] predicts S-palmitoylation of the COOH-terminal Cys (C607) in Lsb6. In addition to the unique structure of its catalytic domain (Figure 3B), membrane targeting via its own post-translational modification would set Lsb6 even further apart from the other yeast PtdIns kinases, which all require accessory proteins for their membrane targeting (Table 1) [109–111,199–202].

Overexpression of Lsb6 weakly suppresses the inviability of a *stt4* null mutation (see 2.2.2), which might be explained by the fact that both Lsb6 and Stt4 are located, in part, at the plasma membrane [200], whereas either deletion or overexpression of *LSB6* in wild-type cells does not cause any obvious phenotype [195,196]. Lsb6 is the only PtdIns 4-kinase in yeast that seems to also localize, in part, to the vacuolar membrane. In this regard, it has been reported

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that PtdIns[4,5]P_2 is somehow involved in homotypic vacuole fusion [203]. The evidence for this claim is that addition of either an anti-PtdIns[4,5]P_2 mAb or Plc1 (the only PtdIns[4,5] P_2-specific phospholipase C in *S. cerevisiae* [204]) interfere with an *in vitro* reaction that purportedly monitors homotypic vacuole fusion, and readdition of PtdIns[4,5]P_2 is sufficient to restore fusion [203]. If true, it is tempting to speculate that Lsb6 might supply the PtdIns [4]P necessary to generate the PtdIns[4,5]P_2 reportedly necessary for homotypic vacuolar fusion.

On the other hand, it has recently been suggested that Lsb6 might play a role in endosome motility via its association with Las17 (yeast WASp) [205], based on the fact The average speed of endosomes is significantly impaired in *lsb6*Δ cells when compared to wild-type yeast. This effect appears to be independent of the kinase activity of the enzyme, as *lsb6*Δ cells expressing a number of different catalytically-inactive forms of Lsb6, or Lsb6 lacking its entire kinase domain, display normal endosome motility. Instead, Lsb6 activates the pathway by the interaction of its NH_2-terminal half with Las17, which in turn activates the Arp2/3 complex. Furthermore, activity of the other two PtdIns 4-kinases in *S. cerevisiae*, Stt4 and Pik1 (see section 2.2.2), and activity of the sole PtdIns[4]P 5-kinase in yeast Mss4 (see section 2.3.2) appear to be dispensable for proper endosome motility, as judged from several strains carrying temperature-conditional alleles of these enzymes upon shift to the restrictive temperature.

2.2.2 Type III PtdIns 4-kinases—Type III PtdIns 4-kinases were originally described in preparations obtained from bovine brain [61]. Unlike Type II enzymes, they are not inhibited by mAb 4C5G [188] and display little or no inhibition by adenosine (Table 1) [61]. Furthermore, Type III enzymes are much larger than Type II kinases (Figure 3B) and possess a catalytic domain that shares a high degree of similarity with that of Vps34 and other PtdIns 3-kinases (Figure 3A). The *S. cerevisiae* genome encodes two Type III enzymes, namely Pik1 and Stt4 [76,206], which are both essential for yeast cell viability [76,193,207]. Moreover, absence of either Stt4 or Pik1 cannot be compensated for by overproduction of the other [207], indicating that each enzyme generates a discrete pool of PtdIns[4]P that regulates a different essential physiological process(es), a hypothesis that is now well supported by the work of a number of groups [193,200,202,208–210]. Together, Stt4 and Pik1 generate the bulk of the PtdIns[4]P in *S. cerevisiae*. Upon shift to restrictive temperature, cells carrying a *stt4-4^{ts}* mutation display severely diminished PtdIns[4]P and PtdIns[4,5]P_2 levels (~40% and ~60% reduction, respectively); similarly, under the same conditions, otherwise isogenic cells carrying a *pik1-83^{ts}* mutation show reductions of ~45% and ~40% respectively; and, cells carrying both mutations reduce both PIPs by >90% [193].

The catalytic domain of Stt4 shares 50% identity with that of its mammalian ortholog PtdIns4KIIIα, and that of Pik1 shares 42% identity with its counterpart PtdIns4KIIIβ [211]. In addition to their catalytic domains, Pik1 and Stt4 both contain an obvious LKU domain (Figure 3B) like that found in Vps34 and its relatives (Figure 3A) [212]. Another motif of unknown function, situated in the central part of the Pik1 primary structure and dubbed the novel homology domain (NHD), is a specific hallmark of Type IIIβ PtdIns 4-kinases (Figure 3B). Stt4, by contrast, possesses an apparent PH domain sandwiched between its LKU and catalytic domains, a diagnostic feature of Type IIIα PtdIns 4-kinases (Figure 3B) [213–215].

Pik1: Like other PtdIns kinases, Pik1 (PtdIns kinase-1) was characterized first biochemically in yeast cell extracts [216] before the corresponding gene was identified. The activity behaved like a soluble protein and had the characteristics of a Type III PtdIns kinase (Table 1). The enzyme was purified 25,000-fold to near homogeneity from yeast lysates and determined to have an M_r of 125 kDa. The purified protein possessed PtdIns kinase activity *in vitro* and was specific for PtdIns as a substrate. Degenerate oligonucleotides corresponding to peptides derived from the purified enzyme were used as probes to clone the cognate gene, *PIK1*, from

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an *S. cerevisiae* genomic DNA library [76]. In good agreement with the biochemical data, the *PIK1* open-reading-frame encodes a protein of 1066 residues (calculated Mr 120,000), and genetic analysis demonstrated that an intact *PIK1* locus is essential for yeast cell viability [76].

Curiously, *PIK1* was cloned independently and nearly contemporaneously by another group using mAb QE5, which was originally raised against NUP135, a component of the human nuclear pore complex. Rather than detecting a corresponding yeast nucleoporin, the protein in yeast genomic expression libraries that cross-reacted most strongly with mAb QE5 was apparently Pik1 [77]. This independent gene isolation confirmed the sequence of *PIK1* and the PtdIns 4-kinase activity of the encoded protein. Moreover, using mAb QE5 for the analysis, this study also reported that Pik1 was associated with the nucleus and that cells carrying certain $pik1^{ts}$ alleles arrested growth at the non-permissive temperature as cells with a large bud, in most of which the nuclei were fully separated [77], a behavior subsequently also observed by others [210]. On this basis, it was suggested that Pik1 may play a role, directly or indirectly, in cytokinesis. Consistent with this conclusion, later work showed that combining defects in a number of genes involved in cytokinesis with certain $pik1^{ts}$ alleles exacerbates their phenotype even at permissive temperature [217]. However, in our hands, mAb QE5 does not cross-react detectably with Pik1 purified from *S. cerevisiae* or with recombinant Pik1 expressed and purified from bacterial cells [77a]. Nonetheless, our subsequent work using epitope-tagged Pik1 and GFP-Pik1 fusions demonstrated that Pik1 does undergo nucleocytoplasmic shuttling and, thus, at any givcn time, some fraction of the Pik1 population resides in the nucleus [202].

We also discovered that Frq1, a small EF-hand-type Ca^{2+}-binding protein that is essential for yeast cell viability, interacts tightly with Pik1 and is required for optimal activity of this enzyme [201], a relationship that was subsequently reported for the mammalian orthologs of both proteins [221–224]. Frq1 is a 190-residue NH_2-myristoylated protein with a M_r of 22 kDa that belongs to a divergent subgroup of the calmodulin superfamily (Figure 3B). Ca^{2+} binding to Frq1 displays cooperativity, but the first EF-hand motif is degenerate and, thus, incapable of coordinating Ca^{2+} ions; consequently, at saturation, Ca^{2+} is bound only to the EF-2, EF-3, and EF-4 motifs [199] (Figure 3B). Frq1 is the functional ortholog of Drosophila frequenin [227] and mammalian neuronal calcium sensor-1 (NCS-1) [201,225,226]. Expression of either frog frequenin [201] or human NCS-1 [228], but not related EF-hand proteins in the same subgroup, such as bovine recoverin [201] or human KChIP2 [228], rescues the lethality of yeast *frq1*Δ mutants. Also, NCS-1 physically associates with Pik1 at the same site as Frq1 does [228, 229]. These results demonstrate the evolutionary conservation of the Frq1-Pik1 interaction. Curiously, in *Schizosaccharomyces pombe*, another small EF-hand-type Ca^{2+}-binding protein, Cdc4 (the fission yeast counterpart of *S. cerevisiae* Mlc1), the ortholog of mammalian myosin light chain (which associates with type V myosins), reportedly interacts physically with the COOH-terminal region of SpPik1 [220]. Because of its role in regulating actin-associated Type V myosin, SpCdc4 was implicated in actin-dependent processes, like cytokinesis [218,219]. Consistent with the view that the COOH-terminal domain of SpPik1 associates with SpCdc4, ectopic overexpression of a COOH-terminal fragment of SpPik1 causes severe morphological defects in *S. pombe* cells, such as elongated and branched cells containing multiple nuclei and septa [220].

In *S. cerevisiae*, the relationship between *FRQ1* and *PIK1* was first identified in a genetic screen designed to find dosage suppressors of a temperature-sensitive mutant of *FRQ1* [201]. Only two suppressors of the conditional lethality of a $frq1^{ts}$ mutant at restrictive temperature could be found, wild-type *FRQ1* and *PIK1*. It was found that *frq1*Δ cells are viable if *PIK1* is overexpressed, suggesting that Pik1 is the sole essential downstream target of Frq1 [201]. The N-myristoyl modification of Frq1 is required for its optimal function, as evidenced by the

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inability of non-myristoylated Frq1(G2A) mutant to fully rescue the conditional lethality of the *pik1-11*^{ts} allele in a *frq1* null background [201]. Pik1 co-purifies stoichiometrically with Frq1 in a 1:1 complex in a Ca^{2+}-independent manner [229]. Frq1 interacts with the NH_2-terminal region of Pik1 at a site spanning residues Phe125-to-Gln136 and Ala157-to-Ala169 [228,229]. Furthermore, both Ca^{2+} binding-induced conformational changes in Frq1 and the presence of its NH_2-myristoyl group contribute to membrane association of Frq1; therefore, Frq1 presumably assists in membrane targeting of Pik1, which itself lacks any obvious membrane- or lipid-binding motifs [199,201]. This conclusion was confirmed subsequently [202] by demonstrating that a derivative of Pik1 lacking its Frq1-binding site [229], GFP-Pik1 (Δ152–191), localizes diffusely throughout the cytoplasm, whereas the corresponding GFP fusion to wild-type Pik1 strongly decorates cytoplasmic puncta that were shown to be Golgi cisternae because they contain Sec7 and Chs5, two well established diagnostic markers for the Golgi compartment [202,210,230,231]. Consistent with a role for Frq1 in targeting Pik1 to the Golgi, Frq1-GFP, which is fully functional *in vivo*, also decorates Golgi cisternae, co-localizing with both Pik1 and Sec7 [202].

In addition to its presence at the Golgi, Pik1 also undergoes nucleocytoplasmic shuttling, so that some Pik1 can be detected in the nucleus in some cells by indirect immunofluorescence microscopy using epitope-tagged Pik1 and an anti-epitope mAb [202,210] or by fluorescence microscopy using a fully functional GFP-Pik1 fusion expressed at near endogenous levels [202,217]. The fact that Pik1 continuously shuttles between the nucleus and the cytoplasm was corroborated by showing that mutants that lack the exportin, Msn5, dramatically accumulate Pik1 in the nucleus and, conversely, that cells with defective importin-β (Kap95) show little or no Pik1 in the nucleus, even when Pik1 is highly over-expressed [202] (Figure 7). Neither Frq1 itself nor its binding to Pik1 is required for Pik1 nucleocytoplasmic transport [202]. The catalytic domain of Pik1 (residues 793–1066) is not required for its high-affinity binding to Kap95 [202].

Although the nuclear function of the PtdIns[4]P generated by Pik1 is still unknown, it is now clear that the presence of catalytically-active Pik1 in the nuclear compartment is essential for yeast cell viability. This conclusion was reached by determining the phenotype caused by restraining Pik1 to different cellular compartments. The COOH-terminal CCAAX motif of yeast Ras2 is both S-palmitoylated and S-prenylated *in vivo* and serves as a constitutive membrane anchor [232]. We found that Pik1-CCAAX localizes to the Golgi apparatus, just like unmodified Pik1 and displays catalytic activity indistinguishable from that of wild-type Pik1 [202]. However, expression of Pik1-CCAAX does not restore viability to *pik1*Δ cells [202], suggesting that Pik1 must have an essential function aside from its role at the Golgi. By contrast, Pik1(Δ10–192), which lacks both its nuclear export sequence and its Frq1-binding site, but retains readily detectable catalytic activity, cannot be tethered at the Golgi and accumulates prominently in the nucleus. Expression of Pik1(Δ10–192) also cannot support the viability of *pik1*Δ cells [202], suggesting that action at the Golgi is also necessary for Pik1 to fulfill its essential functions. In agreement with the conclusion that Pik1 has essential roles both at the Golgi and in the nucleus, co-expression of Pik1-CCAXX and Pik1(Δ10–192) was able to rescue the inviability of *pik1*Δ cells [202]. Moreover, using catalytically-inactive derivatives of each of these compartment-restricted Pik1derivatives, it was demonstrated that the enzyme must be catalytically active in each location to maintain normal cell growth and viability [202], indicating that Pik1 must generate PtdIns[4]P both at the Golgi and in the nucleus. In this regard, it was reported that Pik1 rapidly translocates from the nucleus to the cytoplasm when *sec6-4*^{ts} cells are shifted to the restrictive temperature [210], a reversible effect also seen with other nuclear proteins in other secretory mutants [233]. However, to date, the mechanism and physiological significance of this stress-induced process are not understood.

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In contrast to its still undefined nuclear function, the role that Pik1 serves at the Golgi is better understood. In independent studies by several groups, Pik1 was pinpointed as the enzyme necessary for supplying the pool of PtdIns[4]P needed for the Golgi-to-plasma membrane phase of the secretory pathway [193,208,210] (Figure 7). The first link between Pik1 and secretion was provided when it was found that the cellular PtdIns[4]P level drops markedly upon shift of a *sec14-3^{ts}* mutant to the restrictive temperature [208]. *SEC14* encodes an essential phosphatidylinositol transfer protein (PITP) [41] that had originally been identified in the classical screen for mutations of genes required for proper function of the yeast secretory pathway [40]. Subsequent studies demonstrated that another mutation, *sac1-22*, which suppresses both the growth and secretory defects of *sec14-3^{ts}* mutants, causes an elevation in the cellular level of PtdIns[4]P [234]. Why loss of Sac1 function raises the PtdIns[4]P level became clear when it was shown that Sac1 is a phosphoinositide phosphatase that can act on several PIPs, including PtdIns[4]P [235] (see 3.1.1). Furthermore, like loss of Sac1, it was found that overexpression of Pik1 (but not of Stt4 or Vps34) was able to rescue both the growth and secretory defects of the *sec14-3^{ts}* mutant at a non-permissive temperature [208]. These findings made it plausible that PtdIns[4]P plays some direct and important role in anterograde secretory transport. Indeed, in keeping with this idea, *sac1*Δ mutants display excessive forward transport of the Chs3 chitin synthase to the cell periphery, which leads to specific cell wall defects, an effect that is also seen in cells overexpressing Pik1, but not in cells overexpressing Stt4 [236]. Moreover, upon shift to restrictive temperature, different *pik1^{ts}* alleles display differential decreases in PtdIns[4]P that correlate with the degree to which secretion of two extracellular proteins, invertase and Hsp150, are affected [77a,193,208,210]. Under the same conditions, maturation and delivery of CPY to the vacuole was relatively unaffected [193, 208,210]. Taken together, these findings indicated that the Pik1-generated PtdIns[4]P is necessary for some step(s) in the Golgi-to-plasma membrane stage of exocytosis. Although genetic interactions between a *pik1^{ts}* mutant and genes involved vacuolar function have been reported [217], CPY secretion has never been detected in any *pik1* mutant, arguing that Pik1 is certainly not a Vps factor.

Several proteins require functional Pik1 and, hence, PtdIns[4]P to become selectively recruited to the Golgi *in vivo* [237–239]. In cells carrying a *pik1^{ts}* mutation, recruitment of these proteins to the Golgi can only be observed at the permissive temperature, but not after shift to restrictive temperature, whereas mutations in the gene encoding the sole PtdIns[4]P 5-kinase in yeast, *MSS4* (see 2.3.2), or *lsb6*Δ or *vps34*Δ mutations, have no effect at either temperature and do not display a defect in Golgi morphology or secretion [237–239]. One such effector protein is Kes1 [240,241], a member of the conserved family of oxysterol-binding proteins (OSBP; also termed Osh in yeast) [242]. Oxysterol-binding proteins have been implicated in the maintenance of sphingolipid and sterol composition in membranes, although their molecular function is still only poorly understood [242–244]. Deletion of *KES1* bypasses the requirement of the otherwise essential PtdIns/PtdCho transfer protein (PITP), Sec14, in yeast cells [41, 240], and, hence, was thereby implicated in the formation of Golgi-derived vesicles. Kes1 binds PtdIns[4]P and PtdIns[4,5]P_2 *in vitro*, as judged by competition assays using a range of different phospholipids [241]. Furthermore, this interaction depends on a putative PH domain-like sequence in the central part of the protein, whereas the COOH-terminal region of Kes1 has an inhibitory effect on PIP binding [241]. Sequence comparison of well-characterized PH domains with known binding specificities revealed several residues that appear to be important for PIP binding [56,245]. Mutation of the corresponding residues in the putative PH domain of Kes1 abolishes PIP binding *in vitro* and ablates Kes1 function *in vivo* and prevents its localization to the Golgi [241]. Similarly, full-length Kes1-GFP mislocalizes to the cytoplasm in *pik1-101^{ts}* cells, but not in *stt4^{ts}* or *mss4-2^{ts}* cells, upon shift to restrictive temperature, indicating that Kes1 is an effector of Pik1-generated PtdIns[4]P at the Golgi [241]. However, unlike Pik1, Kes1 is not an essential gene and, hence, cannot be a positive factor centrally important for secretion. Indeed, at a semi-permissive temperature, a *kes1*Δ mutation partially

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alleviates the severity of the phenotype of *pik1-101^{ts}* cells, but not that of a *stt4^{ts}* strain [241], suggesting that Kes1 is a negative regulator. Genetic evidence suggests that these effects could be explained, at least in part, if Pik1-derived PtdIns[4]P is needed for activation (or to prevent inactivation) of Gcs1 and Age2 [241,246], two GTPase-activating proteins (GAPs) for members of the Arf subfamily of small GTPases [247–250]. In this context, *PIK1* displays genetic interactions with Arf family GTPases [193,210,217], and the mammalian ortholog of Pik1, PtdIns4K IIIβ [251] and a number of other proteins are reportedly recruited to the Golgi in an Arf- and PtdIns[4]P-dependent manner [237,252].

In this same regard, EM studies on *pik1^{ts}* cells before and after shift to restrictive temperature revealed an accumulation of abnormal, cup-shaped membranous structures in the cytoplasm [193,202,210]. These structures, termed "Berkeley bodies," were first observed in secretory mutants defective in Golgi-to-plasma membrane transport [40,202]. Interestingly, *frq1^{ts}* cells also form Berkeley bodies and display a block in secretion upon shift to non-permissive temperature, in support of the finding that Frq1 assists with the recruitment of Pik1 to Golgi membranes and that Pik1 action is necessary for exocytosis [202].

In addition to the PtdIns 4-kinases, PtdIns[4]P pools are also regulated by the synaptojanin-like phosphoinositide phosphatases, Inp51/Sjl1, Inp52/Sjl2, and Inp53/Sjl3 (see 3.2). These proteins, which are homologous to the mammalian PtdIns[4,5]P_2 5-phosphatase, synaptojanin, form a subgroup of phosphoinositide phosphatases in yeast and are characterized by two catalytic domains, a COOH-terminal 5-phosphatase domain and an NH_2-terminal domain that resembles the catalytic domain (residues 55–454) of Sac1 [253–255] (see Figure 12). In this regard, *PIK1* displays synthetic genetic interactions with deletions of individual *INP5*/*SJL* genes [256]. Deletion of *INP53/SJL3* in a strain carrying a *pik1^{ts}* mutation is synthetically lethal, whereas deletion of *INP51/SJL1* or *INP52/SJL2* exacerbates the temperature-sensitivity of these cells [256]. In addition, *inp51Δ/sjl1Δ pik1^{ts}* and *inp52Δ/sjl2Δ pik1^{ts}* cells display reduced cellular PtdIns[4]P levels (29% and 11%, respectively), but increased PtdIns[4,5]P_2 levels (62% and 19%, respectively) when compared with the corresponding *pik1^{ts}* cells [256]. Also, these double mutants display even more severe defects in invertase secretion than the corresponding *pik1^{ts}* cells, as well as a hypersensitivity to neomycin [256], a phosphoinositide-masking agent [257,258]. All of these results are fully consistent with the original proposal [208] that PtdIns[4]P, rather than PtdIns[4,5]P_2, is required for anterograde vesicular trafficking of secretory proteins from the Golgi to the plasma membrane in *S. cerevisiae*. Again, in agreement with a role for Pik1 action in exocytosis, a particular temperature-conditional allele, *pik1-101^{ts}*, exhibits synthetic lethality upon overproduction of Gdi1, a protein involved in recycling of the GDP-bound form of the exocyst-associated Rab-like small GTPase, Sec4, from the plasma membrane to secretory vesicles [210]. Given that Sec4 itself regulates Golgi-derived vesicle fusion with the plasma membrane [259], this observation provides yet another link between Pik1 function and secretion. Likewise, several other *pik1^{ts}* alleles display synthetic defects with a number of different mutants known to affect Golgi function or the actin cytoskeleton [210,217]. However, to date it is still unclear whether Pik1-derived PtdIns[4]P plays any direct role in modulating the actin cytoskeleton, in the light of the contradictory data that have been published [193,210].

In addition to genes already known to interact with *PIK1*, Sciorra et al. identified additional genetic interactions of a specific *pik1* allele (*pik1-139^{ts}*) with mutations in the *DRS2*, *TRS33*, *KRE11*, and *YPT31* genes, all of which have previously ascribed roles in Golgi-to-plasma membrane transport [217]. All four genes display serious synthetic defects when combined with a *pik1^{ts}* allele, but not with mutations in either *STT4* or *MSS4* [217]. *YPT31* encodes a Rab-related small GTPase that is essential for secretion in yeast [260,261] and its mammalian ortholog, Rab11, reportedly is recruited to the Golgi apparatus in a manner that depends on PI4KIIIβ [262]. Furthermore, the Pik1 ortholog in *Arabidopsis thaliana*, AtPI4Kβ1, associates

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with a Ypt31 ortholog, AtRabA4, and this interaction is mediated by the novel homology domain (NHD) (see Figure 3B) in AtPI4Kβ1 [263]. Perhaps surprisingly, *YPT32*, another Rab family member that shares 81% sequence identity and 90% similarity with *YPT31*, which has been suggested to perform identical or overlapping functions with *YPT31* [260,261], did not display synthetic defects in combination with *pik1-139*^{ts} [217]. Drs2 is an aminophospholipid translocase [264], and *KRE11* and *TRS33* encode non-essential components of a large Golgi-associated complex, comprised of ten subunits, the so-called TRAPPII complex [265]. Cells carrying temperature-conditional mutations in any of the essential subunits of the complex display serious defects in secretion, as well as aberrant Golgi-derived structures in their cytoplasm upon shift to non-permissive temperature [265]. Closer investigation of the mutant phenotype of a *ypt31*Δ *pik1-139*^{ts} double mutant revealed the formation of some Berkeley bodies even at permissive temperature and mislocalization of certain plasma membrane proteins that continuously recycle back to the Golgi apparatus via early endosomes (i.e. Chs3 and Snc1) and, at restrictive temperature, even more aberrant Golgi structures [217], similar to those seen in cells lacking the Sac1 PIP phosphatase (see 3.1.1) [236]. However, even a *ypt31-101*^{ts} *ypt32*Δ double mutant did not show any perturbation of its PtdIns[4]P level at non-permissive temperature and Pik1 was still localized to the Golgi, indicating that Ypt31 is not necessary to activate Pik1 *in vivo* or to assist with its Golgi recruitment.

In addition to its role in secretion, there are claims that Pik1 also has a function in endocytosis. Membrane protein uptake was followed by monitoring the rate of degradation of an polytopic integral membrane protein (the ABC transporter, Ste6) by pulse-chase analysis and bulk membrane uptake was measured by following movement of a vital fluorescent lipophilic dye, FM4-64. It was observed that, upon shift to the restrictive temperature, *pik1*^{ts} cells seemed unable to degrade Ste6 and failed to accumulate FM4-64 in the vacuolar membrane [193, 210]. Instead, the dye decorated several punctate structures in the cytoplasm, which may represent endosomal membranes, as they do not colocalize with a vacuolar marker, consistent with a block in plasma membrane-to-vacuole transport [210].

Similar to the concerted action of Sec14 and Pik1 in the secretory pathway in vegetatively-growing haploid cells, both proteins play a role in diploid cells in spore formation and meiosis [266]. In addition, Spo14, the major phosphatidylcholine-specific phospholipase D (PLD) isoform in yeast [267–269], is also essential for sporulation [270], whereas it is dispensable during normal vegetative growth and mitosis [271–273]. Efficient initiation and completion of meiosis itself requires functional Pik1 and Sec14, but not the PtdIns[4]P 5-kinase, Mss4 or Spo14, which suggests a specific role for PtdIns[4]P in this physiological process [266]. In contrast, like Pik1 and Sec14, both Mss4 and Spo14 (and, hence, PtdIns[4,5]P_2 and phosphatidic acid, PtdOH, generation) are required for prospore formation around the four meiotic nuclei [266]. In this process, which is basically a developmentally specialized form of secretion that occurs following meiosis, prospore membranes form *de novo* around each meiotic nucleus by the redirection and subsequent fusion of post-Golgi vesicles [274]. Nevertheless, to date the exact contribution at the molecular level of these proteins and, in the case of the enzymes, their products, to the processes of meiosis and spore formation remains unresolved.

STT4: Mutations in the *STT4* (staurosporine- and temperature-sensitive) locus were originally isolated in a genetic screen designed to detect mutations displaying hypersensitivity to a rather non-specific protein kinase inhibitor, staurosporine [206]. Staurosporine is an antifungal alkaloid isolated from *Streptomyces sp.* that was initially thought to inhibit with some selectivity protein kinase C (PKC)-related enzymes [275]. *PKC1/STT1* encodes the only PKC-related protein kinase in yeast [276], which is a known component of the cell wall integrity pathway [277]. In this pathway (Figure 10A) Pkc1 serves as an upstream activator of a MAP kinase cascade comprised of the MEKK Bck1 [278], a pair of redundant MEKs, namely Mkk1

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and Mkk2 [279], and the MAP kinase Slt2/Mpk1 [280,281]. Pkc1 itself, is activated by its interaction with the essential small GTPase Rho1, and likely Rho2 [282–284], which in turn are stimulated by the action of their cognate guanine nucleotide exchange factors (GEF) Rom1 and Rom2 [285]. In addition, Pkc1-mediated activation of the Slt2/Mpk1 Map kinase cascade was shown to be initiated by two transmembrane receptor-like proteins, namely Wsc1 and Mid2, which both interact with Rom2 [285–287]. The staurosporine-sensitive phenotype of *stt4^{ts}* cells can be suppressed by overexpression of *PKC1*/*STT1* [206], suggesting that Stt4 functions in the same pathway upstream of Pkc1 (Figure 8). However, overexpression of *PKC1* only rescues the staurosporine-, but not the temperature-sensitivity of *stt4^{ts}* mutants, indicating additional cellular functions for Stt4, apart from regulating the Pkc1-dependent pathway [206]. Cloning of the corresponding gene by complementation of a *stt4^{ts}* mutation revealed a 1900 residue protein with significant sequence homology to the COOH-terminal regions of Vps34 and mammalian PI 3-kinase, as well as Pik1 (Figure 3B) [206]. Furthermore, *stt4^{ts}* and *stt1/pkc1* mutations display very similar phenotypes: staurosporine hypersensitivity and a cell lysis defect that can be suppressed in certain strain backgrounds by osmotic support, such as the presence of 1M sorbitol in the medium [277,288].

Wortmannin is a steroid-related metabolite of *Talaromyces wortmannii* that functions as an immunosuppressive and anti-inflammatory agent in vertebrates [289]. The physiological effects of wortmannin are at least in part caused by the inhibition of PI 3-kinases [91]. Yeast cells lacking *VPS34*, and, hence, PtdIns 3-kinase activity [42], however, remain wortmannin-sensitive, indicating that this drug, which had been shown to inhibit Vps34 before [89] must have additional targets in *S. cerevisiae* [207]. In addition to PI 3-kinases wortmannin also inhibits the activity of certain PtdIns 4-kinases, although this requires relatively high concentrations of the drug [91]. *In vitro* Stt4, but not Pik1 PtdIns 4-kinase activity is inhibited by this fungal metabolite [207]. Furthermore, overexpression of *STT4* renders cells insensitive to wortmannin and conditions that rescue a *stt4* null mutation in some strain backgrounds also overcome wortmannin toxicity. Nevertheless, in strain backgrounds in which *STT4* was found to be essential for viability, wortmannin is toxic under all conditions [207]. *PKC1*, however, is dispensable in these cells in the presence of osmotic support, again strongly suggesting that Stt4 must have additional essential functions in yeast cells, independent from regulating the cell wall integrity pathway. Moreover, overexpression of *MSS4* (see section 2.3.2) or deletion of *PLC1*, the gene encoding the sole phosphoinositide-specific phospholipase C that hydrolyzes PtdIns[4,5]P_2 in yeast [204], also result in wortmannin resistance, suggesting an important role for PtdIns[4,5]P_2 in the PKC pathway [207].

In this regard, the gene encoding Mss4 (multicopy suppressor of *stt4*, see section 2.3.2), the sole PtdIns[4]P 5-kinase in *S. cerevisiae*, was isolated during the cloning of *STT4* as a dosage suppressor of the *stt4-1^{ts}* mutation, suggesting that Mss4 functions downstream of Stt4 in the same pathway [288]. As Mss4 also suppresses the cell lytic defect (a hallmark of cell wall integrity pathway mutants) of the *stt4-1^{ts}* mutation [288], Stt4 and Mss4 are apparently required for efficacious Pkc1-dependent signaling in the cell wall integrity pathway.

Subsequent work revealed the molecular basis of the interrelationship between *STT4*, *MSS4* (see section 2.3.2) and *PKC1* in the cell wall integrity and heat shock response pathways, which are modulated through the activation of the Slt2/Mpk1 MAP kinase cascade [200,286]. Stt4 localizes to the plasma membrane [200,290], where it generates a pool of PtdIns[4]P that is converted to PtdIns[4,5]P_2 by Mss4, which in turn leads to the activation of the Pkc1-mediated MAP kinase cascade. In line with this, Stt4- and Mss4-dependent PtdIns[4,5]P_2 generation is a prerequisite for Rom2 recruitment to the plasma membrane [200]. This finding is in good agreement with earlier work demonstrating that the elevated intracellular level of PtdIns[4,5]P_2 upon inactivation of the inositol polyphosphate 5-phosphatase Inp51/Sjl1 (see section 3.2.1) results in the hyperphosphorylation and, hence, activation of the MAP kinase Slt2/Mpk1 in the

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absence of an extracellular signal [291]. Further support, for an involvement of PtdIns[4,5]P_2 in the activation of the cell wall integrity and heat shock response pathways came from the finding that mutations in *STT4* and *MSS4* display synthetic defects with a number of mutations in genes encoding known components of the Pkc1 pathway [200]. In this regard, it was shown in a number of independent studies that the bulk of the PtdIns[4,5]P_2 that is generated at the plasma membrane requires functional Stt4, but not Pik1 [237,238,292].

Plasma membrane association of Stt4 depends on another protein, Sfk1 (suppressor of four kinase), a multicopy suppressor of the *stt4-4^{ts}* allele, but not of *pik1-83^{ts}* [200]. Sfk1 is a putative integral membrane protein that colocalizes with Stt4 to small punctate patches at the plasma membrane [200]. In addition, coimmunoprecipitation experiments of covalently cross-linked proteins indicate that both polypeptides physically interact with each other *in vivo* [200]. The significantly reduced level of Stt4 (~ 70%) at the plasma membrane in cells lacking Sfk1, is mirrored by similarly lowered PtdIns[4]P levels. In this respect, it has been reported that the isolated PH-like domain of Stt4 alone is not sufficient for plasma membrane targeting [293].

However, Stt4 must also have an Mss4-independent function in the regulation of cell wall maintenance/synthesis as another temperature-conditional allele, *stt4-4^{ts}*, cannot be rescued by overexpression of *MSS4* [193]. As *stt4-4^{ts}* cells show cell lysis defects upon shift to non-permissive temperature that can be suppressed by 1M sorbitol, almost identical to what had been observed with other mutant alleles of *STT4* and *PKC1* before, it can be argued that this function is related to cell wall integrity sensing [193,207,277].

One function of Stt4, apart from regulating the Pkc1-dependent cell wall integrity and heat shock response is the regulation of the actin cytoskeleton. This is evidenced by the finding that cells carrying either a *stt4-1^{ts}* or a *mss4-2^{ts}* allele (see section 2.3.2), but not those harboring a *pik1-83^{ts}* mutation, fail to properly organize their actin cytoskeletons upon shift to restrictive temperature [193]. This phenotype can be partially suppressed by the inactivation of *SAC1*, which encodes a polyphosphoinositide phosphatase (see section 3.1.1), arguing for a functional connection between Stt4 and Sac1 [294]. Additional evidence confirming this interaction includes the finding that *sac1* null cells display an 8 to 12-fold increase in intracellular PtdIns[4]P, which can be restored to wild-type levels by the inactivation of *STT4*, but not *PIK1* or *LSB6* (see section 2.1) [294,295]. In this regard, *SAC1* had originally been recognized by virtue of its allele-specific genetic interactions with yeast actin defects [296]. Furthermore, cells lacking functional Stt4 or Mss4 shift to isotropic from polarized cell growth and, hence, severely increase in size and adopt a round cell shape [193,297], effects also seen with mutations in actin binding proteins, such as capping protein and profilin [298,299]. Given that evidence for involvement of Mss4 in regulation of the actin cytoskeleton had been obtained before [297,300], these findings collectively suggested a role for PtdIns[4,5]P_2 (rather than PtdIns[4]P) in this process. The heat shock response in *S. cerevisiae* involves the depolarization of both the actin cytoskeleton and certain components of the Pkc1-mediated Slt2/Mpk1 MAP kinase pathway, effects that can be prevented by the addition of osmotic support [301]. Furthermore, both processes require activation of the small GTPase Rho1 [301] suggesting that Stt4 might regulate both processes in a similar way by the PtdIns[4,5]P_2-dependent recruitment of its downstream effector Rom2 to the plasma membrane [200,285].

Stt4 function is also required for the recruitment of the p21-activated kinase, Cla4, to sites of polarized growth [302], in a manner that is independent of Mss4, but requires the Rho-type small GTPase, Cdc42 (Figure 8). Cdc42 has roles in quite a number of processes in *S. cerevisiae*, including the regulation of the mating pheromone and stress response MAP kinase cascades [303,304], septin assembly at the bud neck [305,306], polarization of the actin cytoskeleton [307], it drives exit from mitosis [308–310] and is involved in vacuolar docking

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and fusion [311–313]. Cla4 is one of several members of the p21-activated protein kinase (PAK) family in yeast and is a direct effector of Cdc42, which is important for polarized growth [314,315]. Cla4 binds to Cdc42 via a conserved motif, the p21-binding domain (PBD), whereas its interaction with phosphoinositides is mediated by its PH domain [302,315]. Using a series of GFP-tagged Cla4 truncation and deletion constructs, it has been shown that both domains are necessary and sufficient to target Cla4 to sites of polarized growth, whereas the catalytic domain appears to be dispensable for localization of this protein kinase [302]. Furthermore, it has been established that Cla4 lacking either domain is non-functional *in vivo* and Cla4 point mutants defective in Cdc42- or phosphoinositide-binding fail to suppress the phenotypes of a *cla4* null mutation [302,314]. The phosphoinositide responsible for Cla4 recruitment to sites of polarized growth is PtdIns[4]P generated at the plasma membrane by the action of Stt4, as Cla4 is mislocalized in cells carrying a *stt4^{ts}* allele upon shift to restrictive temperature, but not in cells harboring mutant alleles of *pik1* or *mss4* [314]. Therefore, Cla4 serves as a coincidence sensor for Stt4-generated PtdIns[4]P and Cdc42 at the plasma membrane.

In addition, Stt4 has also been implicated in the regulation of intracellular aminophospholipid transport [209]. In a genetic screen designed to identify novel factors involved in the regulation of intracellular aminophospholipid transport a *stt4* mutation, termed *pstB1* was found to specifically result in the block of phosphatidylserine transport from the endoplasmic reticulum (ER) to the Golgi/vacuole [209]. Measurement of the PtdIns 4-kinase activity of cells carrying the *pstB1* allele under conditions optimized for Stt4 [206] revealed significantly reduced catalytic activity (~ 10%) when compared to the corresponding parental strain [209]. Although both the growth and biochemical defects of the *pstB1* allele could be suppressed by expression of wild-type *STT4* there is no model for the molecular mechanism of Stt4 function in intracellular aminophospholipid transport.

Furthermore, Stt4 function has been connected to activation of phospholipase D (PLD) in vegetatively-growing yeast cells [290]. The major PLD activity in *S. cerevisiae* is encoded by a single gene, *SPO14* [267–269], whose product hydrolyzes phosphatidylcholine to generate phosphatidic acid (PA) and choline [270,316,317] in a fashion that is dependent on the presence of PtdIns[4,5]P_2 [268,269,318]. In yeast Spo14 is dispensable for vegetative growth [271–273], but plays an essential role in prospore formation and is required for increased PA generation during sporulation [319,320] and, as a consequence, *spo14* null cells display severe sporulation defects [268]. However, *SPO14* is transcribed in both sporulating and mitotically-dividing yeast cells and, under certain physiological conditions becomes important for yeast cell viability even in vegetatively growing cells. Spo14 plays an essential role in the secretory pathway under the abnormal condition when the yeast PtdIns/PtdCho transfer protein, Sec14 [41], is dysfunctional and a secondary bypass mutation is also present [272,273]. Recently, a novel class of PtdIns transfer proteins (PITPs), designated Sec14 homolog proteins (SFH), has been identified in yeast [321]. Members of this PITP subfamily (Sfh2 – Sfh5) are atypical in that they confer PtdIns, but not PtdCho, transfer activity *in vitro* [321]. Furthermore, overexpression of any single *SFH* gene rescues the growth and secretory defects associated with non-functional *sec14* alleles, in a process that requires functional Spo14 [321]. Closer investigation of this effect revealed increased bulk PtdIns[4]P levels in cells overexpressing *SFH2* and elevated PtdIns[4,5]P_2 levels in cells overexpressing *SFH2* or *SFH5* under *sec14*Δ bypass conditions [290]. This is likely to be a consequence of increased Stt4 activity, as inactivation of either *SFH2* or *SFH5* exacerbates the temperature-sensitive phenotype of *stt4-4^{ts}*, but not *pik1-83^{ts}* mutant cells [290]. Furthermore, Sfh proteins are required for optimal PLD activity in cells lacking Sec14 [321] as *sfh*Δ cells display a 40% reduction in Spo14 catalyzed PtdCho hydrolysis [321]. Strikingly, this effect is mirrored in cells lacking functional Stt4, but not upon inactivation of Pik1 under the same conditions [290]. In this regard, *sec14* null cells display similar defects in the polarization of the actin cytoskeleton as cells lacking functional Stt4, an effect that can be suppressed by overexpression of *SFH2* and *SFH5* [193,

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290]. Taken together, these data suggest an interaction of Stt4 with certain members of the Sfh subfamily of PITPs in both the regulation of the actin cytoskeleton and Spo14 activation, although the underlying molecular mechanisms remain elusive. In this context, Sfh4, also known as PstB2, was shown to be involved in intracellular aminophospholipid transport, similar to Stt4 [209,322,323].

2.3 PtdInsP kinases

The first indication of the existence of $PtdInsP_2$ in *S. cerevisiae* came from a study by Lester et al., which demonstrated the presence of mono- and diphosphorylated phosphoinositides in lipid extracts of yeast cells [79]. Several years later, it was reported that $PtdIns[4,5]P_2$ is essential for yeast cell viability based on the effect of introducing anti-$PtdIns[4,5]P_2$ antibodies into *S. cerevisiae* cells by electroporation [185]. As bizarre and questionable as those particular experiments were, the conclusion turned out to be correct, as shown by subsequent work [76]. $PtdIns[3,5]P_2$, the second phosphatidylinositol bisphosphate species present in yeast, remained undetected for several years to come [80]. The PtdInsP kinases, the enzymes responsible for generation of diphosphorylated lipid species from their monophosphorylated precursors, form a distinct subfamily of lipid kinases [324,325] because their primary structures do not share any statistically significant similarities with PtdIns 3-kinases and Type III PtdIns 4-kinases, which do display a notable degree of homology in certain domains and even display some similarity to protein kinases [1,326,327] (see sections 2.1 and 2.2). Furthermore, PtdInsP kinases are functionally distinct from the other phosphoinositide kinases in yeast by their preference for PtdInsP over PtdIns. All type I/II PtdInsP kinases, including Mss4, share two conserved regions, a dimerization motif and a COOH-terminal catalytic domain (Figure 9). However, X-ray crystallographic studies on mammalian type IIβ phosphatidylinositol phosphate kinase and structural comparison revealed a certain degree of similarity to protein kinases on the structural level [324,328]. The bipartite kinase domain is separated by a short insert in all PtdInsP kinase family members except for yeast Fab1 and its closest homologs from other species (Figure 9) [324,329]. The *S. cerevisiae* genome encodes two PtdInsP kinases, namely Mss4 and Fab1 [130,288]. They share significant sequence homology with their mammalian counterparts, although Mss4 cannot be classified into subgroups PtdIns5K I and PtdIns5K II like the corresponding enzymes in vertebrates, which are mainly based on different biochemical and antigenic properties [327]. Fab1 also possesses a conserved FYVE domain (Fab1, YGL023, Vps27, and EEA1) (Figure 9) in its NH_2-terminal part, which serves as a PtdIns[3]P-specific binding motif [123]. Moreover, Fab1 contains another conserved region central in its primary sequence (Figure 9), which shares significant homology to a motif present in a subunit of the chaperonin containing T-complex, CCT-1 [330–332]. CCT-1 is an eight subunit chaperone with actin and tubulin-binding specificity that shares notable similarity with the GroEL chaperonin of *E. coli* [333,334]. To date the function of this domain found in type III PtdInsP kinases, however, has not been defined yet. *MSS4* is essential for yeast cell viability and encodes a 779 residue protein, whereas *FAB1* encodes a 2278 residue lipid kinase that is dispensable for vegetative growth at 30° C [130,288].

2.3.1 PtdIns3P 5-kinases—*FAB1* has originally been identified in a genetic screen for abnormal nuclear segregation [130]. Cells with a defective orientation of the mitotic spindle pass both sets of replicated chromosomes on to either the daughter or mother cell [335]. This irregular event results in the formation of aploid and binucleate (FAB) cells, also termed *fab* phenotype. The subsequent cloning of the corresponding *FAB1* gene revealed a 257 kDa product with significant homology to known PtdInsP kinases [130]. Although *FAB1* encodes a non-essential product, cells harboring a *fab1* null allele display slow growth and a temperature-sensitive phenotype [130,336]. Furthermore, $fab1^{ts}$ cells exhibit a cell lysis defect upon prolonged incubation at non-permissive temperature that can be suppressed in the presence of certain osmotic stabilizers [130]. Another striking phenotype of *fab1* null and

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fab1^{ts} cells is the rapid appearance of extremely enlarged vacuoles, as evidenced by an increase in the average vacuolar surface area by approximately 250% [130,336]. In addition, *fab1* null cells display a defect in vacuolar protein sorting, an effect not observed in *fab1^{ts}* cells at restrictive temperature. However, CPY is not secreted in these cells, whereas its maturation is severely inhibited suggesting a block in CPY transport to the vacuole that is different from that in classical *vps* mutants [130,336]. This effect, however, could also be due to slow maturation of certain vacuolar hydrolases owing to reduced activity of proteinase A [337], which plays a key role in the maturation of a number of vacuolar enzymes [337,338] and requires vacuole acidification for its own activity [339]. Likewise, transport of cargo for other trafficking routes to the vacuole, i.e. the Cvt and ALP pathways [179,180], is processed indistinguishable from wild-type cells, indicating that Fab1, unlike Vps34 (see section 2.1) is not directly involved in these processes [336]. Moreover, *fab1^{ts}* cells also display defects in vacuolar acidification, again arguing for a malfunction in vacuolar transport, although proper localization of the vacuolar ATPase is not affected in these cells [130,336,340].

Strains carrying *fab1*Δ or *fab1^{ts}* alleles are defective in spindle position and chromosome segregation as the number of binucleate and aploid cells is increased by more than 100-fold [130] when compared to wild-type [335,341], an effect that is mirrored by the appearance of cells with more than 2C DNA content. Furthermore, cells containing only one nucleus frequently display aberrant chromatin and spindle positions. However, these effects only take place in *fab1^{ts}* cells upon prolonged incubation (> 4h) at a non-permissive temperature, whereas aberrant vacuole morphology and acidification can be observed shortly (~ 30 min) after the temperature shift and irregular chromosomes and bent spindles are always correlated with significantly enlarged vacuoles. Taken together with the finding that the combination of a *fab1* null allele with certain *vps* mutants reduces the aberrant vacuole morphology phenotype also eliminates the chromosomal and cell integrity defects these data suggest that the *fab* phenotype results from steric hindrance due to the enlarged vacuole rather than loss of Fab1 function per se.

Vacuole inheritance in *S. cerevisiae* is a specialized form of membrane traffic during cell division where the mother donates both vacuolar membrane and lumenal contents to the daughter cell [342–344]. This process is initiated in early S phase by the projection of membranous structures from the mother cell vacuole into the emerging bud [345], in an actin- and Myo2-dependent process [346]. Mutants with defects in vacuolar inheritance are categorized into three classes according to vacuole morphology [347]. The class III mutants *fab1-2^{ts}* [130,348], *vac7-1^{ts}*, and *vac14-1^{ts}* [347] are characterized by dramatically enlarged, unlobed vacuoles that frequently fill up the entire cytoplasm, whereas wild-type cells typically contain multilobed vacuoles of smaller size. In addition, class III mutants display defects in vacuole acidification and fail to segregate their vacuoles during cell division, resulting in a characteristic 'open figure eight' vacuole morphology [340,349]. Furthermore, overexpression of *FAB1* suppresses the mutant phenotype of *vac14-1^{ts}* cells and combination of two or more mutant class III alleles results in the appearance of synthetic defects, such as slower growth rates and even further enlarged vacuoles [340,349]. Introduction of a class B *vps* mutation, which are characterized by highly fragmented vacuoles [93], into cells harboring the *vac7-1^{ts}* allele specifically suppresses the vacuole inheritance defect while retaining other characteristics of the single mutants, suggesting that the failure to properly inherit the vacuole is a secondary effect of the membrane fission defect.

In a genetic screen it was found that hyperosmotic shock leads to a rapid (5 – 15 min) and transient (30–60 min) increase in cellular PtdIns[3,5]P_2 in *S. cerevisiae* [80]. Surprisingly, this effect is independent of the Hog1 (high osmolarity glycerol response) MAP kinase cascade, which plays an important role in the adaptation of yeast to conditions of high osmolarity [350,351], as cells harboring mutations in single components of this pathway do not display

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any changes in the hyperosmotically evoked synthesis of PtdIns[3,5]P_2. The temporary elevation of cellular PtdIns[3,5]P_2 levels is mirrored by a simultaneous increase in PtdIns[3]P that depends on functional Vps34 (see section 2.1), as cells carrying a *vps34^{ts}* allele fail to show this effect at a non-permissive temperature [352]. Hence, PtdIns[3,5]P_2 is produced sequentially by the action of Vps34 to generate PtdIns[3]P, which is then further converted to PtdIns[3,5]P_2 by a PtdIns[3]P 5-kinase.

Subsequently, Fab1 was identified as the PtdIns[3]P 5-kinase mediating this hyperosmotic stress response [353], which also requires functional Vac7 and Vac14 [349,352–354]. Astoundingly, the hyperosmotically induced transient increase of PtdIns[3,5]P_2 is also dependent on functional Fig 4, a polyphosphoinositide phosphatase (see section 3.1.2), that specifically acts on PtdIns[3,5]P_2 [354–357]. Deletion of *FAB1*, *VAC7* or *VAC14* renders cells incapable of generating readily detectable levels of PtdIns[3,5]P_2 [336,349,352], independent of osmotic conditions, and that this effect can be reversed by the reintroduction of *FAB1* on a plasmid in *fab1*Δ and *vac14*Δ cells [352,353]. Similarly, *fab1^{ts}* cells display significantly decreased PtdIns[3,5]P_2 levels under all conditions and fail to induce PtdIns[3,5]P_2 production upon hyperosmotic shock at restrictive temperature [336,353]. Likewise, cells expressing mutant versions of Fab1, carrying point mutations in conserved residues predicted to be important for catalytic activity as their sole source for the enzyme display severely decreased cellular PtdIns[3,5]P_2 levels [336]. Furthermore, absence of Fab1 has no effect on the cellular PtdIns[4,5]P_2 levels, indicating that it does not serve as a PtdIns[4]P 5-kinase *in vivo* [336, 353]. Moreover, *in vitro* lipid kinase assays revealed that Fab1 has a strong selectivity for catalyzing the phosphorylation of the hydroxyl group in the D5 position of the inositol ring of PtdIns[3]P to PtdIns[3,5]P_2 [353,358]. Intriguingly, *vac7*Δ and *vac14*Δ cells also lack readily detectable PtdIns[3]P 5-kinase activity, suggesting that Vac7 and/or Vac14, which do not display any homology to known lipid kinases, might act as upstream activators of Fab1 [336, 349,352]. In this regard, as revealed by using the yeast two-hybrid screen, Vac14 interacts with the PtdIns[3,5]P_2 phosphatase, Fig 4 (see section 3.1.2), and a number of other proteins likely to play roles in membrane trafficking and vacuolar function [349,356,357], whereas other evidence suggests that Vac7 is involved in vacuole inheritance and retrograde membrane trafficking between the vacuole and the PVC [340,359]. Furthermore, the phenotypes of a *vac7* null mutation are suppressed in cells expressing the *fab1-5* allele that is characterized by a dramatic increase in the cellular PtdIns[3,5]P_2 levels [356]. Moreover, in a screen designed to identify *vac7*Δ bypass mutants FIG 4 and *SAC1*, both encoding polyphosphoinositide phosphatases (see section 3.1), have been identified as negative regulators of Fab1-dependent PtdIns[3,5]P_2 levels [356]. In addition, cells expressing the mutant *fab1-20* allele, which retains residual PtdIns[3]P 5-kinase activity, as their sole source for this enzyme display less severe defects in vacuolar protein sorting, morphology and acidification as *fab1* null cells [360]. Taken together these findings strongly suggest that the phenotypes shared by the class III mutants *fab1*, *vac7* and *vac14* are due to a deficiency in cellular PtdIns[3,5]P_2 levels. In line with this, overproduction of PtdIns[3,5]P_2 due to the simultaneous overexpression of Fab1 and Vac14 results in both an increase in the number and a decrease in the volume of vacuole lobes, similar to what is observed in cells exposed to osmotic stress [352,354]. Along these lines, cells harboring hyperactive alleles of *FAB1* produce elevated levels of PtdIns[3,5]P_2 under basal and hyperosmotic conditions. Likewise, the defects in vacuolar lobe size associated with *vac7*Δ and/or *vac14*Δ and even *vac7*Δ *vac14*Δ fig 4Δ triple mutations are also suppressed in these cells, whereas the hyperosmotic response is abolished in the triple mutant. In addition, overexpression of both *VAC7* or *VAC14* result in elevated basal PtdIns[3,5]P_2 levels, whereas only overexpression of *VAC7* leads to a further increase of the cellular level of this phospholipid during hyperosmotic response [355].

Cell fractionation studies reveal that Fab1 is enriched in fractions that contain vacuolar and endosomal markers and indirect immunofluorescence experiments demonstrate predominant

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localization of Fab1 and Vac7 to the vacuolar membrane [336,340], which is in good agreement with the reported phenotypes of *fab1* mutants. In addition, Vac14 also associates with vacuolar membranes in a manner that depends on Fab1, but not Vac7 [357] as demonstrated by cell fractionation studies [352] and detection of a Vac14-GFP fusion *in vivo* [357]. Furthermore, the subcellular distribution pattern of GFP-Fab1 is unaltered in cells lacking either *VAC7* or *VAC14* when compared to wild-type, demonstrating that Fab1 localization to endosomal and vacuolar membranes is independent of its upstream activators [349,352]. In addition, the subcellular distribution patterns of Fab1, Vac7 and Vac14 do not change during the hyperosmotic stress response [354], although all three proteins are clearly involved in this process.

In this regard, PtdIns[3,5]P_2, which is generated sequentially by the sequential action of Vps34 and Fab1, plays an important role in the multivesicular body (MVB) sorting pathway in yeast and mammalian cells [361,362]. In eukaryotic cells the MVB pathway mediates the delivery of transmembrane proteins and lipids destined to be degraded in the vacuole/lysosome and of a subset of hydrolases native to this compartment [363–365]. MVBs are formed by budding or invagination of vesicles into the lumen of the endosomal compartment. Subsequently the mature MVBs are transported to and fuse with the vacuole/lysosome, thereby delivering the internal vesicles and associated transmembrane proteins into the vacuolar lumen for further processing or degradation by vacuolar hydrolases [363–365]. The MVB sorting pathway is dependent on the class E subset of *VPS* genes [100], which for the most part encode subunits of one of three different ESCRT (endosomal sorting complex required for transport) complexes [163–165,363,365], as null mutations in these genes lead to the mislocalization of transmembrane proteins to the limiting vacuolar membrane thereby forming an aberrant membranous structure termed the class E compartment [131,362,366]. Likewise, cells expressing catalytically inactive Fab1 mislocalize carboxypeptidase S (CPS), a resident vacuolar hydrolase, and Ste3, a plasma membrane mating pheromone receptor, to the peripheral vacuolar membrane rather than delivering it into the lumen of this organelle, suggesting that Fab1 is involved in MVB protein sorting of both biosynthetic and endocytic cargo [360,362]. This effect not seen in cells expressing *FAB1* carrying mutations in the catalytic domain leading to significantly decreased yet still detectable PtdIns[3,5]P_2 levels [362]. Further support for a role of Fab1 in the MVB sorting pathway comes from the finding that *fab1* null cells exhibit a dramatically reduced number of intravacuolar vesicles [336]. In addition, the vast majority of *vac14* null but not *vac7*Δ cells display a similar MVB protein sorting defect as *fab1* mutants [349].

To date a number of different PtdIns[3,5]P_2 effectors have been identified in yeast, including Ent3, Ent5, and Atg18 (Figure 11) [159,367,368]. Ent3 is a homolog of mammalian epsin and possesses an evolutionarily conserved ENTH (epsin NH_2-terminal homology) domain, a known phosphoinositide-binding motif [48,49,369,370]. Ent5 is another yeast epsin containing an ANTH (AP180 NH_2-terminal homology) domain that is highly related to, yet distinct from the ENTH domain [48,49,370]. Both proteins specifically bind PtdIns[3,5]P_2 but not other phosphoinositides both *in vitro* and *in vivo*, in a manner that depends on their ENTH/ANTH domains [367,368]. In addition, Ent3 and Ent5 localize to a prevacuolar endosomal compartment in wild-type cells, but not in cells defective for Fab1 and/or Vps34 function [367,368].

Both proteins share a redundant cellular function, as double null mutants, but not individual deletion of either gene alone, leads to defects in CPS and α-factor maturation [369]. In line with this, cells lacking both Ent3 and Ent5 display a defect in the MVB sorting of biosynthetic and endocytic cargoes, such as Ste2 and Cps1, downstream of cargo ubiquitination [367]. This effect is specific for MVB sorting pathway as *ent3*Δ *ent5*Δ cells properly process DPAP B (dipeptidyl aminopeptidase B), a type II transmembrane protein that reaches the limiting

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vacuolar membrane via the Vps pathway [367]. Similarly, Ent3 and Ent5 appear to play no role in vacuole acidification [367], unlike Vac7 and Vac14 [340,349], a process known to require functional PtdIns[3,5]P_2 [130,336]. In this regard, both Ent3 and Ent5 have been shown to interact with clathrin [368,369] and, hence, could function in a manner analogous to Ent1/2 upon PtdIns[4,5]P_2-binding during the internalization step of endocytosis at the plasma membrane [370,371].

In addition to PtdIns[3,5]P_2 generation MVB sorting also depends on the tagging of cargoes by monoubiquitination, in a manner that requires the three ESCRT complexes and the FYVE domain protein Vps27, but not Fab1 (see section 2.1) [372–375]. In *S. cerevisiae* the class E Vps protein Vps27 forms a heterodimeric complex with Hse1, which directly interacts with ubiquitin via ubiquitin interaction motifs (UIMs) [372,375]. In this regard, it has been shown that Ent3 and Ent5, which lack any known ubiquitin-binding motif, directly interact with Vps27 *in vitro* and *in vivo*, thereby bridging the gap between these yeast epsins and monoubiquitination of MVB cargo proteins [367]. Taken together, these findings suggest that the Ent3/5-Vps27 complex may provide a direct connection between monoubiquitinated cargo proteins and phosphoinositide signaling in the MVB-sorting pathway.

Atg18, a protein implicated in regulation of both autophagy/pexophagy and the Cvt pathway [376,377], was first identified as a PtdIns[3,5]P_2 effector owing to the fact that disruption of *ATG18* phenocopies the extreme enlargement of the single-lobed vacuole observed in *fab1* mutants [130,159]. Subsequently, it could be demonstrated that Atg18 localizes to the vacuolar membrane in a Fab1-dependent manner and specifically binds to PtdIns[3,5]P_2 *in vitro* [159]. This interaction is mediated by a seven-bladed β-propeller structure of Atg18 that is formed by multiple WD40 repeats, as other regions of the protein are dispensable for phosphoinositide binding (Figure 9) [159]. Moreover, expression of Atg18 constructs incapable of binding PtdIns [3,5]P_2 do not suppress the vacuolar morphology phenotype associated with an *atg18* null mutation, indicating an essential role for this phospholipid in Atg18 function [159]. Furthermore, cells harboring an *atg18* null mutation display a block in the vacuole-to-late endosome trafficking [159], suggesting that Atg18 functions together with Fab1 in the recycling of proteins and membrane from the vacuole to the endosome. Other cellular functions of Fab1-generated PtdIns[3,5]P_2, however, do not depend on Atg18, as vacuolar acidification and MVB sorting are not affected in *atg18*Δ cells [159].

In *Schizosaccharomyces pombe* the vacuole serves similar physiological roles as in *S. cerevisiae* and most proteins required for vacuolar protein transport and biogenesis are conserved between both yeasts [172]. In this context the *S. pombe* homolog of Fab1, namely SpSte12, restores basal levels of PtdIns[3,5]P_2 when expressed in *S. cerevisiae* harboring a *fab1* null mutation, whereas it does not complement the temperature-sensitive growth phenotype or restore normal morphology of these cells [358]. This result suggests that SpSte12 is unable to interact properly with certain Fab1 regulators in *S. cerevisiae* or that both proteins have different functional properties independent of their lipid kinase activities. In this context, and unlike its *S. cerevisiae* counterpart, evidence for involvement of the *S. pombe* enzyme in the pheromone response pathway in this organism has been obtained [378].

In summary, these findings strongly suggest that Fab1-generated PtdIns[3,5]P_2 exerts its physiological roles through multiple effectors that at least partially act independently of each other.

2.3.2 PtdIns4P 5-kinases—The first PtdInsP kinase gene to be cloned from *Saccharomyces cerevisiae* was *MSS4* [288]. Mss4 displays PtdIns[4]P 5-kinase activity *in vitro* [297,300] and also functions as a PtdIns[4]P 5-kinase *in vivo*, as the expression of different mammalian isozymes, but not of a PtdIns[5]P 4-kinase isoform, restores the viability of yeast cells

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harboring a *mss4* null mutation [297]. Similarly, the temperature-sensitive growth defect of two different conditional alleles, namely *mss4-1*^{ts} and *mss4-2*^{ts}, which is mirrored by a decreased cellular PtdIns[4]P 5-kinase activity [297,300], is equally suppressed when Mss4 is expressed from a plasmid and cells overexpressing *MSS4* display elevated PtdIns[4,5]P_2 levels [300]. In cell fractionation experiments about 80% of the enzyme is found in the membrane fraction, whereas indirect immunofluorescence microscopy reveals an almost exclusive and uniform localization of Mss4 at the plasma membrane [200,297].

Mss4 was initially isolated as a multicopy suppressor of the temperature-sensitive growth of the *stt4-1*^{ts} (MSS4) mutation in an attempt to clone the authentic wild-type *STT4* gene (see section 2.2.2) [288]. This strongly suggested a role for Mss4 in one or more Stt4-mediated pathways (Figure 10A). This hypothesis received further support when it was demonstrated that overexpression of *MSS4* and *STT4*, but not *FAB1* (see section 2.3.1) conferred wortmannin resistance to otherwise sensitive cells [207]. Moreover, cells lacking Plc1, a phospholipase C isozyme that specifically hydrolyses PtdIns[4,5]P_2 to generate DAG and Ins[1,4,5]P_3 [204, 379,380], are viable and resistant to growth inhibition by wortmannin, again strongly suggesting a phosphatidylinositol cascade involving both Stt4 and Mss4 and an essential function for PtdIns[4,5]P_2 itself rather than its breakdown products [207].

However, cells lacking functional Mss4 are inviable irrespective of osmotic support, even in strains where Stt4 is dispensable in the presence of an osmotic stabilizer. Moreover, *mss4* null cells do not display a lytic phenotype in some strain backgrounds, unlike cells lacking either *STT4* or *PKC1* and overexpression of *MSS4* only partially suppresses the lytic phenotype of a *stt4-1*^{ts} mutant [288]. In addition, overexpression of *MSS4* does not suppress the staurosporine-sensitive phenotype caused by *stt4-1*^{ts} at all [288]. In summary, these findings indicate additional physiological functions for Mss4 apart from its role in the Pkc1-mediated signaling pathway downstream of *STT4* [288].

In this context, cells lacking functional Mss4 display a phenotype reminiscent to that of mutations of actin-binding proteins, namely enlarged cells with a rounded shape [298,299] and eventually undergo lysis, similar to cells lacking *STT4* or *PKC1* [200,300]. Furthermore, Boi1 and Boi2 are recruited to the bud via their PH domains in a PtdIns[4,5]P_2–dependent manner, where they play an important role in polarized growth [381,382]. A closer investigation of a possible function of Mss4 in the regulation of the actin cytoskeleton by several groups using temperature-conditional alleles of *MSS4* and fluorescently labeled phalloidin, showed that proper organization of actin cables and polarized cortical actin patches is lost upon shift to non-permissive temperature. Instead these cells display randomly distributed cortical actin patches throughout the cells, clearly indicating that functional Mss4, and, hence, PtdIns[4,5]P_2, is required for proper organization of the actin cytoskeleton in *S. cerevisiae* [193,297,300,383].

In this regard, evidence suggests that Mss4 acts downstream of calmodulin (Figure 10B), a small Ca^{2+}-binding protein that is involved in controlling a variety of physiological processes, including actin cytoskeleton organization [384–386]. Temperature-conditional alleles of calmodulin, which is encoded by the essential gene *CMD1* [387], can be classified into a number of different intragenic complementation groups according to their mutant phenotypes [386, 388]. Mss4 specifically suppresses the mutant phenotype of calmodulin alleles defective in actin cytoskeleton organization (*cmd1-226*^{ts}), but not those inoperative in calmodulin localization (*cmd1-228*^{ts}) or nuclear division (*cmd1-239*^{ts}) [389]. Moreover, at restrictive temperature *cmd1-226*^{ts} cells exhibit severe deficiencies in actin cytoskeleton polarization and growth defects, reminiscent of the *mss4*^{ts} phenotype, that can both be suppressed by the overexpression of *MSS4*, but not *PIK1* or *STT4* (see section 2.2.2) [389]. Furthermore, unlike wild-type cells [200, 300], strains carrying the *cmd1-226*^{ts} allele fail to induce PtdIns[4,5]P_2 production in response to mild heat shock [389]. The finding that overexpression of various

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components of the Pkc1-dependent Slt2/Mpk1 MAP kinase cascade or of upstream activators, such as Rho2 and Rom2 only weakly suppress the mutant phenotype of *cmd1-226*^{ts} and *mss4*^{ts} alleles or do not have an effect at all indicates that Mss4 and Cmd1 signal at least in part independently of the Pkc1-activated pathway [389]. Moreover, Mss4 and Cmd1 are not required for activation of the Pkc1-dependent MAP kinase pathway, as cells harboring either *cmd1-226*^{ts} or *mss4*^{ts} alleles display normal activation of Slt2/Mpk1 at restrictive temperature [389]. Taken together, this suggests that calmodulin functions upstream of Mss4 in a pathway that controls actin cytoskeleton organization via PtdIns[4,5]P_2 (Figure 10B) and is at least in part independent of the Slt2/Mpk1 MAP kinase cascade. This hypothesis is supported by the observation that there is some functional redundancy of the Slt2/Mpk1- and Ca^{2+}-mediated regulation of the actin cytoskeleton [390].

In addition, *MSS4* plays a role in endocytosis, as cells lacking functional Mss4 are defective in fluid phase endocytosis and in receptor-mediated uptake [389]. Calmodulin has also been implicated in endocytosis and *cmd1*^{ts} mutants display defects in endocytic uptake, very similar to those seen in *mss4*^{ts} mutants at restrictive temperature [385,391]. In this regard, it has been established that a number of key components of the clathrin-mediated endocytic machinery are PtdIns[4,5]P_2-binding proteins [392,393].

These findings received further support when it was demonstrated that *MSS4* functions as a multicopy suppressor of certain *tor2*^{ts} mutants [394]. *TOR2* (target of rapamycin) [395] was implicated previously in regulation of the actin cytoskeleton because cells carrying a *tor2*^{ts} allele fail to properly organize their actin cytoskeleton at restrictive temperature [396,397]. Tor1 and Tor2 are two highly homologous (67% identity) phosphoinositide kinase-related Ser/Thr-specific protein kinases [398,399] that share overlapping physiological functions [395, 400]. However, whereas both Tor1 and Tor2 play a role in the control of cell growth in response to nutrient conditions [401], Tor2 also has a second, unique function in the cell cycle-dependent control of the polarized distribution of the actin cytoskeleton [394,397,402]. In addition to *MSS4*, *RHO2*, *ROM2* and *PKC1* have been identified as suppressors of a mutant deficient specifically in the unique function of Tor2 [394]. All these genes have been demonstrated independently to function in the cell wall integrity pathway, as well as in heat shock-induced reorganization of the actin cytoskeleton downstream of both *STT4* (see section 2.2.2) and *MSS4* [200,282,284,300,396,403]. Moreover, Rom2, a GEF specific for Rho1, and likely Rho2, [285] and upstream activator of the Pkc-dependent Slt2/Mpk1 MAP kinase pathway [282,284], is recruited to the plasma membrane in a PtdIns[4,5]P_2-dependent manner that requires both Stt4 and Mss4 (Figure 10A) [200].

Furthermore, in a genome-wide synthetic lethality screen designed to detect novel regulators and effectors of PtdIns[4,5]P_2 in yeast, a direct connection between Tor2-dependent signaling and Mss4 has been established [383] (Figure 10B). Here the *mss4-102*^{ts} allele was combined with each of approximately 4700 deletion mutants and the resulting double mutants were tested for synthetic growth defects. In addition to genes already known to interact with *MSS4*, such as *ROM2* [200], several additional factors were identified that had not been implicated previously in PtdIns[4,5]P_2 signaling. Most of the genes displaying synthetic defects with *mss4-102*^{ts}, including *API4*, *AVO2*, *BEM4*, *BOI2*, *CAP1*, *CAP2*, *CYK3*, *ILM1*, *MYO5*, *SPA2*, and *VAN1*, as well as multiple components of the GimC complex, which acts as a chaperone for the assembly of actin and tubulin [404], had previously been suggested to serve as regulators of polarized growth and actin cytoskeleton organization. However, a number of genes with known roles in membrane transport and heat shock response, in addition to genes encoding proteins of unknown function were also identified in this screen.

Subsequently, the authors chose to investigate the interaction of *mss4-102*^{ts} with a novel gene termed *SLM1* (synthetically lethal with *mss4*^{ts}) in more detail. In addition to the inviability

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observed for *slm1*Δ *mss4-102^{ts}* mutants [383], combination of a *slm1* null mutation with a *stt4^{ts}* allele (see section 2.2.2) results in a synthetic lethal defect [405]. Slm1 and its closest homolog in the yeast genome Slm2 (53% identity) share an essential function, as cells lacking either gene grow normally and display no obvious phenotype, whereas a *slm1*Δ *slm2*Δ double mutant is inviable [383,405]. Sequence comparison reveals that both proteins possess a coiled-coil domain, as well as a COOH-terminal pleckstrin homology (PH) domain [383,405] that is able to interact with phosphoinositides *in vitro* [405,406]. Surprisingly, a *slm2* null mutation does not display any synthetic growth defects in combination with *mss4-102^{ts}* [383] and only shows mild synthetic defects together with the *mss4-2^{ts}* allele [405], findings that could be explained by the fact that Slm1 is much more abundant than Slm2 in wild-type cells. Expression of fully functional GFP-fusion proteins of Slm1 and Slm2 *in vivo* uncover an association with punctate structures present at the cell periphery that are independent of F-actin [383,405]. In cells lacking functional Mss4, however, Slm1 partially mislocalizes to the cytoplasm, although cortical punctate structures can still be detected [383,405]. On the other hand, Slm1 localization in the absence of either functional *PIK1* or *FAB1* (see section 2.3.1) is indistinguishable from that in wild-type cells, whereas strains harboring a *stt4^{ts}* mutation display redistribution of a Slm1 pool from the plasma membrane to the cytoplasm [405]. Introduction of point mutations in residues critical for phosphoinositide binding of the PH domain [406] or COOH-terminal truncations, lacking this domain altogether, have a similar effect on the subcellular distribution of Slm1 as inactivation of *MSS4*, suggesting an interaction of Slm1 with PtdIns[4,5]P_2 via its PH domain *in vivo* [383,405]. Furthermore, cells lacking *INP51/SJL1*, encoding a non-essential polyphosphoinositide phosphatase (see section 3.2.1), display a two- to four-fold increase in PtdIns[4,5]P_2 levels [255,291] and disruption of *INP51/SJL1* suppresses the inviability of cells expressing *SLM1* at very low levels as their sole source for Slms [405]. Another indication that Slm1 acts downstream of PtdIns[4,5]P_2 comes from the finding that at restrictive temperature *slm1^{ts} slm2*Δ cells display the same phenotypes as *mss4^{ts}* cells, namely depolarization of the actin cytoskeleton with randomly distributed cortical actin patches throughout both mother and daughter cells and a cell lytic defect upon extended incubation at elevated temperature [383, 405].

In a subsequent screen both *SLM1* and *SLM2*, as well as *MSS4*, *PKC1* and *SEC4* were detected as dosage-suppressors of a *slm1^{ts} slm2*Δ double mutant at restrictive temperature [383,405]. *SEC4* encodes a member of the Ypt/Rab subfamily of small GTPases that plays a role in the polarized transport of post-Golgi vesicles to and fusion with the plasma membrane [407–409]. Sec4 function had already been implicated in the transport of Rho1 to sites of polarized growth and *sec4* mutants had been reported to display defective organization of the actin cytoskeleton [410,411]. Furthermore, all genes that suppressed the growth defect of the *slm1^{ts} slm2*Δ double mutant have also been found to alleviate the actin defects of this mutant. In addition, expression of a constitutively active version of Pkc1 or deletion of *SAC7*, encoding a GTPase-activating protein (GAP) for Rho1 [403] also allowed growth of cells carrying the *slm1^{ts} slm2*Δ double mutation [383]. Taken together these findings strongly suggest an essential role for Slm1/2, and, hence, Mss4 in the regulation of growth and the polarization of the actin cytoskeleton.

In addition, Slm1 and Slm2 are phosphoproteins *in vivo* [383,405]. In this regard, Slm1 and Slm2 have been demonstrated to interact with Avo2 [383,405,412,413], a component of the TORC2 complex, which contains Tor2, but not Tor1, and mediates the essential unique function of Tor2 in actin cytoskeleton organization [402,414]. Unlike in wild-type cells, the phosphorylated species of Slm1 are not present in *tor2^{ts}* or *mss4-102^{ts}* cells upon shift to restrictive temperature [383]. Moreover, mutant versions of Slm1 that are unable to interact with PtdIns[4,5]P_2, display residual membrane localization that is completely dependent on TORC2, as it cannot be detected at the cell periphery in a *tor2^{ts}* strain or in cells lacking individual TORC2 components [383,405]. In this regard, defects associated with non-

functional *avo3*^{ts}, an essential TORC2 component [402], can be suppressed by overexpression of *SLM1* [415]. Intriguingly, Tor2 and other TORC2 components display localization to punctate clusters at the plasma membrane [416,417], similar to Slm1, Slm2 and Mss4. Taken together with the earlier finding that *AVO2* had been identified in the original genetic synthetic analysis this strongly suggests that both Tor2 and Mss4 are required for proper localization and phosphorylation of Slm1 and Slm2, which serve to integrate both inputs, in a pathway that regulates actin cytoskeleton polarization.

Recently, evidence for a function of Mss4 in post-Golgi secretory trafficking was obtained, in that overexpression of *MSS4* specifically alleviated the growth and secretory defects of a number of late secretion (*sec*^{ts}) mutants [290]. Among the *sec*^{ts} mutants that are rescued by an increased dosage of *MSS4* are various components of the exocyst (*SEC8*, *SEC10*, and *SEC15*), an octameric multiprotein complex required for tethering of secretory vesicles to the plasma membrane in preparation for fusion [408,418,419]. In this context, another exocyst component, Sec3, is a known effector of Rho1 [420], which is itself activated by Rom1/2 in a PtdIns[4,5]P_2-dependent manner [200]. Furthermore, *MSS4* also functions as a dosage suppressor of mutations in the structural genes for the syntaxin-binding protein Sec1 [421–423], the plasma membrane t-SNARE (target-membrane soluble *N*-ethylmaleimide-sensitive fusion protein attachment receptor) Sec9 [424–426], and Sec2, a guanine nucleotide exchange factor (GEF) specific for the Ypt/Rab small GTPase Sec4 [259,427]. Elevated expression of *SEC9* also rescues the inviability of cells carrying temperature-conditional alleles of the exocyst components, *SEC8*, *SEC10*, and *SEC15*, at a non-permissive temperature, suggesting that *SEC9* functions downstream of the exocyst. This finding receives further support by the cumulative genetic evidence of a number of studies indicating that Sec2/4 function upstream of both the exocyst and the yeast *lgl* (lethal giant larvae) ortholog, Sro7 (also known as Sop1) [428,429], which in turn function upstream of Sec1 and Sec9 [430]. Taken together, these findings suggest that *MSS4* somehow regulates the fusion of secretory vesicles with the plasma membrane, possibly by the lipid-mediated activation of Sec9 function. In this regard, Spo20, a sporulation-specific t-SNARE that is homologous to Sec9 depends on the presence of acidic phospholipids to execute its function at the prospore membrane [431].

Another effector for Mss4-generated PtdIns[4,5]P_2 is Spo14 [432], the major phosphatidylcholine-specific phospholipase D activity in yeast [267–269]. Usage of a GFP-Spo14 chimera, which serves as a reporter for elevated PtdIns[4,5]P_2, uncovers Spo14 as an effector for Mss4 at the plasma membrane of vegetatively growing yeast cells overexpressing *MSS4* [432]. Furthermore, this Mss4-dependent relocalization of Spo14 depends on the integrity of its PH domain [432], which is known to influence the localization of Spo14 [318, 433].

Furthermore, in *S. cerevisiae*, Mss4 sits at the nexus of a newly uncovered relationship between phosphoinositide and sphingolipid metabolism. Accumulating evidence places Mss4 as a downstream effector of sphingolipid turnover [434]. Sphingolipids constitute an essential class of abundant lipids, whose backbone is formed by ceramide rather than diacylglycerol, that play important roles as precursors for signaling molecules and as structural components of biomembranes [435–438]. Myriocin is a potent inhibitor of serine-palmitoyltransferase, encoded by *LCB1* and *LCB2* in yeast, and, hence, blocks the de novo sphingolipid biosynthesis pathway and leads to cell death upon extended exposure [439]. In this regard, *MSS4* was identified as a multicopy suppressor of the myriocin-induced inviability in a number of yeast strains [434]. This effect depends on the catalytic activity of Mss4, as kinase-dead versions of the enzyme do not restore viability in the presence of myriocin. Moreover, disruption of *PLC1*, the structural gene encoding the sole phosphoinositide-specific phospholipase C activity in *S. cerevisiae* [204], but not overexpression of the PtdIns 4-kinase Stt4 (see section 2.2.2), causes myriocin resistance [434], indicating an important role for PtdIns[4,5]P_2 in this process.

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Strikingly, overexpression of *MSS4* does not lead to a restoration of sphingolipid biosynthesis in myriocin-treated cells, as sphingolipid levels are indistinguishable from those of cells expressing *MSS4* at physiological levels. Myriocin, however, leads to a reduction of Mss4 activity *in vivo*, which can be restored by the supply of phytosphingosine (PHS) to the culture medium [434]. In addition, myriocin treatment of cells results in the mislocalization of Mss4 to the cytoplasm [434], which in turn prevents its phosphorylation by casein kinase I, a process known to be important for proper Mss4 function [440]. However, external supply of PHS or overexpression of *MSS4* reverses this effect even in the presence of myriocin. Analysis of a number of strains carrying mutations in different sphingolipid biosynthetic genes revealed mislocalization of Mss4 in *csg2*Δ cells, suggesting that the mannosylated inositol-phosphorylceramide species is required for plasma membrane-targeting of the enzyme [434]. Similar to what had been observed before for dosage suppressors of *tor2*^{ts} mutants [394] the growth defects associated with the myriocin-treatment of cells can be suppressed by elevated levels of *MSS4*, *RHO2*, *ROM2* and *PKC1* [434]. The aberrant cytoplasmic localization of Mss4 in myriocin-treated cells is mirrored by the mislocalization of the Rho1/2 GEF, Rom2, under the same conditions. In summary, this observation suggests that *MSS4* functions upstream of *ROM2*, *RHO2* and *PKC1* in a sphingolipid-dependent pathway that is essential for yeast cell growth. However, the exact mechanism by which sphingolipids might influence this pathway remains elusive.

In addition to its peripheral association with the plasma membrane a pool of Mss4 also localizes to the nucleus [440], similar to mammalian type I and II PtdInsP kinases [441,442]. Temperature-conditional mutants of *MSS4* that either specifically accumulate in the nucleus (class I) or remain mainly localized at the plasma membrane (class II) upon shift to non-permissive temperature have been isolated. Representative alleles, namely *mss4-1*^{ts} (class I) and *mss4-6*^{ts} (class 2), display severely reduced cellular levels of PtdIns[4,5]P_2 and defects in actin cytoskeleton organization at restrictive temperature. The finding that the *mss4-1*^{ts} allele only contains two point mutations in the NH_2-terminal non-catalytic part of the enzyme suggests that mislocalization, rather than impaired kinase activity is responsible for its mutant phenotype [440].

Nuclear entry of Mss4 is mediated largely by the karyopherin, Kap123, as cells harboring a *kap123* null allele display a dramatic accumulation GFP-Mss4 in the cytoplasm, whereas nuclear localization is undetectable in normal cells, even when *MSS4* is overexpressed. Deletion of *KAP123* rescues the temperature-sensitivity of the *mss4-1*^{ts} allele, as does membrane-tethering of the Mss4-1 mutant protein. The authors of this study interpreted these results as indicating that PtdIns[4,5]P_2 generation at the plasma membrane (rather than in the nucleus) is essential for yeast cell survival [440]. However, it is possible that the *mss4-1* allele specifically prevents efficient Mss4 association with and action at the plasma membrane, but does not interfere with Mss4 entry into and function within the nucleus (the strategy to tether Mss4-1 to the plasma membrane was not likely to be 100% effective). Moreover, the inositol-phosphates derived from Plc1-dependent hydrolysis of Mss4-generated PtdIns[4,5]P_2 are highly water-soluble and presumably can diffuse rapidly into (and out of) the nucleus. In this regard, it is important to point out that Plc1 undergoes nucleocytoplasmic shuttling [599]. Furthermore, Pik1 also undergoes nucleocytoplasmic shuttling and is responsible for generating the nuclear pool of PtdIns[4]P (which Mss4 in the nucleus can presumably convert to PtdIns[4,5]P_2) and lack of nuclear entry of Pik1 is lethal [202]. Thus, it seems reasonable to assume that nuclear Mss4, and the nuclear PtdIns[4,5]P_2 pool derived from its action, also are necessary for yeast cell viability.

Furthermore, like several PtdIns[4]P 5-kinase from other organisms [443], Mss4 is subject to phosphorylation, which only takes place at the plasma membrane, as the enzyme is phosphorylated in *mss4-1*^{ts} cells at the permissive, but not at restrictive temperature. In contrast,

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this effect cannot be observed in cells harboring the class II allele *mss4-6^{ts}* where Mss4 is equally phosphorylated under both conditions. The kinase responsible for Mss4 phosphorylation is casein kinase I, a plasma membrane-associated enzyme that is encoded by two separate genes, namely *YCK1* and *YCK*2 [85]. Newly synthesized Mss4 largely fails to associate with the plasma membrane in cells lacking functional casein kinase I (5% in *yck1*Δ *yck2^{ts}* cells versus 65% in the corresponding wild-type cells) indicating an important role for Mss4 phosphorylation in membrane targeting although additional factors may be involved in this process [440]. Nuclear export of Mss4 depends on the expression of *BCP1* [440], an uncharacterized essential gene that shares significant homology with a nuclear protein in mammals encoded by *BCCIP* [444,445]. *BCP1* overexpression restores plasma membrane association of Mss4-1, leads to an increase in the cellular PtdIns[4,5]P_2 levels and alleviates the actin cytoskeleton organization defect at restrictive temperature in *mss4-1^{ts}* cells, but not in cells harboring *mss4-6^{ts}* [440]. In addition, *bcp1^{ts}* cells completely lack the heat shock-induced increase in PtdIns[4,5]P_2 production [440]. Strikingly, these cells also show defects in Mss4 phosphorylation, which typically parallels plasma membrane localization and *bcp1^{ts} yck1*Δ *yck2^{ts}* display significant synthetic growth defects. Therefore, Bcp1 appears to promote both nuclear export and plasma membrane targeting of Mss4. This effect is unlikely to be solely due to increased membrane binding, and, hence, amplified phosphorylation of Mss4, as the unrelated large 60S ribosomal subunit also failed to become efficiently exported from the nucleus in *bcp1^{ts}* cells at restrictive temperature [440]. This notion receives further support from the finding that fully functional GFP-fusion of Bcp1 preferentially localizes to the nucleus [440].

3. Phosphoinositide phosphatases

Phosphoinositides cannot only be modified by lipid kinases, but also by a range of different phosphatases. Early studies demonstrating that phosphoinositides from animal brain are rapidly broken down with the liberation of inorganic phosphate suggested the presence of phosphoinositide phosphatases in these preparations [446,447], a hypothesis that could be verified several years later [448]. Similar to the conservation of PI kinases most genes encoding phosphoinositide phosphatases have been highly conserved during evolution.

The *S. cerevisiae* genome encodes seven known polyphosphoinositide phosphatases that belong to three different subgroups based on their catalytical domains (Figure 12). The first group possesses a catalytic domain related to that of yeast Sac1, which is thus referred to as Sac1-like domain [296]. Although *S. cerevisiae* Sac1 is the founding member of this protein family [296], the lipid phosphatase activity of Sac1-like domains was first demonstrated in mammalian cells [449]. Members of this family, including mammalian synaptojanin and the yeast enzymes Sac1, Fig 4 [450], Inp51/Sjl1, Inp52/Sjl2, and Inp53/Sjl3 have been conserved from lower eukaryotes to vertebrates [4]. The second group is characterized by an inositol polyphosphate 5-phosphatase domain that specifically hydrolyzes phosphates in the D5 position of the inositol ring [7]. The *S. cerevisiae* genome encodes four enzymes carrying an inositol 5-phosphatase domain, namely Inp51/Sjl1, Inp52/Sjl2, Inp53/Sjl3, and Inp54 [254, 255, 291, 451]. The last group of inositol phosphatases in yeast is represented by a single gene, *YMR1* [452], which shows substantial homology to mammalian myotubularins [453]. Unlike the phosphoinositide kinases most of the phosphatases have partially overlapping physiological functions in yeast cells, as Sac1-like domains use phosphate groups in different positions of the inositol ring as a substrate in a promiscuous fashion and Inp52/Sjl2 shares a role in several cellular processes with both Inp51/Sjl1 and Inp52/Sjl2 (Table 2).

3.1 PtdIns4P- and PtdIns3P-specific phosphatases

Sac1 and Fig 4 are characterized by a single Sac1-like phosphatase domain at their NH_2-termini (Figure 12), which retains catalytic activity when excised from the parent protein [235,454].

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In addition, this domain catalyzes the dephosphorylation of a number of different phosphoinositide species in both yeast and animal cells [235,449,454]. In *S. cerevisiae* Sac1 displays phosphatase activity against both PtdIns[3]P and PtdIns[4]P [235,454], whereas Fig 4 appears to specifically dephosphorylate PtdIns[3,5]P_2 at the D5 position of the inositol ring (Table 2) [357]. Both Sac1 and Fig 4 are encoded by non-essential genes presumably owing to the fact that most polyphosphoinositide phosphatases in yeast share somewhat redundant functions [124,239,255,294,455]. In line with this, overexpression of either *INP52/SJL2* or *INP53/SJL3* restores several of the phenotypes associated with *sac1* null mutations and the combination of a number of different null alleles of genes encoding polyphosphoinositide phosphatases display synthetic genetic interactions [294,454–457]. Sac1 is a 623 residue type II membrane protein that possesses two transmembrane domains in its COOH-terminal part [296,458], whereas FIG 4 encodes an 879 residue enzyme lacking any other known sequence elements (Figure 12) [450].

3.1.1 Sac1—The *SAC1* (suppressor of actin) gene was originally identified and cloned as a mutant suppressor of a temperature-conditional mutation in the single gene encoding actin in *S. cerevisiae* [296]. More than ten years later, owing to the fact that it shares considerable homology with mammalian synaptojanin, Sac1 had been demonstrated for the first time to possess polyphosphoinositide phosphatase activity [235,454]. *In vitro* Sac1 dephosphorylates a number of different phosphoinositides, including PtdIns[3]P, PtdIns[4]P, and with much lower activity PtdIns[3,5]P_2 (Table 2) [235,454]. Sac1 is a type II membrane protein that localizes to both the ER and the Golgi apparatus in a manner that depends on two transmembrane domains in its COOH-terminal part [294,458–460]. Furthermore, ER localization of Sac1 depends on Dpm1 [459], an ER transmembrane dolichol phosphate mannose synthase [461,462]. Likewise, functional Sac1 is required for efficient dolichol oligosaccharide synthesis in the ER [459]. In line with this, Sac1 exclusively localizes to the Golgi apparatus when Dpm1 is inactivated [459]. Moreover, both proteins interact in the ER in a manner that requires the transmembrane domain of Dpm1 [459]}. Subcellular localization of Sac1 depends on the growth conditions, as both its interaction with Dpm1 and ER localization is lost under nutrient limiting conditions in a reversible fashion [459]. In this regard, a PtdIns[4]P-specific probe that is exclusively found at the Golgi apparatus in exponentially growing cells displays perinuclear ER localization in glucose-starved cells, an effect also seen in *sac1* null strains [295,459]. Taken together this suggests that PtdIns[4]P is one of the major targets of Sac1 phosphatase activity *in vivo*, in good agreement with the finding that *sac1*Δ cells accumulate this phospholipid at the ER and vacuolar membranes [238,295]. Although *SAC1* encodes a non-essential product, *sac1* mutants are frequently inositol auxotrophs and display multiple drug- and cold-sensitive phenotypes [234,454,459,460,463–466].

Initially a mutant *sac1* allele was recovered as a spontaneous heat-resistant revertant from cells harboring the conditional-lethal temperature-sensitive *act1-1* mutation [296]. In this regard, *sac1* mutants display phenotypes reminiscent to those of the *act1-1* allele itself (Table 2), such as the failure to properly polarize the actin cytoskeleton and aberrant chitin deposition at the cell wall [236,296,463,464,467]. However, Sac1 does not colocalize with components of the actin cytoskeleton in *S. cerevisiae* [460]. These findings can now be explained, as Sac1 specifically acts on the pool of PtdIns[4]P at the plasma membrane that is generated by Stt4, but not Pik1 or Lsb6 [294,295] (see section 2.2). In line with this, cells harboring a *sac1* null mutation or mutant alleles compromised in their catalytic activity display significantly elevated levels of PtdIns[4]P, whereas the levels of other phosphoinositide species are only modestly affected or not changed at all [234,235,294,295,458,466]. Furthermore, it has been demonstrated [302] that Stt4 functions upstream of the p21-activated protein kinase, Cla4, and the small GTPase, Cdc42 (Figure 8), known regulators of polarized growth in *S. cerevisiae* [307,314,315]. In this regard, it has also been demonstrated that inactivation of Sac1 in cells

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harboring a temperature-conditional allele of *STT4* leads to the suppression of the actin cytoskeleton defects at restrictive temperature [294]. Likewise, deletion of *SAC1* in cells harboring temperature-conditional alleles of the genes encoding PtdIns 4-kinases in yeast, alleviates the defects associated with *stt4*^{ts}, but not *pik1*^{ts} or *lsb6*Δ mutations [295]. Furthermore, Stt4 generates a pool of PtdIns[4]P at the plasma membrane [200,290], which is converted to PtdIns[4,5]P_2 by the action of the PtdIns[4]P 5-kinase Mss4 (see section 2.3.2; Figure 10A) [237,238,292]. To date there is accumulating evidence indicating the importance of PtdIns[4,5]P_2 and, hence, Mss4 in the regulation of the actin cytoskeleton (Figure 10B) [193,297,300,383,389,394]. In this regard, *sac1* mutants display severe synthetic defects in combination with *slt2/mpk1* null alleles [236], again suggesting a role for Sac1 in the regulation of the actin cytoskeleton, since the Slt2/Mpk1 MAP kinase pathway has been implicated in the regulation of cell wall integrity and polarized growth [286]. Taken together, these results provide adequate evidence that Sac1 affects the actin cytoskeleton by regulating a pool of PtdIns[4]P, and, in turn also PtdIns[4,5]P_2, at the plasma membrane that are generated by Stt4 and Mss4, respectively (Table 2). These findings also suggest that Sac1, and hence the ER, must be in very close proximity to the plasma membrane in order for Sac1 to be able to act on the PtdIns[4]P pool generated by Stt4 at this location.

In addition, *sac1*Δ cells display impaired trafficking in the endocytic pathway of both the lipophilic dye FM4-64 and the chitin synthase Chs2 (Table 2) [295]. The expression of Chs2, which plays a role in septum formation, is cell cycle regulated [468], as it is rapidly endocytosed and degraded in the vacuole after completion of septum formation in wild-type cells [469]. In cells lacking Sac1, however, it accumulates in an endosomal compartment [295]. Moreover, this specific defect can be partially suppressed by the inactivation of Stt4, providing another link between the cellular functions of this PtdIns 4-kinase and Sac1 [295].

A role for Sac1 in regulating ATP uptake into the ER has been reported [470,471] (Table 2), a finding in good agreement with the reported localization of Sac1 to this compartment [294, 458–460]. In *S. cerevisiae*, the post-translational translocation of proteins destined for the secretory and vacuolar protein sorting (Vps) pathways depends on an HSP70 chaperone family member, Kar2 [472–474]. Kar2 and its mammalian homolog, BiP (immunoglobulin heavy chain-binding protein) [475], are located in the lumen of the ER and undergo cycles of peptide binding and release in a manner that requires the presence of ATP [476,477]. In this process, the ATP-bound form of Kar2/BiP, which is characterized by an open peptide-binding pocket, binds to polypeptides that are imported into the ER lumen [477]. Subsequently, the interaction of Kar2/BiP with Sec63 triggers rapid ATP hydrolysis thereby leading to the closure of the peptide-binding pocket. This in turn prevents the incoming polypeptides from sliding back to the cytoplasm, resulting in an overall forward movement. Therefore a sufficient supply of ATP in the ER lumen is crucial for the import of secretory and vacuolar proteins, such as prepro-α-factor and prepro-CPY (Figure 4), into this organelle [476]. In this regard, a *sac1* null allele displays synthetic defects together with mutations in a number of genes required for ATP-dependent protein translocation into the ER, including *KAR2*, *SEC61*, *SEC62*, and *SEC63* [470]. Moreover, reconstituted microsomes from strains harboring a *sac1* null mutation display impaired prepro-CPY and prepro-α-factor translocation, when compared to reconstituted proteoliposomes prepared from wild-type cells [471]. This effect is mirrored by the drastically reduced levels of ATP import into microsomal membranes prepared from cells harboring a *sac1* null allele (~ 85% reduction), while overexpression of *SAC1* or addition of recombinant Sac1 causes an increase in the rate of ATP uptake that is directly proportional to the amount of Sac1 protein present [470,471]. As a direct consequence of the lower rate of ATP import into the ER the Kar2/BiP-polypeptide complexes are stabilized and translocated proteins are retained in the lumen for a prolonged time, an effect also observed in *sac1*Δ cells [470,471]. Likewise, Kar2 is readily co-immunoprecipitated with pro-CPY from extracts of *sac1* null cells using an antiserum directed against CPY, whereas almost no Kar2 is found to be associated

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with pro-CPY when extracts prepared from wild-type cells are used [470]. Furthermore, accumulation of misfolded proteins in the ER of *S. cerevisiae* and other eukaryotes leads to the activation of a signal transduction cascade termed unfolded protein response (UPR) [478–481]. Ultimately this pathway leads to transcriptional activation of genes encoding resident ER proteins, such as molecular chaperones and protein folding catalysts, thereby counteracting the protein folding/transport defect. In line with this, the UPR pathway is constitutively activated in *sac1* null cells, again arguing for a reduced transport rate out of the ER in these cells [470]. The finding that purified recombinant Sac1 restores the impaired ATP transport activity in reconstituted detergent extracts from *sac1*Δ microsomes, but not in reconstituted proteoliposomes [470] indicates that Sac1 does not represent the ATP transporter itself, but rather a crucial cofactor for this process. The molecular basis for this function of Sac1, however, still remains elusive. Cells harboring the *sac1-22* allele as their sole source for this enzyme display defects in the Golgi-specific functions of Sac1, whereas the ER-specific ATP uptake is not affected [458]. In this regard, Sac1-22, which, unlike wild-type Sac1, accumulates at the ER *in vivo*, displays severely reduced phosphatase activity and, hence, causes alterations in the cellular levels of phosphoinositides, most prominently PtdIns[4]P [458]. Taken together, these findings suggest that Sac1 serves important independent functions in both the ER and the Golgi, and, hence, that substantial pools of PtdIns[4]P exist in both organelles.

In addition, Sac1 appears to play a role in cell wall maintenance (Table 2), as evidenced by the finding that a number of *sac1* mutants are hypersensitive to calcofluor white [236,458,467], which interferes with cell wall assembly and, hence, is frequently used to detect cell wall deficiencies in yeast [482]. Moreover, *sac1*, but not *inp51/sjl1*, *inp52/sjl2*, or *inp53/sjl3* (see section 3.2) single mutants show strong synthetic defects with mutations in *SLT2/MPK1* [236,467], which encodes a MAP kinase that plays an essential role in the cell wall integrity pathway [280,281,483]. Likewise, *sac1*Δ *slt2*Δ do not display any obvious phenotype in the presence of osmotic support, a condition known to suppress the defects associated with various cell wall mutants. Moreover, both proteins appear to regulate different aspects of cell wall maintenance, as overexpression of each gene does not suppress the defects associated with the loss of the other [467]. As cells carrying the mutant allele *sac1-22*, which does not display any defects in ATP uptake and protein transport in the ER [470], are sensitive towards calcofluor white it can be concluded that Sac1 serves its role in cell wall maintenance at the Golgi apparatus rather than the ER. In *S. cerevisiae* the chitin synthase 3 (Chs3) is rapidly redistributed to the plasma membrane from internal stores within the Golgi apparatus and endosomal compartments under conditions of cell wall stress [484,485]. This process ultimately leads to an increased chitin deposition in the cell wall, a process that has been proposed to provide a mechanism of cell wall repair [486,487]. In this regard, Chs3 is missorted to the vacuolar lumen in *sac1* null cells in a manner that requires a functional endocytic pathway [236]. Under normal growth conditions Chs3 is continuously recycled back from the plasma membrane to the Golgi/endosome, resulting in a steady-state localization in the storage compartments [484]. Overexpression of the PtdIns 4-kinase, Pik1, but not Stt4 (see section 2.2.2), also leads to mislocalization of Chs3 to the vacuole in a wild-type strain and causes a pronounced osmo-remedial defect in *slt2/mpk1* null cells [236].

Moreover, *sac1*Δ *mpk1/slt2*Δ double mutants also display morphological defects when grown without osmotic support, as the majority of these cells arrest as large budded cells with drastically elongated bud necks [467]. This effect is due to incomplete cell separation, as mitosis and cytokinesis are not affected in these mutants and the morphological defect can be reversed in the presence of zymolyase, an enzyme that digests the yeast cell wall [467]. In vegetatively growing cells a chitin ring is formed at the base of the emerging bud, which through further chitin deposition develops into the disc-shaped primary septum as the cell cycle progresses [488]. In addition to its part in the repair of cell wall damage Chs3, together with

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Chs2, also serves a role in chitin ring and septum biogenesis in dividing yeast cells [468]. In this regard, unlike each of the single mutants, *sac1*Δ *mpk1/slt2*Δ cells form extremely enlarged septa indicating abnormal chitin deposition at this location, an effect that is mirrored by the mislocalized accumulation of Chs2 at the cell periphery [467]. In conclusion, both Sac1 and Mpk1/Slt2 are required for transport and proper localization of the two chitin synthases Chs2 and Chs3 in yeast. In this regard, Pik1 has been shown to localize to the Golgi apparatus where it is involved in forward trafficking of secretory cargo destined for the plasma membrane [193,202,208,210]. Taken together, this suggests that the increased cellular PtdIns[4]P levels cause the Chs3 mislocalization and cell wall defects in cells compromised for Sac1 function. Moreover, these findings indicate that Sac1 and Pik1 functionally interact at the Golgi apparatus in the regulation of PtdIns[4]P levels in an antagonistic manner. Likewise, the localization of the oxysterol-binding protein family member Kes1 to the Golgi apparatus is regulated by PtdIns[4]P generated by Pik1 [240–242]. In contrast, Kes1 mislocalizes to the ER in *sac1* mutants, again indicating that Sac1 counteracts Pik1 function at this location [241].

The finding that inactivation of *SAC1* suppresses the defects associated with *sec14^{ts}* and *sec14*Δ mutations provided another link between Sac1 function and the secretory pathway [460,463]. *SEC14* encodes an essential phosphatidylinositol/phosphatidylcholine transfer protein (PITP) that had originally been identified in a genetic screen to detect novel secretory mutants [40,489]. In this regard, Sec14, which has functionally been linked to the PtdIns 4-kinase Pik1, localizes to the Golgi apparatus where it plays an important role in secretory trafficking [41,208,240,489–491]. In addition, mutations that interrupt the CDP-choline pathway that consumes DAG to generate phosphatidylcholine also lead to a bypass for the requirement of functional Sec14 [491]. Consistently, expression of bacterial diacylglycerol kinase, an enzyme that uses DAG to generate phosphatidic acid abolishes the Sec14-bypass phenotype of *sac1* strains [465]. Taken together, these findings suggest that a Golgi DAG pool is somehow required for Sec14-dependent secretion and that Sac1 negatively regulates this pool in a manner that still remains elusive.

In addition, Sac1 has also been implicated in vacuolar function, as *sac1* mutant cells display a fragmented vacuole phenotype and show synthetic defects with a number of Vps genes (Table 2) [295]. Interestingly, *vps34*Δ *sac1*Δ, *vps15*Δ *sac1*Δ (see section 2.1), and *fab1*Δ *sac1*Δ (see section 2.3.2) double mutant cells are all inviable, strongly suggesting a role for Sac1 in PtdIns [3]P and/or PtdIns[3,5]P_2 turnover [295].

3.1.2 Fig 4—The FIG 4 (factor induced gene) was originally identified in a screen for genes induced by mating pheromone in *S. cerevisiae* [450]. In this regard, FIG 4 expression is upregulated more than forty fold upon treatment of cells with α-factor [450]. In line with this, Fig 4 is required for normal mating projection formation and fig 4 mutants show polarization defects in mating mixtures [450]. Consistently, cells harboring a fig 4 null allele show an aberrant distribution of actin in mating projection tips [450]. To date the exact cellular function of Fig 4 during mating in yeast cells, however, remains elusive.

In another study, a function for Fig 4 in regulating the Fab1 lipid kinase (see section 2.3.1) and its upstream regulator, Vac7, has been suggested [356]. In line with this conclusion, Fig 4 is a peripheral membrane protein that predominantly localizes to the limiting membrane of the vacuole, but not endosomal compartments, in a manner that requires the COOH-terminal portion of the protein [354,356,357]. In addition, Fig 4 possesses polyphosphoinositide phosphatase activity and that PtdIns[3,5]P_2 functions as its major substrate both *in vitro* and *in vivo* (Table 2) [354,356,357]. Consistently, deletion of either FIG 4 or *SAC1* restore the cellular PtdIns[3,5]P_2 levels in a *vac7* null background [356]. Moreover, inactivation of Fig 4 or Sac1 reverses the mutant phenotypes associated with the *vac7* deletion, such as temperature-sensitive growth and the enlarged vacuole morphology, suggesting some degree of functional

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redundancy between these two phosphatases *in vivo* [356]. Moreover, Inp52/Sjl2 and Inp53/Sjl3 (see section 3.2) also regulate cellular PtdIns[3,5]P_2, as the levels of this lipid are significantly increased in *inp52/sjl2*Δ *inp53/sjl3*Δ fig 4Δ triple mutant cells when compared to fig 4 null mutants [356].

In addition to Vac7, Vac14 also serves as positive regulator of the PtdIns[3]P 5-kinase activity of Fab1 (see section 2.3.1) [340,349,352]. In this regard, Fig 4 physically associates with Vac14 and this interaction is required for the recruitment of Fig 4 to the vacuolar membrane [349, 357]. Vac14 localizes to the vacuolar membrane more efficiently when bound to Fig 4 [357]. Furthermore, the localization of both Vac14 and Fig 4 to the vacuole also relies on Fab1, but not Vac7 [357]. Nevertheless, Fig 4 does not suppress the *vac14* mutant phenotypes, suggesting that Vac7-dependent activation of Fab1 is weakened in the absence of Vac14 [349,357]. In contrast, Vac14 is required for the fig 4Δ-mediated suppression of the phenotypes associated with a *vac7* mutation [349,357].

Unexpectedly, Fig 4 is also involved in the upregulation of cellular PtdIns[3,5]P_2 levels upon hyperosmotic shock, in a manner that depends on its association with Vac14, but not Vac7 (see section 2.3.1) [354,355]. Consistently, the highest basal and hyperosmotically evoked levels of PtdIns[3,5]P_2 are observed in cells coexpressing all three regulators of Fab1 activity [354, 355]. Furthermore, Fig 4 and Vac14 also function together in the turnover of PtdIns[3,5]P_2, as mutants in either gene lead to a similar delay in the turnover of this phospholipid [355]. Likewise, cells expressing a catalytically inactive version of Fig 4 display elevated levels of PtdIns[3,5]P_2 under basal conditions and a decreased rate of both the hyperosmotic shock-induced increase and turnover of this phosphoinositide [355].

Taken together Fig 4 appears to be the primary means for PtdIns[3,5]P_2 turnover in *S. cerevisiae*, although other phosphatases, such as Inp52/Sjl2, Inp53/Sjl3 and Sac1 (see sections 3.1.1, 3.2.2 and 3.2.3, respectively), might be involved in this process too. Surprisingly, Fig 4 also plays a role in the generation of this phospholipid under basal and its upregulation upon hyperosmotic shock. To date the molecular basis for these findings, however, still remains elusive.

3.2 Synaptojanin orthologs

In addition to polyphosphoinositide phosphatases carrying a Sac1-like catalytic domain yeast cells also express enzymes that are characterized by an inositol polyphosphate 5-phosphatase domain (Figure 12). The *S. cerevisiae* genome encodes four members of this family, including Inp51, Inp52, Inp53, and, Inp54 (inositol polyphosphate 5-phosphatase 1–4) [254, 255, 291, 451]. The 5-phosphatase domains of all four Inp5 family members in yeast have been demonstrated to preferentially use PtdIns[4,5]P_2 as a substrate *in vitro*, but do not act on soluble inositol phosphates [235, 255, 492–494]. Three members of this family also possess a second catalytic domain similar to mammalian synaptojanin, the Sac1-like domain (Figure 12), and, hence, are also referred to as synaptojanin-like (Sjl) proteins [253, 254]. All four *INP5/SJL* genes are not essential for viability of vegetatively growing yeast cells, suggesting functional some degree of redundancy between these enzymes, although single and double null mutants display a range of abnormal phenotypes and the triple mutant *inp51/sjl1*Δ *inp52/sjl2*Δ *inp53/sjl3*Δ is inviable under regular growth conditions [253–255, 495]. The inviability of these cells can be suppressed by the expression of a mammalian inositol polyphosphate 5-phosphatase, indicating the evolutionary conservation of function between the mammalian and yeast enzymes [495]. Furthermore, this also demonstrates the physiological importance of the 5-phosphatase domain, as the mammalian enzyme used in these studies is lacking a Sac1-like domain [495]. Consistently, cells expressing Inp52/Sjl2 carrying a nonfunctional Sac1-like domain as their sole source for synaptojanin-like enzymes are viable, whereas expression of this phosphatase harboring mutations in residues essential for its 5-phosphatase activity does

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not allow growth in the same genetic background [239]. In line with this, expression of Inp53/Sjl3 carrying a non-functional Sac1-like domain restores the viability of *inp51/sjl1*Δ *inp52/sjl2*Δ *inp53/sjl3*Δ triple mutants [496]. The phenotypes associated with mutations in any pair of the *INP5/SJL* genes do not directly correlate with the cellular levels of PtdIns[4,5]P_2, although each of these double mutant strains display significantly elevated levels of this phosphoinositide [235, 454, 495].

Inp51/Sjl1 (108.4 kDa), Inp52/Sjl2 (133.3 kDa), and Inp53/Sjl3 (124.6 kDa) are similar in size and are characterized by proline-rich regions in their COOH-terminal parts, in addition to the 5-phosphatase domain (39%–66% identity) central in their primary sequences and the Sac1-like domain (34%–66% identity) in their NH_2-termini (Figure 12). In contrast to the other three 5-phosphatases in yeast Inp54 (43.8 kDa) completely lacks a Sac1-like domain, which is in good agreement with the finding that it cannot compensate for the loss of the other *INP5/SJL* genes [253–255, 495]. *In vitro* the Sac1-like domains of both Inp52/Sjl2 and Inp53/Sjl3 dephosphorylate PtdIns[3]P and PtdIns[4]P, and, with significantly lower activity PtdIns[3,5]P_2, whereas that of Inp51/Sjl1 does not exhibit phosphatase activity due to absence of the cysteine, arginine and serine/threonine residues in the highly conserved CX_5R(T/S) motif [235, 454], which is also present in a range of other lipid and protein phosphatases [497–501].

3.2.1 Inp51/Sjl1—The *INP51/SJL1*, together with *INP52/SJL2* and *INP53/SJL3*, genes had first been identified due to its close sequence homology to a number of mammalian polyphosphoinositide 5-phosphatases, including the synaptic vesicle associated PtdIns[4,5]P_2 5-phosphatase synaptojanin [253–255,291]. Inp51/Sjl1 differs from Inp52/Sjl2 and Inp53/Sjl3, as it possesses only inositol polyphosphate 5-phosphatase activity but does not dephosphorylate monophosphorylated derivatives of PtdIns [235,254,255,291]. This effect can be explained by the finding that its Sac1-like domain (Figure 12) is non-functional, which is attributable to mutations of essential residues in the CX_5R(T/S) motif [235]. In line with this *inp51/sjl1* null mutants and cells expressing catalytically inactive Inp51/Sjl1 as their sole source for this enzyme display increased PtdIns[4,5]P_2 levels *in vivo* (Table 2), in good agreement with the finding that it prefers this phosphoinositides a substrate over PtdIns[3,5]P_2 *in vitro* [235,254,255,291]. Consistently, overexpression of Inp51/Sjl1 decreases the cellular levels of PtdIns[4,5]P_2 and also leads to a corresponding increase in PtdIns[4]P [235,291].

Phenotypic analysis of cells carrying an *inp51/sjl1* null allele indicated that the enzyme is not essential for yeast cell viability under regular growth conditions [253–255,291]. However, these cells display a cold-tolerant phenotype, as they are able to grow significantly faster at temperatures below 15°C, when compared to the corresponding wild-type strain [255,291]. This effect, which is much weaker in *inp52/sjl2*Δ strains and cannot be observed in *inp53/sjl3*Δ cells at all, is attributable to the lack of 5-phosphatase activity, as expression of a catalytically inactive mutant of Inp51/Sjl1 as the sole source of this enzyme is sufficient to confer cold-tolerance to yeast cells [291]. The molecular basis of this phenotype of *inp51/sjl1* null cells, however, remains elusive, although some evidence points to the upregulation of a tryptophan permease in these cells [291]. In this regard, mutations in the tryptophan biosynthesis machinery are frequently associated with cold-sensitive growth, even in the presence of tryptophan in the culture medium [502,503].

In addition, Inp51/Sjl1 has been implicated in regulation of the cell integrity pathway [504] (Table 2). This finding is adding further support to the notion that Inp51/Sjl1 acts on PtdIns[4,5]P_2 *in vivo*, as this phospholipid and the enzymes responsible for its synthesis, such as Stt4 and Mss4 (see sections 2.2.2 and 2.3.2, respectively) are known regulators of this pathway [200,206,288]. In this regard, disruption of *INP51/SJL1* is sufficient to suppress the temperature-conditional growth defects of *wsc1* and *rom2* mutants [504], known positive

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regulators of cell wall integrity sensing [200,285,287,505,506]. Consistently, mutations in *SAC7*, encoding a GTPase-activating protein (GAP) for Rho1 that functions as an antagonist of Rom2 [285,507,508], lead to synthetic defects in combination with an *inp51/sjl1* null allele [504].

Moreover, Inp51/Sjl1 also genetically interacts with Tor2, as deletion of *INP51/SJL1*, but not *INP52/SJL2* or *INP53/SJL3* restores growth of cells carrying a *tor2^{ts}* allele at restrictive temperature [504]. In this regard, Tor2 plays an important role in proper polarization of the actin cytoskeleton, together with the PtdIns[4]P 5-kinase, Mss4 [383,394,405] (see section 2.3.2 and Figure 10B). One of the functions of PtdIns[4,5]P_2 generated by Mss4 and Tor2 in the regulation of the actin cytoskeleton is the recruitment of their shared effector proteins Slm1 and Slm2 to sites of polarized growth in a synergistic manner [383,405]. In line with this, disruption of *INP51/SJL1* suppresses the inviability of cells expressing *SLM1* at very low levels as their sole source for Slm1/Slm2, again supporting the notion that Inp51/Sjl1 acts on a pool of PtdIns[4,5]P_2 important for polarized growth of *S. cerevisiae* [405].

Furthermore, Inp51/Sjl1 interacts with two redundant proteins, namely Tax4 and Irs4, which function as positive regulators of its polyphosphoinositide 5-phosphatase activity [504]. Both proteins possess a COOH-terminal Eps15 homology (EH) domain, a protein-protein interaction module that has been highly conserved throughout evolution [509–511]. The majority of EH domains preferentially interact with a short motif containing an asparagine-proline-phenylalanine (NPF) core in their target proteins [509,512,513]. In line with this, such an NPF motif is present in the COOH-terminal portion of Inp51/Sjl1 (residues 932–934), but not in any of the other 5-phosphatases in yeast [514]. Consistently, Inp51/Sjl1 lacking this motif is unable to interact with either Irs4 or Tax4, which do not interact with each other and bind separately to Inp51/Sjl1 [504]. The finding that deletion of both *IRS4* and *TAX4* restores growth of *tor2^{ts}* mutants at a non-permissive temperature, similar to inactivation of *INP51/SJL1*, indicates that they also function in the regulation of the actin cytoskeleton [504]. Moreover, both proteins appear to also play a role in cell wall integrity sensing, as *irs4*Δ *tax4*Δ double mutants show synthetic interactions with *sac7* alleles [504]. Along these lines, activity of the MAP kinase, Slt2/Mpk1, a known component of the cell wall integrity pathway [280,281], is increased in the absence of functional Sac7 [504,507,508]. Consistently, this effect is much more pronounced in cells additionally lacking either *INP51/SJL1* or *IRS4* and *TAX4* [504]. In line with this, deletion of *INP51/SJL1* suppresses the temperature-conditional growth defects associated with *sac6* mutations [253], encoding a homolog of the mammalian actin bundling protein fimbrin [515–518]. In addition, cells lacking Inp51/Sjl1 also display aberrant chitin disposition to the entire cell surface, presumably due to mislocalization of chitin synthase 3 [504], an enzyme involved in cell wall repair (Table 2) [484,486,487]. Consistently, *inp51/sjl1 inp52/sjl2* double mutant cells display a markedly thickened cell wall, suggesting partially redundant functions for Inp51/Sjl1 and Inp52/Sjl2 [253–255,495]. Furthermore, these findings provide a potential link to Sac1 activity, which has also been implicated in the regulation of the actin cytoskeleton (see section 3.1.1). Taken together, these findings indicate that Inp51/Sjl1, together with its activators Irs4 and Tax4, controls a pool of PtdIns[4,5]P_2 that is involved in the regulation of the actin cytoskeleton and/or cell wall integrity sensing (Table 2).

Strikingly, Inp51/Sjl1 also interacts genetically with yet another EH domain-containing protein, Pan1 [510,519]; the combination of an *inp51/sjl1* null mutation with the *pan1-20* allele results in a synthetic lethal defect [514]. Pan1 itself is essential for yeast cell viability and functions as a plasma membrane-tethered nucleator of Arp2-Arp-3 complex-dependent actin filament polymerization for endocytic vesicle movement [510,519–525]. In addition to its two EH domains Pan1 also possesses a coiled-coil region and a proline-rich motif, and, hence, may serve as a multivalent adaptor to mediate interactions among a number of factors that act on or near the plasma membrane [514]. To date, it remains elusive, however, whether Inp51/Sjl1

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and Pan1 physically interact or localize to the same cellular compartments as part of a multimeric complex, although both proteins are clearly involved in the regulation of both endocytosis and the actin cytoskeleton. Likewise, deletion of *INP51/SJL1* suppresses the growth and endocytic internalization defects associated with the *sla2(4K-A)* mutation, similar to overexpression of *MSS4* (see section 2.3.2) [526]. In this regard, Sla2 specifically interacts with PtdIns[4,5]P_2 via its ANTH (AP180 NH_2-terminal homology) domain [526], a known phosphoinositide-binding motif [48,49,370]. Consistently, deletion of *INP51/SJL1* in cells does not rescue the actin and endocytic defects observed in cells expressing a version of Sla2 lacking its ANTH domain [526] and proper subcellular localization of the protein depends on Inp5/Sjl activity [527]. Taken together, these findings suggest that the PtdIns[4,5]P_2 levels at the plasma membrane, which are, at least in part, controlled by the action of Mss4 and Inp51/Sjl1, play a key role during endocytic internalization (Table 2).

The subcellular localization of Inp51/Sjl1, however, remains unknown, although it is predominantly found associated with the pellet in cell fractionation studies [255,291]. This notion is in good agreement with the finding that *inp51/sjl1 inp52/sjl2* double mutants exhibit prominent defects in plasma membrane structure and actin cytoskeleton organization (Table 2) [239,253–255,495]. Furthermore, this suggests that Inp51/Sjl1 and Inp52/Sjl52 share some overlapping functions, as the combination of *inp51/sjl1* and *inp53/sjl3* mutations does not lead to the manifestation of any prominent phenotypes in addition to those seen in the respective single mutants [253–255,495]. Consistently, *inp51/sjl1*Δ *inp52/sjl2*Δ double mutants display defects in fluid-phase and receptor-mediated endocytosis, an effect not seen in the corresponding single mutants [253]. Moreover, these cells accumulate the lipid dye FM4-64 in aberrant invaginated structures at the plasma membrane, indicating defects in the scission/transport of endocytic vesicles to the vacuole [253]. Likewise, electron microscopic studies demonstrated that the plasma membrane of cells lacking both Inp51/Sjl1 and Inp52/Sjl2 forms irregular deep invaginations [253,255,495], which originally had been misinterpreted as aberrant vacuolar structures [254]. Furthermore, these cells also display an altered budding pattern and actin organization, as well as chitin deposition defects, again indicating a role for Inp51/Sjl1 in the regulation of the actin cytoskeleton [253,254,495]. In summary, these findings suggest that Inp51/Sjl1 and Inp51/Sjl2 have several overlapping physiological roles in yeast, whereas Inp51/Sjl1 and Inp53/Sjl3 for the most part serve different cellular functions. In this regard, *inp51/sjl1 inp52/sjl2* double mutants also display defects in vacuolar and mitochondrial morphology, as well as an increased sensitivity to hyperosmotic conditions (Table 2), although the molecular basis for these observations remains elusive [253–255,492,495]. Furthermore, mutations in *INP51/SJL1* display synthetic defects in combination with *pik1^{ts}* alleles, including exacerbated temperature- and neomycin-sensitivity, and impaired secretion, suggesting a role for Inp51/Sjl1 in the regulation of these processes [256].

3.2.2 Inp52/Sjl2—In addition to its inositol polyphosphate 5-phosphatase domain, Inp52/Sjl2 also possesses a functional Sac1-like domain in its NH_2-terminal part (Figure 12) and, hence, holds two independent lipid phosphatase activities, similar to Inp53/Sjl3 (see section 3.2.3) [235]. In this regard, Inp52/Sjl2 shares several substrates with Inp51/Sjl1 and Inp53/Sjl3 (see sections 3.2.1 and 3.2.3, respectively) and all three enzymes have partially redundant cellular functions (Table 2) [235, 255]. Consistently, *inp51/sjl1*Δ *inp52/sjl2*Δ and *inp52/sjl2*Δ *inp53/sjl3*Δ, but not *inp51/sjl1*Δ *inp53/sjl*Δ*3* double mutants display a growth defect at 30°C [255]. In addition, unlike *inp53/sjl3* null cells, *inp52/sjl2* mutants are modestly cold-tolerant, an effect seen more pronounced in *inp51/sjl1*Δ cells [255, 291]. Moreover, cells lacking both Inp51/Sjl1 and Inp52/Sjl2 display gross morphological defects, such as irregular plasma membrane invaginations, abnormal cell wall depositions, small fragmented vacuoles and aberrant mitochondrial and actin cytoskeleton organization, whereas *inp52/sjl2 inp53/sjl3* double mutants are characterized by a very thick cell wall, abnormal plasma membrane invaginations and distortions, as well as fragmented internal membranous structures and an

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irregular actin cytoskeleton morphology (Table 2) [239, 253–255, 492, 495]. Furthermore, *inp52/sjl2*Δ *inp53/sjl3*Δ double mutants display impaired fluid phase and receptor-mediated endocytosis, as well as budding polarity defects and increased sensitivity to hyperosmotic conditions (Table 2), similar to cells lacking both Inp51/Sjl1 and Inp52/Sjl2 [253, 254, 492]. In line with this, *inp51/sjl1*Δ *inp52/sjl2^{ts} inp53/sjl3*Δ cells display defects in actin cytoskeleton organization at a non-permissive temperature [239]. Strikingly, this phenotype is not observed in this strain carrying an additional *mss4^{ts}* allele (see section 2.3.2) under the same conditions [239]. Moreover, the finding that *inp51/sjl1*Δ *inp52/sjl2^{ts} inp53/sjl3*Δ *mss4^{ts}* cells synthesize PtdIns[4,5]P_2 levels similar to wild-type cells, indicates that the cellular levels of this phosphoinositide directly affect the organization of the actin cytoskeleton [239]. Similarly, *inp51/sjl1*Δ *inp52/sjl2^{ts} inp53/sjl3* cells also exhibit impaired rates of endocytosis and form large irregular plasma membrane invaginations in a manner that requires ongoing actin polymerization at a non-permissive temperature when compared to the corresponding wild-type strain [239, 527]. These mutant phenotypes can also be attributed directly to the cellular levels of PtdIns[4,5]P_2, as they are suppressed when Mss4 is inactivated in cells carrying null alleles of *inp51/sjl1* and *inp52/sjl2* [239]. In addition, Inp52/Sjl2 and Inp53/Sjl3 have been implicated in the regulation of trans-Golgi/endosomal sorting of clathrin-coated vesicles (Table 2), in a manner that specifically requires the 5-phosphatase activity of the enzymes, whereas vacuolar protein sorting and secretory pathways are not affected in *inp52* and/or *inp53* mutant cells [239, 528]. Consistent with this conclusion, *inp51/sjl1*Δ *inp52/sjl2^{ts} inp53/sjl3*Δ cells display aberrant subcellular distribution of PtdIns[4,5]P_2 upon shift to a non-permissive temperature [239].

Furthermore, extracts prepared from cells expressing Inp52/Sjl2 as their sole source for an inositol polyphosphate 5-phosphatase hydrolyze 80% of all phosphoinositide species present in yeast [235]. Under conditions that allow activity of its inositol polyphosphate 5-phosphatase, but not of the Sac1-like domain, however, the enzyme only converts PtdIns[4,5]P_2 to PtdIns [4]P, but does not utilize PtdIns[3]P, PtdIns[4]P or PtdIns[3,5]P_2 as substrates anymore [235], whereas *in vitro* its isolated 5-phosphatase domain dephosphorylates both PtdIns[4,5] P_2 and PtdIns[3,5]P_2 to form PtdIns[4]P and PtdIns[3]P, respectively (Table 2) [492]. *In vivo*, cells lacking both Inp52/Sjl2 and Inp53/Sjl3 display increased levels of PtdIns[4,5]P_2 and PtdIns[3,5]P_2, although to varying degrees depending on the time period of inositol labeling and growth conditions, whereas the levels of the two monophosphorylated derivatives of PtdIns are unchanged when compared to the corresponding wild-type strains [235,495]. Consistently, cells carrying a temperature-sensitive allele of *INP52/SJL2* as their sole source for any inositol polyphosphate 5-phosphatase display a specific increase in PtdIns[4,5]P_2 levels at a non-permissive temperature, whereas PtdIns[3,5]P_2 levels are equally elevated at both permissive and restrictive temperatures [239]. However, overexpression of Inp52/Sjl2 and Inp53/Sjl3, but not Inp51/Sjl1 or Fig 4, suppresses the multiple-drug-sensitive phenotype and the growth defects associated with *sac1* null alleles, in a manner that requires their Sac1-like domains [454]. In good agreement with this finding, overexpression of *INP52/SJL2* also restores the levels of all polyphosphoinositides present in *S. cerevisiae* in cells lacking Sac1 [454]. In summary, these findings point to somewhat redundant roles for Inp52/Sjl2 and Sac1 in yeast cells, in addition to the overlapping physiological functions of the inositol polyphosphate 5-phosphatase activities of Inp52/Sjl2, Inp51/Sjl1 and Inp53/Sjl3 (Table 2).

In good agreement, with partially redundant physiological functions for Inp52/Sjl2 and Inp53/Sjl3 both enzymes show similar subcellular localization patterns, namely a diffuse distribution in the cytoplasm and in the case of Inp53/Sjl3 exclusion from the nucleus (Table 2) [492]. Upon shift to hyperosmotic conditions, however, Inp52/Sjl2 rapidly and transiently translocates to cortical actin patches in both mother and daughter cells, in a manner that depends on both its NH_2-terminus including the Sac1-like domain and the proline-rich region in its COOH-terminal part (Figure 12) [492]. Nevertheless, as the isolated proline-rich region of

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Inp53/Sjl3 is required and sufficient to mediate its hyperosmotically evoked translocation [492], this might reflect improper folding of Inp52/Sjl2 lacking its NH_2-terminal part, rather than a direct role of this region in targeting the protein to actin patches. Furthermore, although this process requires the redistribution of the actin cytoskeleton the translocation of Inp52/Sjl2 to patch-like structures in hyperosmotic conditions may not directly depend on actin, as it can still be observed upon treatment of cells with the actin-depolarizing drug latrunculin-A in certain genetic backgrounds, but not in others [492,527]. Correspondingly, in a different strain background, Inp52/Sjl2 localizes to cortical actin patches at the plasma membrane, even in untreated cells, in a manner that depends on its proline-rich domain [527]. The finding that the recruitment of Inp52/Sjl2 to cortical structures depends on Abp1 [527], which is itself an actin-binding protein, might explain the apparent discrepancy about whether actin is required for this process. Consistently, Inp52/Sjl2 and Abp1 physically interact with each other and colocalize *in vivo* [527]. Strikingly, Inp52/Sjl2 also physically interacts at actin patches with Bsp1, which itself genetically interacts with *ABP1* and other genes encoding components of cortical patches [529]. This interaction is mediated by the NH_2-terminal part of Inp52/Sjl2 containing its Sac1-like domain, whereas the COOH-terminal portion of the protein is dispensable for the association of both proteins [529]. Furthermore, the recruitment of Bsp1 to cortical actin patches requires functional Pik1 (see section 2.2.2), suggesting that it binds to PtdIns[4]P and or PtdIns[4,5]P_2 [529]. Interestingly, *in vitro* both Inp51/Sjl1 and Inp52/Sjl2 bind to the SH3 domain of Sla1 [530], a protein required for the assembly of the cortical actin cytoskeleton in yeast that also interacts with regulators of endocytosis and actin cytoskeleton organization [531–533]. Likewise, *inp51/sjl1*Δ *inp52/sjl2*Δ and *abp1*Δ *inp51/sjl1*Δ double mutants display similar phenotypes, including the formation of abnormal plasma membrane invaginations and endocytic internalization defects [527]. In this regard, overexpression of *INP52/SJL2* results in a dramatic acceleration of the repolarization of actin patches upon hyperosmotic treatment of yeast cells, in a manner that depends on a functional 5-phosphatase, but not its Sac1-like domain, suggesting an important role for PtdIns[4,5]P_2 in this process [492]. Moreover, this behavior is in good agreement with the reported defects in actin cytoskeleton organization observed in *inp52/sjl2* mutants (Table 2) [239,253,254,495], as hyperosmotic treatment of yeast cells is known to provoke a rearrangement of the actin cytoskeleton [534,535]. In addition, Sla2, an ANTH domain containing clathrin- and F-actin-binding protein [536–538] localizes to cortical actin patches, in a manner that requires Inp5/Sjl activity [527]. In this regard, Sla2 has also been reported to bind PtdIns[4,5]P_2 and to interact genetically with both *INP51/SJL1* and *MSS4* (see sections 3.2.1 and 2.2.2, respectively) and physically with Sla1 (see above) [526,531].

Furthermore, Inp52/Sjl2 possesses a candidate CAAX-box [232] at its COOH-terminus, a motif that may be S-prenylated *in vivo* and, hence, could serve as a constitutive membrane anchor. However, this potential CAAX-box does not conform to the consensus sequence for such a motif, and does not seem to play a role in subcellular localization of Inp52/Sjl2 [492]. Therefore, in good agreement with its predominantly cytoplasmic distribution in vegetatively-growing yeast cells, this motif is unlikely to be modified in Inp52/Sjl2 and play a role in the membrane targeting of this enzyme. Inp52/Sjl2 lacking its NH_2-terminal part, including the Sac1-like domain, also localizes to the nucleus in addition to the cytosol (Table 2) [492]. As this truncated version of the enzyme is still far too large (83 kDa) to be able to freely diffuse in and out of the nucleus (exclusion limit for passive diffusion in *S. cerevisiae* ~ 50 kDa), this suggests that full-length Inp52/Sjl2 normally shuttles between the cytoplasm and the nucleus and that it carries a nuclear export signal (NES) in its NH_2-terminal part. Thus, deletion of the NH2-terminus including this signal sequence leads to an accumulation of the protein in the nucleus, whereas the full-length enzyme displays a predominantly cytoplasmic subcellular distribution pattern at steady-state due to an export rate exceeding that of its nuclear import.

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In summary, a number of the mutant phenotypes of *inp52/sjl2* mutant cells, including aberrant actin cytoskeleton organization and cell wall depositions, defects in endocytosis and irregular plasma membrane invaginations are likely attributable to loss of PtdIns[4,5]P_2 5-phosphatase activity. In this regard, PtdIns[4,5]P_2 is predominantly generated at the plasma membrane in *S. cerevisiae* by the action of Stt4 and Mss4 (see sections 2.2.2 and 2.3.2, respectively), where it is bound by a several actin-regulatory proteins and the organization of the actin cytoskeleton is controlled by the regulation of cellular PtdIns[4,5]P_2 levels [298,299,389,539]. Furthermore, actin patches have been demonstrated to be associated with plasma membrane invaginations, which have also been suggested to represent sites of cell wall synthesis [540–542]. Consistently, the phenotypes of null mutations in actin patch components, including defects in actin cytoskeleton organization and endocytosis, as well as aberrant cell wall depositions and increased osmosensitivity [411,485,510,543–548] are intriguingly similar to those observed in double inositol polyphosphate 5-phosphatase mutants [253–255,492,495].

In addition, an *inp52/sjl2* mutant was identified in a screen designed to detect novel components of the machinery that mediates proper recombination partner choice during meiosis (Table 2), using a transposon insertion approach [549]. Specifically, the disrupted *inp52/sjl2* allele identified exhibits an increase in unequal meiotic sister-chromatid recombination (*msc* phenotype), similar to *inp52/sjl2* null cells [549]. Likewise, this mutant displays a dominant synthetically lethal phenotype when crossed to a congenic *SPO13* strain monosomic for chromosome VIII [549], an effect not seen in other strain backgrounds [255]. In this regard, Spo13 is a meiosis-specific protein that functions in maintaining sister chromatid cohesion during meiosis I and promotes proper attachment of kinetochores to the spindle during meiosis I and II [550–556]. The molecular basis for this genetic interaction, however, remains elusive, although it has been suggested that Inp52/Sjl2 might function in regulation of Dmc1 (disruption of meiotic control) [549], another meiosis-specific protein required for pairing between homologous chromosomes and repair of double strand breaks [557–561]. In this regard, cells harboring a *dmc1* null allele have been demonstrated to display meiotic defects similar to those seen in *inp52/sjl2* mutants [549].

3.2.3 Inp53/Sjl3—Inp53/Sjl3 and Inp52/Sjl2 share a high degree of sequence homology in their NH_2-terminal parts, comprising both the Sac1-like and inositol polyphosphate 5-phosphatase domains (66% identity), whereas their COOH-termini, including the proline-rich domains, are less related (Figure 12). Similar to Inp52/Sjl2 and mammalian synaptojanin both catalytic domains of Inp53/Sjl3 are functional [235]. In this regard, the isolated 5-phosphatase domain of Inp53/Sjl3 dephosphorylates PtdIns[4,5]P_2 to generate PtdIns[4]P *in vitro*, whereas its Sac1-like domain converts PtdIns[3]P, PtdIns[4]P and with weaker activity PtdIns[3,5]P_2 to PtdIns (Table 2) [235, 493]. Furthermore, *inp52/sjl2 inp53/sjl3* double mutants display elevated PtdIns[4,5]P_2 levels under certain conditions, although to a lesser degree than *inp51/sjl1 inp53/sjl3* or *inp51/sjl1 inp52/sjl2* null cells [495]. As deletion of *INP53/SJL3* is synthetically lethal in combination with temperature-sensitive alleles of *PIK1* (see section 2.2.2) [256], it is conceivable that Inp53/Sjl3 generates PtdIns[4]P by acting on PtdIns[4,5]P_2, and, hence, controls the cellular levels of both phosphoinositides *in vivo*.

Likewise, overexpression of Inp53/Sjl3 or Inp52/Sjl2, but not Inp51/Sjl1 (see sections 3.2.2 and 3.2.1, respectively), restore the vacuole morphology defect, slow growth and multiple-drug sensitive phenotypes of *sac1* null mutants, in a manner that partially depends on their Sac1-like domains (Table 2) [294,454,496]. Consistently, the combination of *sac1* and *inp53/sjl3* null alleles results in a synthetically lethal defect and *sac1*^{ts} *inp53/sjl3*Δ cells manifest a synthetic growth defect when compared to the corresponding single mutants [294]. Furthermore, this phenotype is mirrored by dramatically increased PtdIns[4]P levels in *sac1*^{ts} *inp53/sjl3*Δ, whereas *sac1*^{ts} *inp52/sjl2*Δ *inp53/sjl3*Δ mutants exhibit elevated levels of both PtdIns[4]P and PtdIns[3,5]P_2 [294]. The increased PtdIns[4]P levels in *sac1*^{ts} *inp52/*

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*sjl2*Δ *inp53/sjl3*Δ mutants expressing an Inp53/Sjl3 derivative lacking 5-phosphatase activity are partially restored at restrictive temperature, whereas they are even further elevated upon expression of this enzyme lacking a functional Sac1-like domain [496]. In addition, *sac1^{ts} inp53/sjl3*Δ and *sac1^{ts} inp52/sjl2*Δ *inp53/sjl3*Δ cells display defects in Golgi function at a non-permissive temperature, as protein transport and glycosylation is impaired in these mutants [294]. However, elevated cellular levels of any *INP5* gene product does not confer a Sec14-bypass phenotype, indicating that Sac1 and the Inp5 phosphatases have only partially redundant physiological functions [465].

In line with overlapping physiological functions for Inp52/Sjl2 and Inp53/Sjl3 mutant cells lacking both phosphatases display compromised cell growth, whereas an *inp51/sjl1 inp53/sjl3* double mutant does not [255]. In addition, unlike the corresponding single mutants, cells lacking both Inp52/Sjl2 and Inp53/Sjl3 display a cold-resistant phenotype, similar to *inp51/sjl1*Δ mutants [255]. Furthermore, these cells also manifest defects in both fluid-phase uptake and receptor-mediated endocytosis (Table 2), again an effect not seen in *inp51/sjl1 inp53/sjl3* double mutants [253]. Moreover, cells lacking Inp52/Sjl2 and Inp53/Sjl3 are also characterized by a loss in actin cytoskeleton organization and in bud-site selection during polarized growth, as well as a pronounced thickening of the cell wall and fragmented internal structures [253–255]. Furthermore, similar to Inp52/Sjl2, in response to conditions of hyperosmotic stress Inp53/Sjl3 rapidly relocalizes from a predominantly cytosolic location to cortical actin patches in a transient fashion (Table 2) [492]. This effect is mediated by the proline-rich domain in the COOH-terminal part of Inp53/Sjl3, whereas both of its catalytical domains are dispensable for this process [492]. In this regard, Inp52/Sjl2 and Inp53/Sjl3 have been found to associate with Bsp1 (binding of Sac1 domain protein) via their Sac1-like domains both *in vitro* and *in vivo*, whereas no interaction with either Inp51/Sjl1 or Sac1 could be detected [529]. Consistent with a function of all three proteins in the regulation of the actin cytoskeleton Bsp1 also localizes to cortical actin patches and cells overexpressing *BSP1* exhibit defects in the polarized arrangement of the actin cytoskeleton [529]. Furthermore, *BSP1* genetically interacts with genes encoding components cortical actin patches [529]. Similar to a fraction of Inp53/Sjl3, Bsp1 behaves like a membrane-associated protein in cell fractionation studies, in a manner that requires functional Pik1 (see section 2.2.2), suggesting that it interacts directly with PtdIns[4]P and/or PtdIns[4,5]P_2 [529]. In line with these findings, it has been proposed that Bsp1 may serve as an adaptor that links Inp52/Sjl2 and Inp53/Sjl3 to the actin cytoskeleton [529]. Furthermore, *inp52/sjl2*Δ *inp53/sjl3*Δ *ymr1*Δ mutants are not viable, which is in good agreement with the finding that Inp53/Sjl3 regulates a pool of PtdIns[3]P that is also targeted by Ymr1 (see section 3.3) [457]. Growth can be restored to this triple mutant by the expression of a PtdIns[3]P-targeted Sac1-like domain [457]. Taken together, these findings indicate that Inp53/Sjl3 shares a number of substrates/cellular roles with the other *INP5* gene products, as well as Sac1 and Ymr1.

Nonetheless, Inp53/Sjl3 must have independent cellular functions on its own, as cells lacking *INP53/SJL3* display irregularly shaped and enlarged vacuoles, aberrant plasma membrane invaginations, a slightly thickened cell wall and a germination defect, phenotypes that are not observed in *inp51/sjl1*Δ or *inp52/sjl2*Δ cells [255,456]. Moreover, *inp53/sjl3* mutant cells display a defect in the recycling of the trans-Golgi protease Kex2 from an endosomal compartment, another effect not seen in the other polyphosphoinositide 5-phosphatase single mutants (Table 2) [451,528]. In this regard, an *inp53/sjl3*Δ mutation exhibits a synthetically lethal defect in combination with *chc1-521^{ts}* [528]. In *S. cerevisiae*, a single gene, *CHC1*, encodes the heavy chain of clathrin [562,563], which serves as a vesicular coat protein that has been evolutionarily conserved from yeast to humans. The formation of clathrin-coated vesicles is necessary for a number of protein- and lipid-trafficking processes in yeast [563–567], including those between the endosome and the late Golgi apparatus [568–570]. One of the proteins affected by this pathway is Kex2 [571,572], thus providing a direct link between Inp53/

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Sjl3 function and clathrin-dependent vesicular transport between the Golgi and the endosome [572,573]. In this regard, the isolated proline-rich domain of Inp53/Sjl3 physically interacts with Chc1 *in vitro*, in a manner that partially depends on a short motif, namely L(L/I)(D/E/N)(L/F)(D/E), present in this region that is frequently found in clathrin-binding proteins [496, 574,575]. In good agreement with these findings, an *inp53/sjl3* mutant was identified in a genetic screen designed to identify novel factors involved in the regulation of transport processes between the late Golgi and the prevacuolar complex (PVC) [456]. More specifically *inp53/sjl3* mutants exhibit an increased rate of anterograde transport of several cargoes, including Kex2, from the Golgi to the endosome, whereas the retrieval from the PVC to the trans-Golgi network is unaffected [456]. Consistently, a portion of the total cellular Inp53/Sjl3 (20%–25%) associates with membranes in cell fractionation studies [456,496]. As the Inp53/Sjl3-containing fraction is also enriched in Kex2 and Vph1, this indicates that a fraction of Inp53/Sjl3 associates as a peripheral protein with membranes of the Golgi and/or endosomal compartments [456]. Taken together with the finding that *inp53/sjl3*Δ cells exhibit impaired α-factor processing [239,456,528] this points to a role for Inp53/Sjl3 in clathrin-dependent trafficking of certain proteins between the Golgi apparatus and prevacuolar endosomes. This function of Inp53/Sjl3 requires both functional Sac1-like and 5-phosphatase domains, in addition to the presence of the proline-rich region in its COOH-terminal part (Figure 12) [456,496], suggesting that several different phosphoinositides play a role in this process, a notion that is in good agreement with the finding that the PtdIns 3-kinase Vps34 (see section 2.1) also acts in Golgi-endosome transport processes.

3.2.4 Inp54—Although Inp54 carries a inositol polyphosphate 5-phosphatase domain that is highly homologous to that of the other Inp5/Sjl gene products in yeast it completely lacks a Sac1-like domain and a proline-rich region (Figure 12), and, hence, is much smaller in size (43.8 kDa) [255, 493, 494]. In this regard, it must also be functionally distinct from the other 5-phosphatases in yeast as it cannot compensate for their loss [253–255, 495], whereas cells lacking *INP54* are viable [494]. However, Inp54 possesses inositol polyphosphate 5-phosphatase activity towards PtdIns[4,5]P_2 [493, 494] (Table 2). In addition, Inp54 contains a short very hydrophobic region at its COOH-terminus (Figure 12) [494], similar to membrane targeting motifs of a number of COOH-terminal tail-anchored proteins [576–579]. In good agreement with the finding that these proteins frequently are associated with mitochondrial and ER membranes, Inp54 localizes predominantly to the ER *in vivo* [494]. In this regard, Inp54 interacts tightly with ER membranes (Table 2) with the bulk of the protein oriented towards the cytoplasm, in a manner that depends on the last 13 residues (Leu^{371}-Leu^{384}) within the hydrophobic COOH-terminal domain (Figure 12) [494].

Cells lacking *INP54* are viable and grow with similar rates as the corresponding wild-type strain and do not display any significant morphological phenotypes [494]. Cells harboring an *inp54* null allele, however, are characterized by a marked increase in secretion (Table 2) [494]. In summary, these findings suggest a negative role for Inp54 in the secretory pathway at the ER level, which is likely to be exerted by the regulation of the levels of PtdIns[4]P and/or PtdIns[4,5]P_2 at this organelle.

3.3 Myotubularin ortholog (Ymr1)

The founding member of the family of myotubularin-related proteins is the human gene *MTM1*, which is mutated in X-linked myotubular myopathy, a severe congenital disorder that is characterized by small rounded muscle fibers with varying percentages of centrally located nuclei that resemble fetal myotubes [580–587]. Owing to the fact that it contains a number of conserved motifs, then known as hallmarks for dual specificity protein tyrosine phosphatases, Mtm1 has long been thought to serve as a protein phosphatase [581,588]. The finding that another member of the protein tyrosine phosphatase superfamily, namely PTEN (phosphatase

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and tensin homolog) [589,590], acts on the phosphoinositide PtdIns[3,4,5]P_3 *in vivo* rather than on protein substrates [591,592] led to the discovery that Mtm1 also functions as a lipid phosphatase [452,593]. The *S. cerevisiae* genome encodes a single myotubularin homolog, namely *YMR1* [581,588], which has also been demonstrated to act as a polyphosphoinositide phosphatase [452]. Ymr1 is an 80.1 kDa protein that is characterized by an NH_2-terminal GRAM (glucosyltransferase, Rab-like GTPase activator and myotubularin) domain and a bipartite catalytic domain central in its primary sequence (Figure 12) [580,581]. *YMR1* is not essential for yeast cell viability and gene disruptant mutants do not display any obvious phenotypes, possibly owing to partially overlapping functions with other lipid phosphatases in this organism (Table 2) [452,594].

In this regard, Ymr1 exhibits PtdIns[3]P 3-phosphatase activity *in vitro* and cells lacking *YMR1* display elevated levels of PtdIns[3]P *in vivo* [452]. Consistently, cells overexpressing Ymr1 have significantly lower levels of PtdIns[3]P than the corresponding wild-type cells, whereas the cellular levels of other polyphosphoinositides remain mainly unchanged [457]. In good agreement with the reported role for PtdIns[3]P and Vps34 in the regulation of the vacuolar protein sorting (Vps) pathway (see section 2.1), overexpression of *YMR1* causes a partial defect in carboxypeptidase Y (CPY) sorting from the Golgi to the vacuole [457].

Moreover, the pool of PtdIns[3]P that is regulated by Ymr1 can also be targeted by Inp53/Sjl3 (Table 2), as double mutants lacking both phosphatases display elevated levels of this phosphoinositide over either single mutant, whereas the combination of *ymr1*Δ with null alleles of any of the other genes encoding polyphosphoinositide phosphatases in yeast (see sections 3.1 and 3.2) does not lead to any synthetic effects [457]. Furthermore, the elevated levels of PtdIns[3]P in *ymr1*Δ *inp53/sjl3*Δ double mutants are mirrored by an accumulation of this lipid at vacuolar membranes, in contrast to the predominantly punctate perivacuolar pattern, resembling endosomal compartments, seen in wild-type cells [457]. Furthermore, *ymr1*Δ *inp53/sjl3*Δ manifest a fragmented vacuole phenotype, suggesting a role for both phosphatases in the maintenance of vacuolar homeostasis (Table 2) [457]. In line with a role for Ymr1 in the Vps pathway and overlapping functions with Inp53/Sjl3, cells harboring null alleles of both genes exhibit a more severe defect in CPY and carboxypeptidase S (CPS) sorting than the *ymr1*Δ single mutant [457]. Consistently, two components of the retromer complex, namely Vps17 and Vps5, that are recruited to the prevacuolar endosomal compartment (PVC) by PtdIns [3]P via their PX domains [155] mislocalize to the cytoplasm and vacuolar membranes in *ymr1*Δ *inp53/sjl3*Δ mutants [457]. In this regard, mutations in *VPS5* or *VPS17*, which encode components of the retromer coat complex that is necessary for recycling of the CPY receptor, Vps10, from endosomes back to the Golgi, lead to a fragmented vacuole phenotype [145, 156,157,595].

Another effector of PtdIns[3]P is Vps27, an endosomal protein harboring a FYVE (Fab1, YGL023, Vps27, and EEA1) domain (see section 2.1), a Zn^{2+}-binding fold that serves as a PtdIns[3]P-binding domain in yeast and other organisms [121–124]. Vps27 functions in the formation of multivesicular bodies (MVBs), structures that are generated at a late endosomal compartment during vacuolar protein sorting (see section 2.1) [160–162]. More specifically Vps27 serves as a membrane-docking site for the ESCRT-I complex (endosomal complex required for transport) at endosomal membranes to which it becomes recruited in a in a PtdIns [3]P-dependent fashion [163]. Consistently, Vps27 is redistributed from perivacuolar endosomal structures to the vacuolar membrane in *ymr1*Δ *inp53/sjl3*Δ mutants, an effect that is mirrored by a defect in MVB sorting of CPS in these cells [457].

Likewise, the cytosol to vacuole (Cvt) transport pathway serves as an additional route for the delivery of certain cargoes to the vacuolar lumen, in a manner that depends on PtdIns[3]P [133,140]. Two PX domain-containing, and, hence, PtdIns[3]P-binding proteins, namely

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Cvt13 and Cvt20, have been implicated in regulation of this pathway [151]. In this regard, cells lacking both *YMR1* and *INP53/SJL3* display strong defects in the processing of Ape1, a protein known to follow the Cvt pathway for vacuolar delivery, and mislocalize Cvt13 to the cytoplasm [457]. In summary, these findings suggest that Ymr1 serves as a PtdIns[3]P specific phosphatase *in vivo* that plays an important role in the regulation of various endosomal/vacuolar protein sorting pathways.

Combination of *ymr1*Δ, *inp52/sjl2*Δ and *inp53/sjl3*Δ alleles yields a synthetic lethal defect, demonstrating that all three phosphatases must share an essential physiological function [457]. Strikingly, viability can be restored in these cells by the expression of a mutant form of Inp52/Sjl2 carrying a non-functional inositol polyphosphate 5-phosphatase domain, indicating that the Sac1-like domain activity of the *INP5* gene products becomes essential in cells lacking Ymr1 [457]. In this regard, *ymr1*Δ *inp52/sjl2^{ts} inp53/sjl3*Δ cells exhibit increased cellular levels of both PtdIns[3]P and PtdIns[3,5]P_2 at restrictive temperature [457]. Furthermore, expression of a Sac1-like domain targeted to PtdIns[3]P, but not those targeted to PtdIns[4]P or PtdIns[4,5]P_2, allows growth of *ymr1^{ts} inp52/sjl2*Δ *inp53/sjl3*Δ triple mutant cells at a non-permissive temperature [457]. This strongly suggests that accumulation of PtdIns[3]P is toxic in *S. cerevisiae*, as the above mentioned effect depends on the catalytic activity of the targeted Sac1-like domain, whereas the generation of this lipid is dispensable for yeast cell growth (see section 2.1) [42,43]. In this regard, deletion of *VPS30*, encoding a subunit of Vps34 PtdIns 3-kinase complexes (see section 2.1 and Figure 5) [132], leads to a marked decrease in the cellular levels of PtdIns[3]P and, hence, restores growth of *ymr1*Δ *inp52/sjl2*Δ *inp53/sjl3*Δ triple mutants [455]. Likewise, a genetic screen designed to identify suppressors of the lethal phenotype of *YMR1* depletion (SYD) in an *inp52/sjl2*Δ *inp53/sjl3*Δ background yielded four groups of proteins based on their cellular function, including complementing phosphatase domains of Ymr1, Inp52/Sjl2 and Inp53/Sjl3, the oxysterol-binding protein Osh7, regulators of vesicle transport such as Ypt1 and Vam6 and several components of the cell wall integrity MAP kinase pathway [455]. The most frequently isolated suppressors identified were truncated versions of Pkc1 lacking their COOH-terminal kinase domains [455]. In this regard, Pkc1 is a protein serine/threonine kinase that is essential for the control of the highly conserved in the cell wall integrity pathway [276,277,596]. This pathway is comprised of a series of phosphorylation events that are initiated at the plasma membrane by the activation of Pkc1 and involve the Slt2/Mpk1 MAP kinase cascade (Figure 10A) [278–280,597]. Consistently, cells carrying mutations in genes encoding components of this pathway loose their osmotic stability and frequently display a cell lytic defect [277,281,596].

In addition to truncated versions of Pkc1, two negative regulators of the cell wall integrity pathway, namely *SAC7* and *MSG5*, were also identified as SYD genes [455]. Consistently, *ymr1*Δ *inp52/sjl2^{ts} inp53/sjl3*Δ triple mutants are viable in the presence of osmotic support at a non-permissive temperature [455]. Likewise, *ymr1^{ts} inp52/sjl2*Δ *inp53/sjl3*Δ mutants display a potentiated response to heat stress, a potent activator of the cell wall integrity pathway [598], and exhibit higher levels of Slt2/Mpk1 phosphorylation under basal conditions [455], indicating an upregulation of the cell wall integrity pathway in these cells. Cells lacking *VPS34*, encoding the sole PtdIns 3-kinase in *S. cerevisiae* (see section 2.1) [42], also display severe defects in the regulation of cell integrity signaling [455]. Moreover, overexpression of an isolated Rho1-GTP interacting domain of Pkc1 also restores growth in *ymr1^{ts} inp52/sjl2 inp53/sjl3* triple mutant cells at a restrictive temperature [455], indicating that the truncated versions of Pkc1 that were identified originally in the genetic screen interfere with the cell wall integrity pathway by blocking Rho1 signal propagation. Consistently, deletion of *ROM2*, encoding a major upstream activator of Rho1 [285], also allows a bypass of the essential requirement PtdIns 3-phosphatase activity in yeast, as it allows growth of *ymr1*Δ *inp52/sjl2^{ts} inp53/sjl3*Δ cells at a non-permissive temperature [455]. This suggests that the loss of the guanine exchange factor (GEF) Rom2 reduces the ability of cells to activate Rho1 and, hence,

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other events further downstream in the pathway, as the PtdIns[3]P levels do not significantly change upon deletion of *ROM2* in *ymr1*Δ *inp52/sjl2*^{ts} *inp53/sjl3*Δ cells [455]. Taken together, these findings strongly suggest a role for PtdIns[3]P in the regulation of the cell wall integrity pathway in yeast and that the toxic effect of an accumulation of this phospholipid is at least partially mediated by the hyperactivation of Rho1/Pkc1-mediated signaling.

4. Summary and Perspectives

In this review, we summarized our current knowledge about PIPs in the yeast *Saccharomyces cerevisiae*. We specifically focused on the kinases and phosphatases involved in the generation and interconversion of this group of phospholipids. During the last decade, study of PIPs in yeast has provided important and rapidly expanding insight into the physiological processes affected by PIPs in eukaryotes. Genetic screens, biochemical analysis, and cell biological studies have greatly enhanced our knowledge of this subject. One major conclusion of the work accomplished is that certain PIPs appear to be restricted to specific membranes (Figure 13), and that aberrant generation of any species at other cellular locations leads to serious physiological consequences. In this regard, we know now that PtdIns[4]P is the major phosphoinositide of the Golgi apparatus, where it plays a role in the regulation of secretion. Attempts to detect the pool of this phospholipid generated at the plasma membrane have been unsuccessful so far making it very likely that it becomes rapidly converted there to PtdIns[4, 5]P_2, the main phosphoinositide species at this location. In line with this, illicit accumulation of PtdIns[4]P at the ER and plasma membrane in certain phosphatase mutants is accompanied by growth defects and a number of other phenotypes. Likewise, accumulation of PtdIns[3]P, a phosphoinositide that is dispensable for normal growth and the major species at endosomal membranes and the PVC, leads to hyperactivation of the small GTPases, Rho1 and Rho2, and, hence, inappropriate and adventitious stimulation of the cell wall integrity MAPK signaling pathway. Moreover, cells completely lacking PtdIns[3]P phosphatase activity are inviable, demonstrating that amassing of this phosphoinositide is incompatible with yeast cell growth and that regulation of its level is an essential function. However, many cellular processes regulated by phosphoinositides are still not fully understood, whereas others such as the nuclear function of these lipids remain largely unresolved. Future studies of these phospholipids and the enzymes that regulate their metabolism will be required to address these questions.

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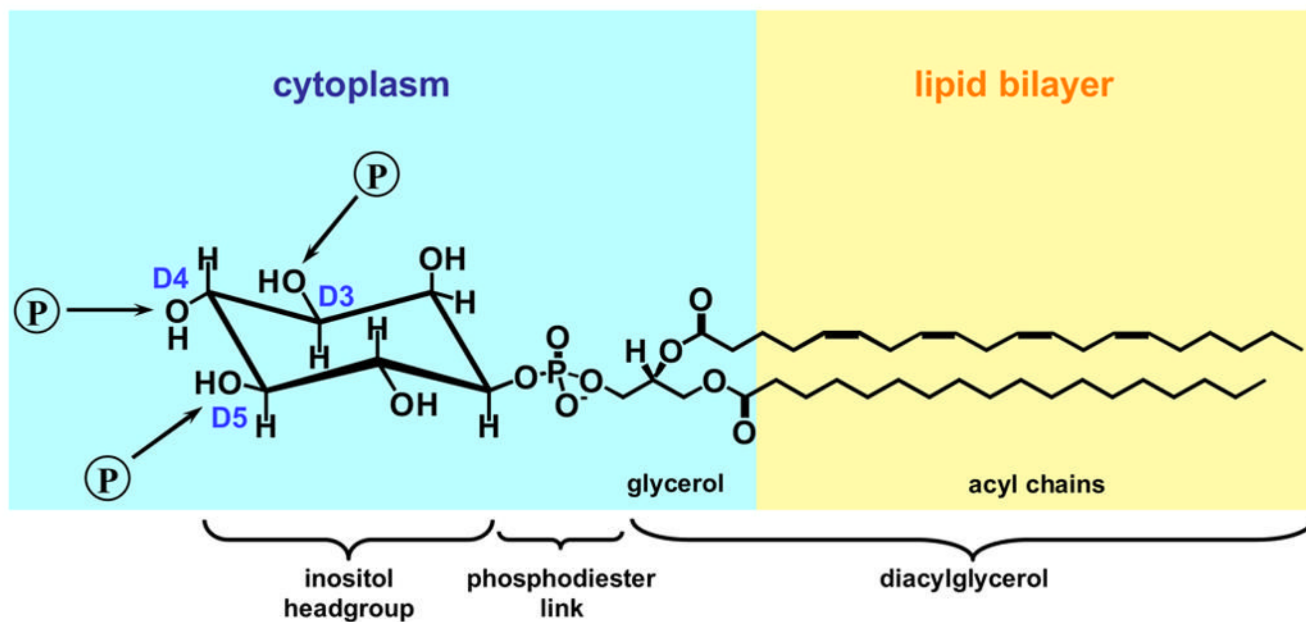

Figure 1. Chemical structure of phosphatidylinositol (PtdIns)

The inositol headgroup is esterified to inorganic phosphate, which is in turn esterified to diacylglycerol thereby forming a phosphodiester link. *In vivo* the lipid portion of the molecule is directly inserted into the lipid bilayer, whereas the hydrophilic headgroup protrudes into the cytosol on the cytoplasmic surface of biological membranes. This leaves the hydroxyls in positions D3, D4 and D5 accessible for cytoplasmic and membrane-bound enzymes such as lipid kinases and phosphatases.

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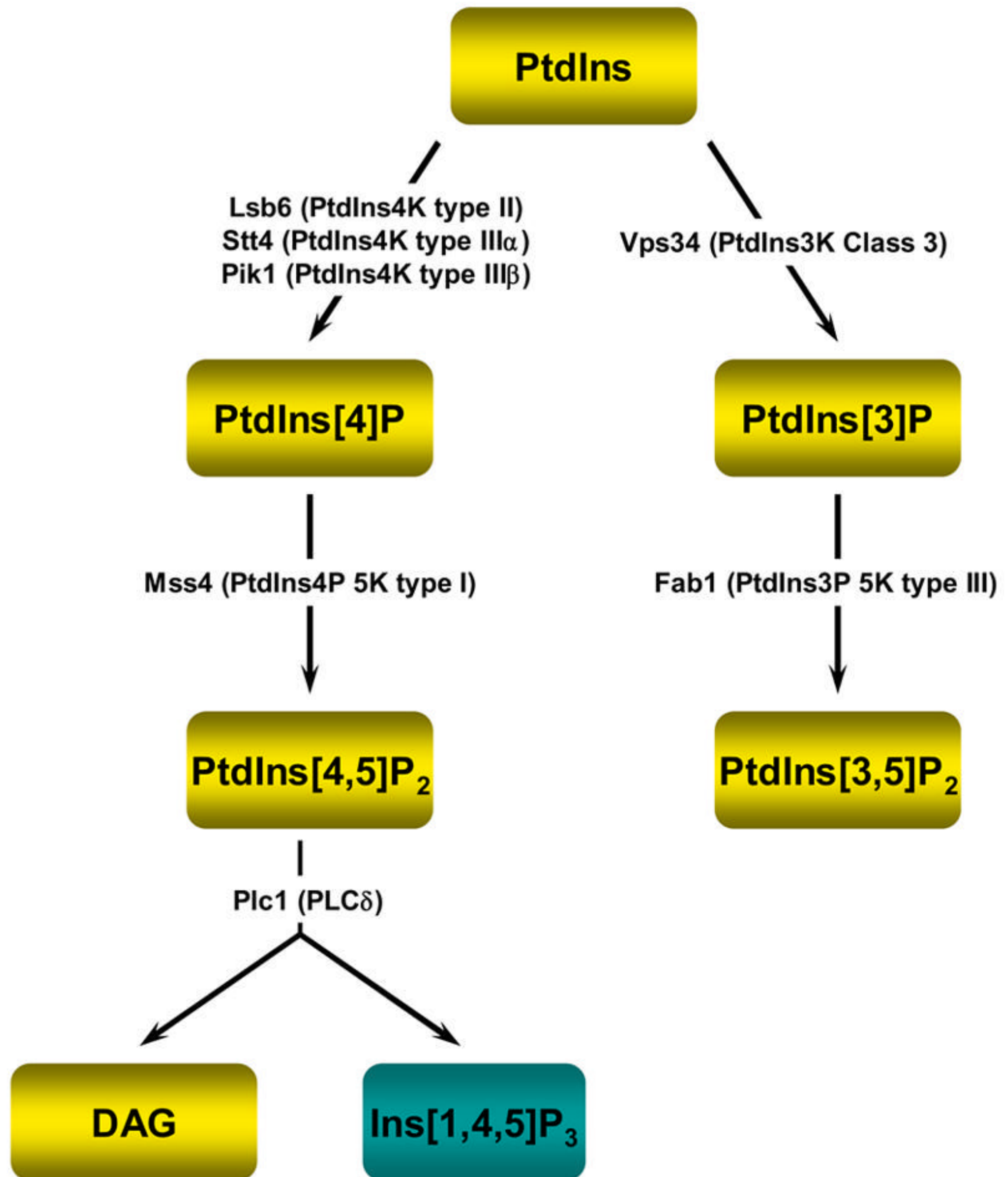

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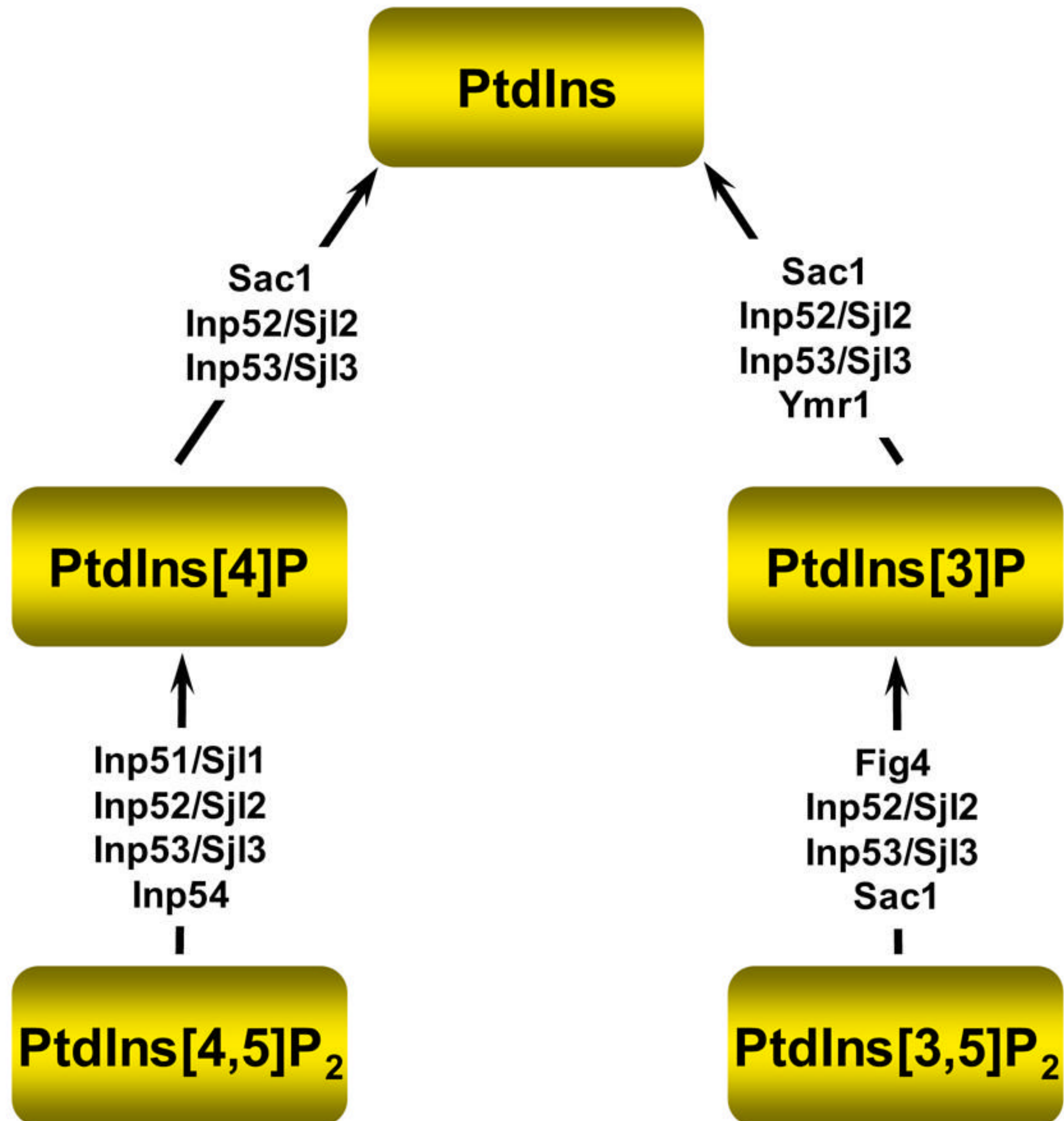

Figure 2. Interconversion of phosphoinositides in yeast

(A) Phosphoinositide kinase reactions in *S. cerevisiae*. The individual steps in the synthesis of phosphoinositides catalyzed by lipid kinases, as well as the breakdown of PtdIns[4,5]P_2 by phosphoinositide-specific phospholipase C (Plc1) are depicted. All phosphoinositide kinases that have been identified in yeast exhibit specificity for a single substrate. The phosphorylation of PtdIns to PtdIns[4]P can be catalyzed by three different enzymes, namely Lsb6, Pik1 and Stt4, whereas all other reactions are catalyzed only by one kinase. The classification for each enzyme is also illustrated. (B) Phosphoinositide phosphatase reactions in yeast. In contrast to the phosphoinositide kinases, the lipid phosphatases of *S. cerevisiae* show a high degree of redundancy, as most of them dephosphorylate a number of substrates with relatively little specificity. Only Ymr1, Fig 4, Inp51/Sjl1 and Inp54 are highly selective for a single substrate, whereas Inp52/Sjl2 and Inp53/Sjl3 can convert most phosphoinositides that have been identified in yeast to PtdIns [235].

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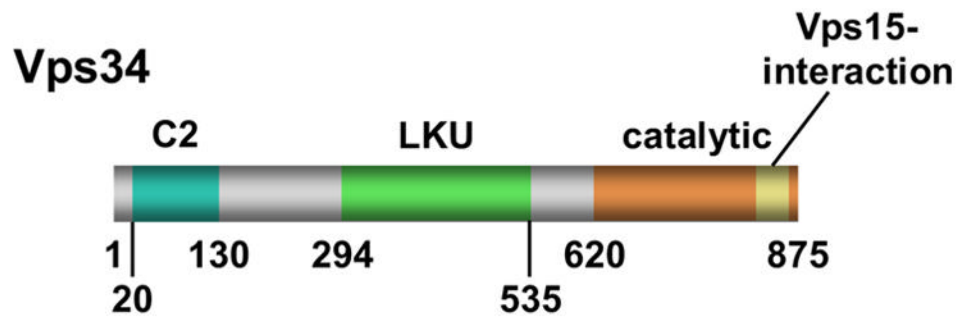

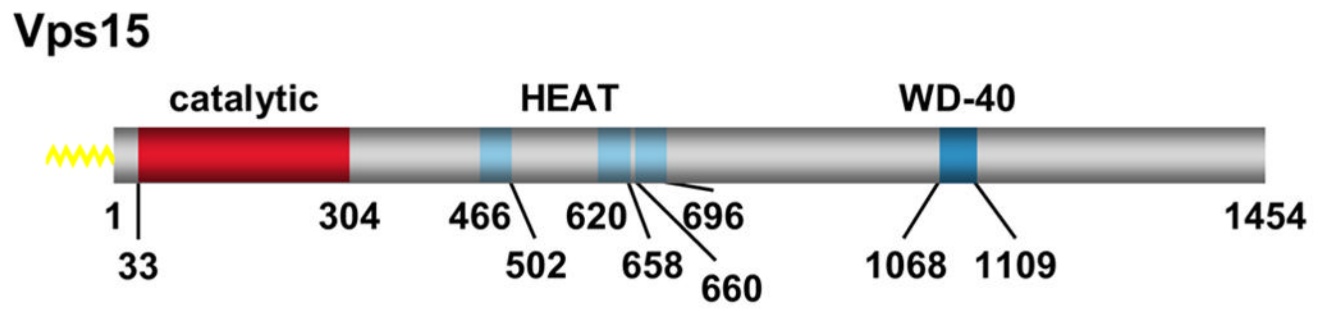

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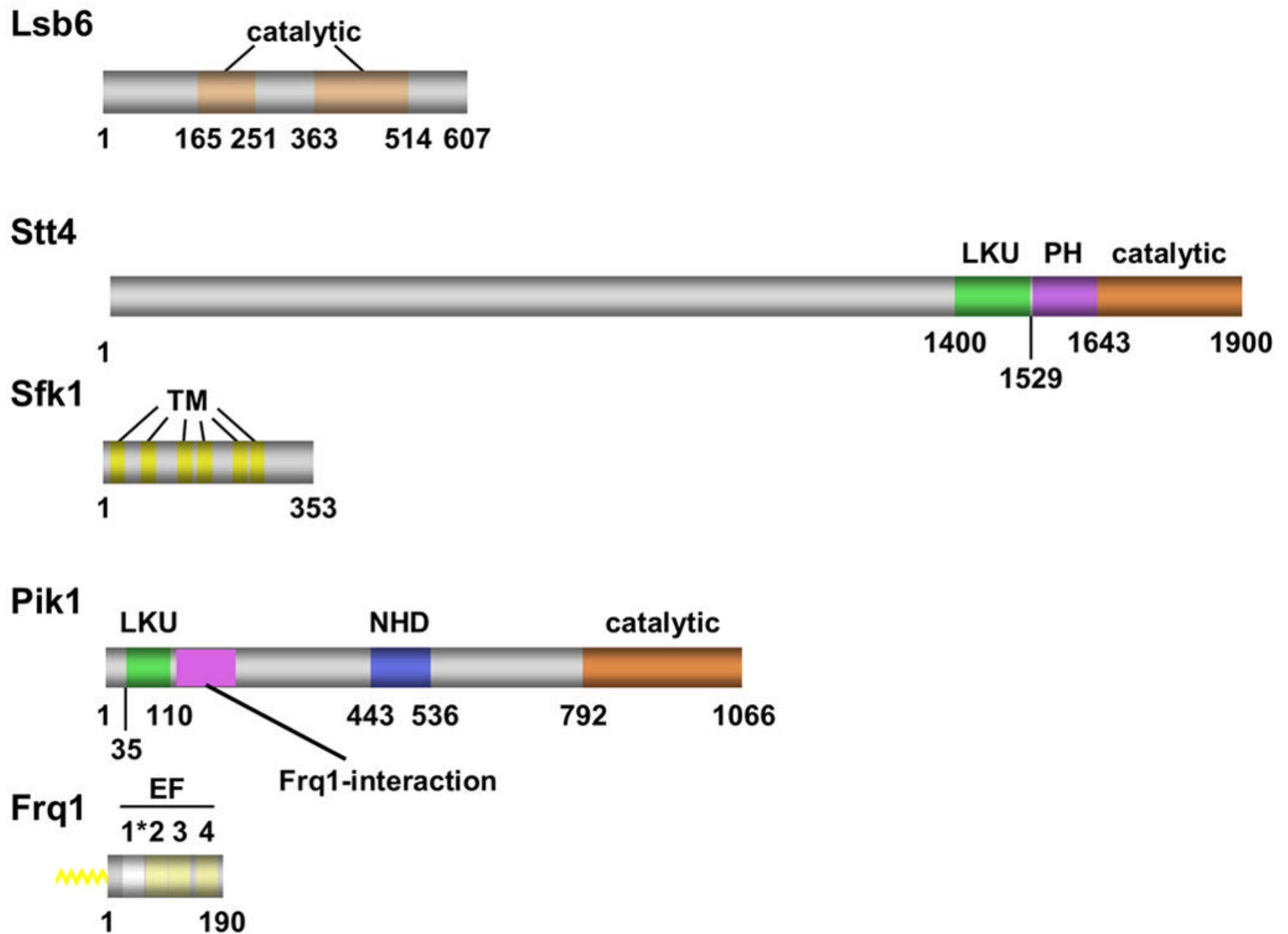

Figure 3. Domain structure of the PtdIns kinases and their accessory proteins in yeast

The different conserved domains are represented by colored boxes and their positions within the primary sequences of the proteins are also indicated. Domains that share a significant degree of homology, such as the lipid kinase unique (LKU) domain and the catalytic domains of Vps34, Pik1 and Stt4, are shown in the same color. (A) Domain structure of the PtdIns 3-kinase Vps34 and its accessory protein Vps15. The interaction of both proteins is mediated by a short 28 residues-spanning region in the COOH-terminal catalytic domain of Vps34 and the three HEAT repeat motifs located in the center of Vps15. The C2 domain of Vps34 and the WD-40 motif of Vps15 are also depicted. (B) Domain structure of the PtdIns 4-kinases of yeast and their accessory factors, Sfk1 and Frq1. The bipartite catalytic domain of Lsb6 is shown in a different color, as it is unrelated to those of the other lipid kinases in yeast. The region of Stt4 that is sandwiched between its LKU and catalytic domains is similar to the pleckstrin homology (PH) domains found in other proteins. The non-functional EF-hand motif in Frq1, which contains substitutions in key residues necessary for Ca^{2+} coordination, is indicated with an asterisk and demarcated with a different color than the other three EF-hand motis. C2, C2 domain; EF, EF-hand motif; HEAT, HEAT repeat motif; LKU, lipid kinase unique domain; NHD, novel homology domain; PH, pleckstrin homology-like domain; TM, transmembrane domain; WD-40, WD-40 motif.

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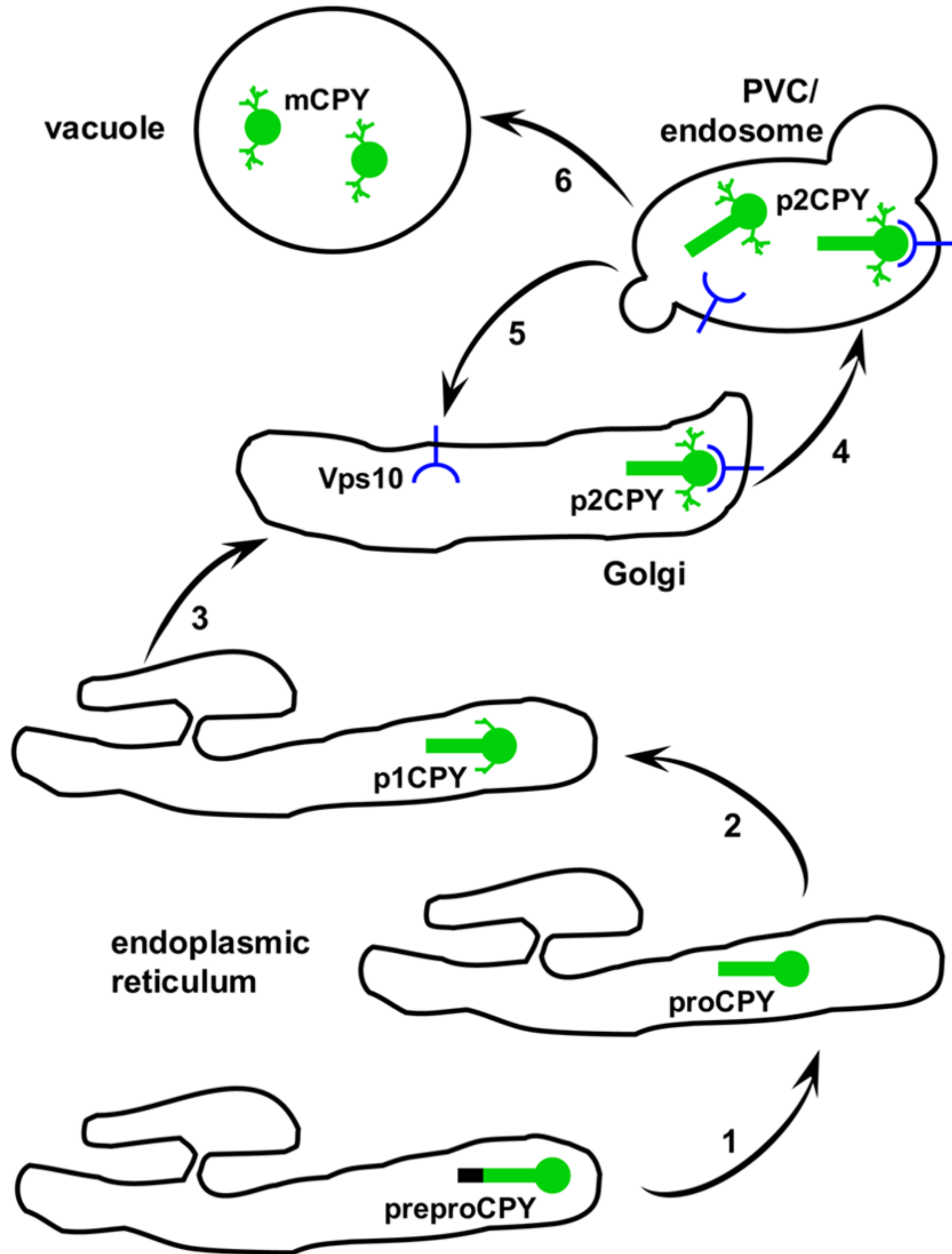

Figure 4. Vacuolar protein sorting of carboxypeptidase Y (CPY)

CPY is synthesized at the ER as an inactive precursor, prepro-CPY. Upon release into the ER lumen its NH_2-terminal signal sequence is removed proteolytically, thereby generating pro-CPY (1). Subsequently, it undergoes NH_2-linked glycosylation to become the ER-modified 67 kDa form p1CPY (2). In a next step p1CPY is transported to the Golgi apparatus where it receives further oligosaccharide modifications yielding another intermediate form, the 69 kDa p2CPY (3). In the late Golgi p2CPY is bound by Vps10, which serves as a transport receptor for regulated trafficking to a prevacuolar endosomal compartment (PVC), away from secretory cargo destined for the plasma membrane (4). In the PVC p2CPY is released from Vps10, which itself is recycled back to the Golgi apparatus for another round of receptor-mediated transport

between these two compartments (5). Following its delivery to the vacuole p2CPY is cleaved by lumenal proteases into the active, mature 61 kDa form mCPY (6).

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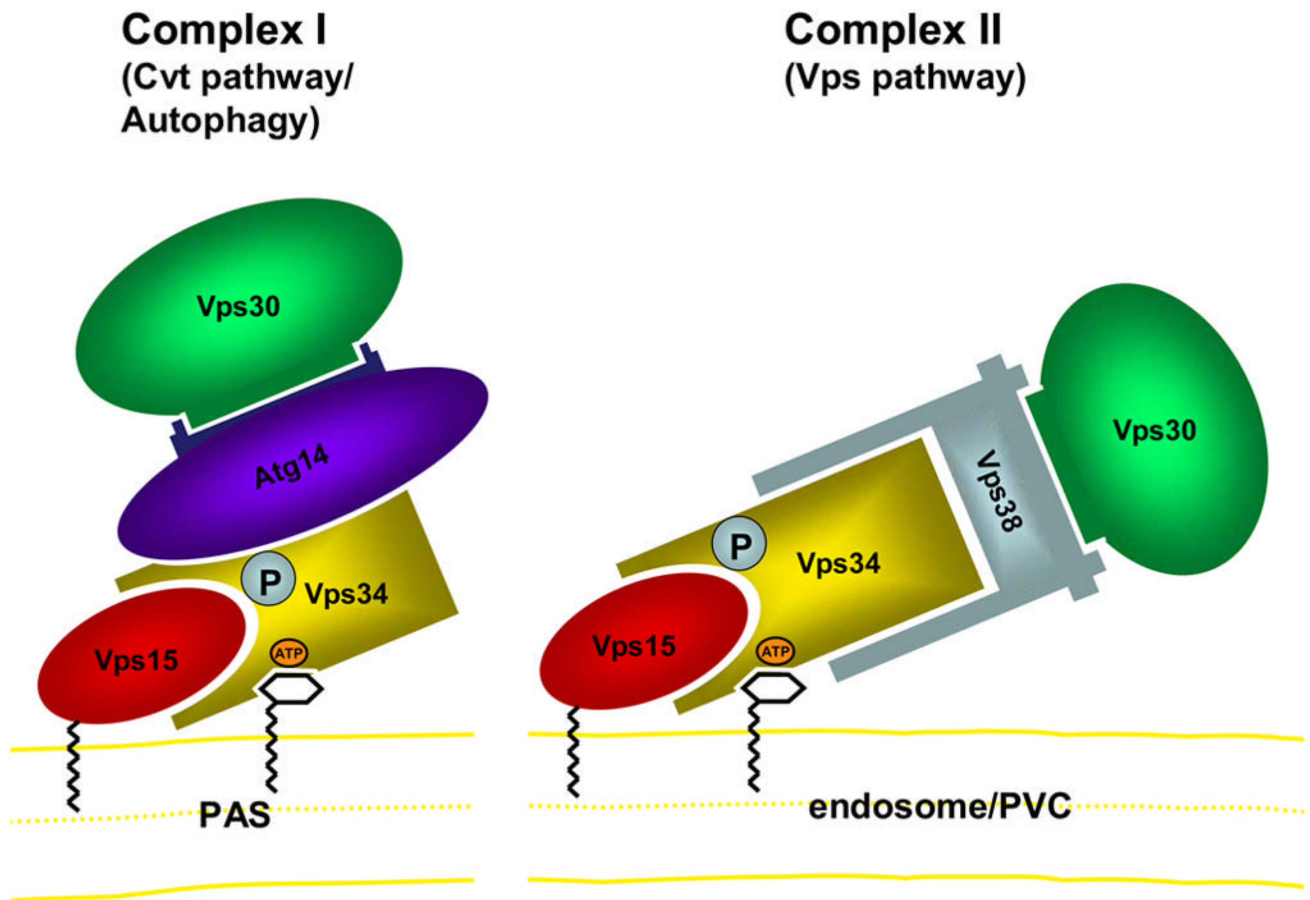

Figure 5. Vps34 containing PtdIns 3-kinase complexes

The yeast PtdIns 3-kinase, Vps34, forms two different multi-subunit complexes (Complex I and Complex II), which function in distinct biological processes, namely the autophagy/cytosol-to-vacuole transport (Cvt) and vacuolar protein sorting (Vps) pathways. Both complexes share the Vps15, Vps34 and Vps30 subunits, whereas Atg14 is limited to complex I and Vps38 is specific for complex II. Vps15 is NH_2-myristoylated, and, hence, mediates the recruitment of complexes I and II to the pre-autophagosomal structure (PAS) and the prevacuolar endosomal compartment (PVC), respectively. In addition, Atg14 is essential for the recruitment of complex I to the PAS, whereas complex II localizes to the PVC in a manner that does not depend on the presence of Vps38. Phosphorylation of Vps34 by Vps15, which plays a role in the interaction of both proteins, as well as in the binding of Vps34 to Atg14 and Vps38, is also depicted.

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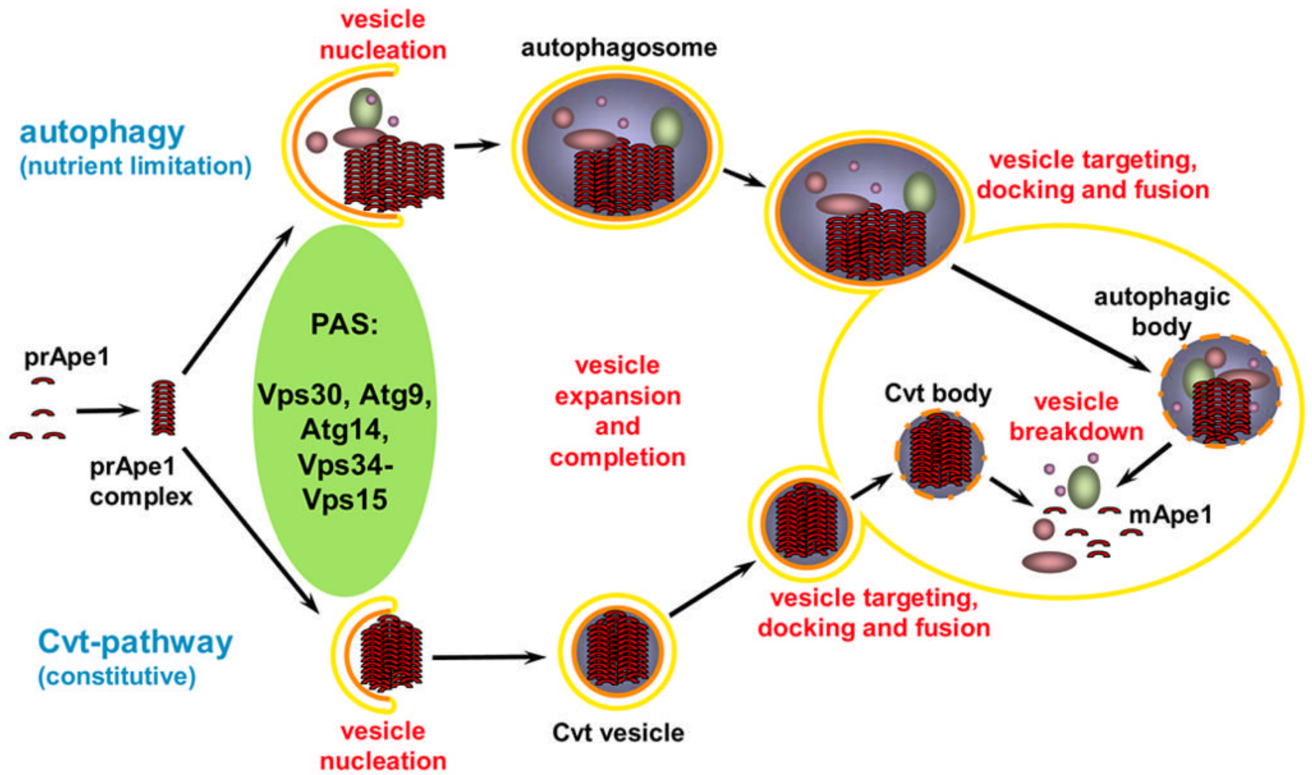

Figure 6. Schematic representation of the autophagy and cytosol-to-vacuole (Cvt) pathways in yeast
Aminopeptidase 1 (Ape1), which is synthesized as an inactive precursor (prApe1) in the cytoplasm follows both pathways for its delivery to the vacuolar lumen where it is proteolytically processed to yield the mature form of the enzyme (mApe1), is shown as a model substrate. The commitment of yeast cells to either pathway depends on nutrient conditions. In both pathways, autophagy and cytosol-to-vacuole transport, cytosol and in the case of autophagy also entire organelles are engulfed and sequestered by a double membrane. The initial step of this process, which also referred to as vesicle nucleation, takes place at the pre-autophagosomal structure (PAS). Factors known to be involved in the function of this organelle, including the PtdIns 3-kinase Vps34, are also shown. Upon completion, autophagosomes and Cvt vesicles are targeted to and fuse with the vacuolar membrane. Ultimately, the resulting autophagic and Cvt bodies are degraded in the vacuolar lumen.

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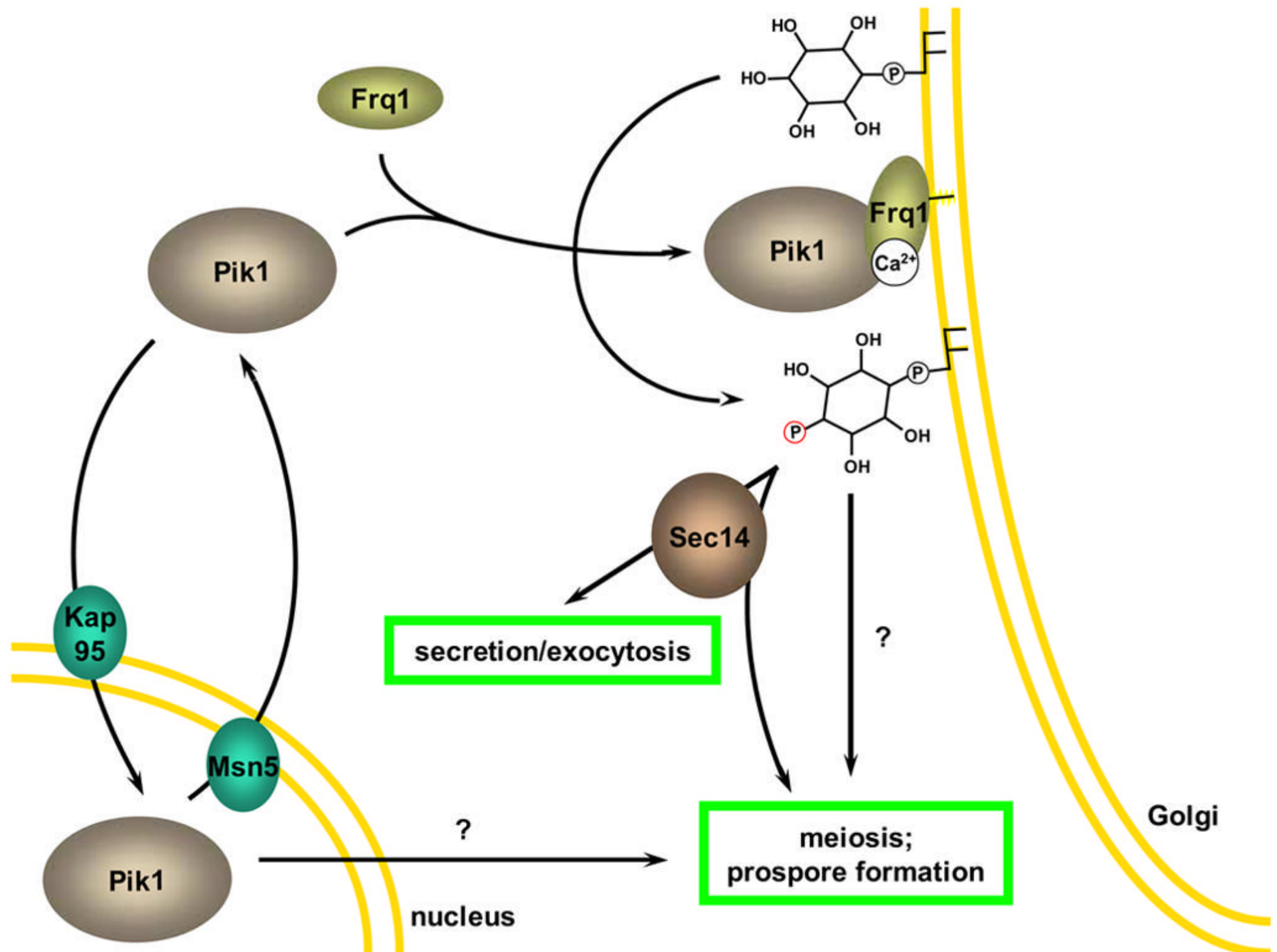

Figure 7. Schematic representation of the cellular functions of Pik1

Pik1 and Frq1 both localize to Golgi membranes, where they, together with Sec14, play a role in the regulation of secretion and, in sporulating cells, prospore formation. Pik1 also shuttles between the cytosol and the nucleus in a manner that depends on the karyopherins Kap95 and Msn5. One possible nuclear function of Pik1 is the regulation of one or more processes required for successful completion of meiosis.

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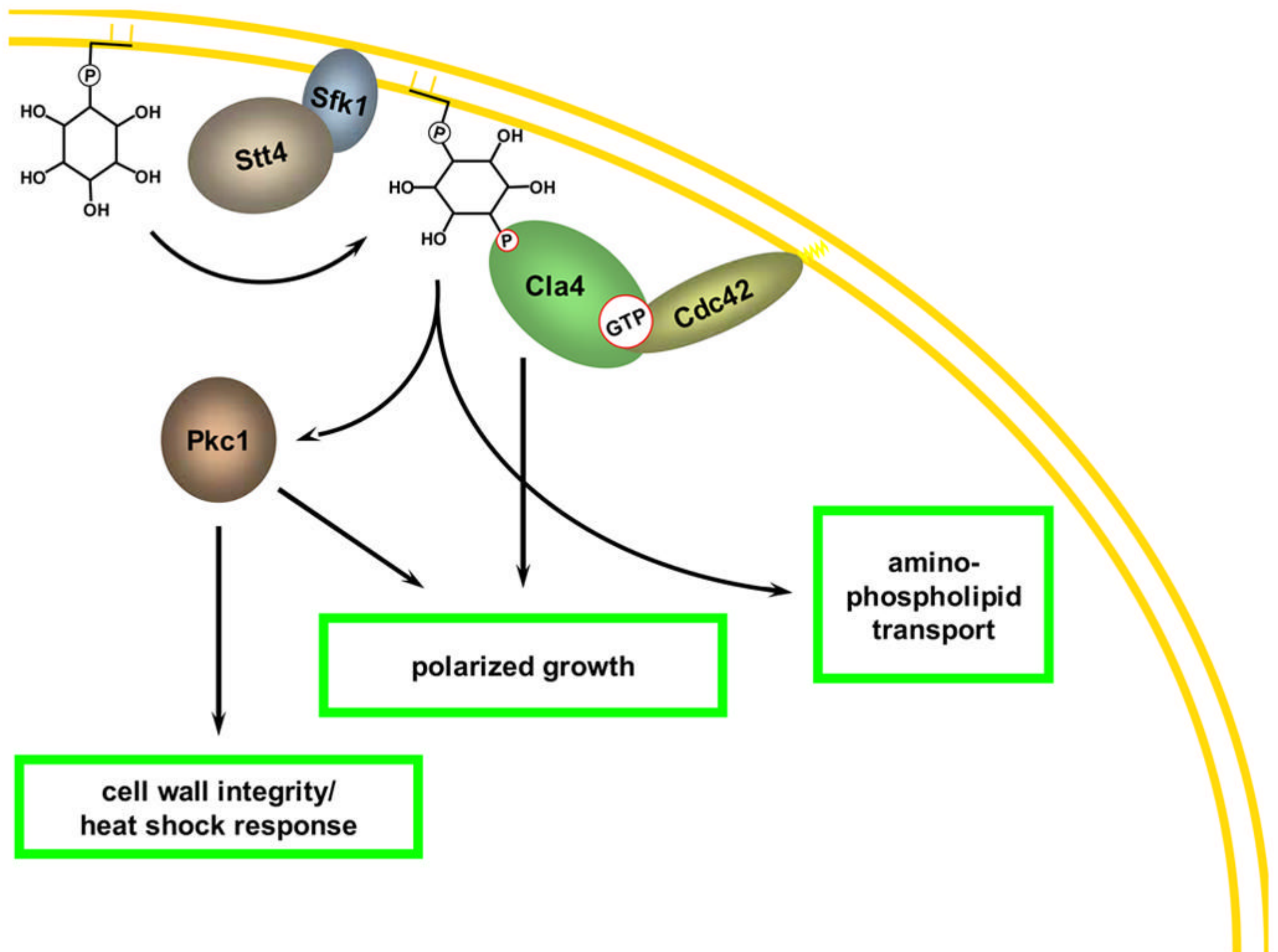

Figure 8. Schematic representation of the cellular functions of Stt4

Stt4 localizes to the plasma membrane in a manner that requires its accessory protein Sfk1. Stt4 functions upstream of both Pkc1 and Cla4 in the regulation of polarized growth and the cell wall integrity/heat shock response pathways. Furthermore, evidence for a role of Stt4 in aminophospholipid transport from the ER has been obtained [209].

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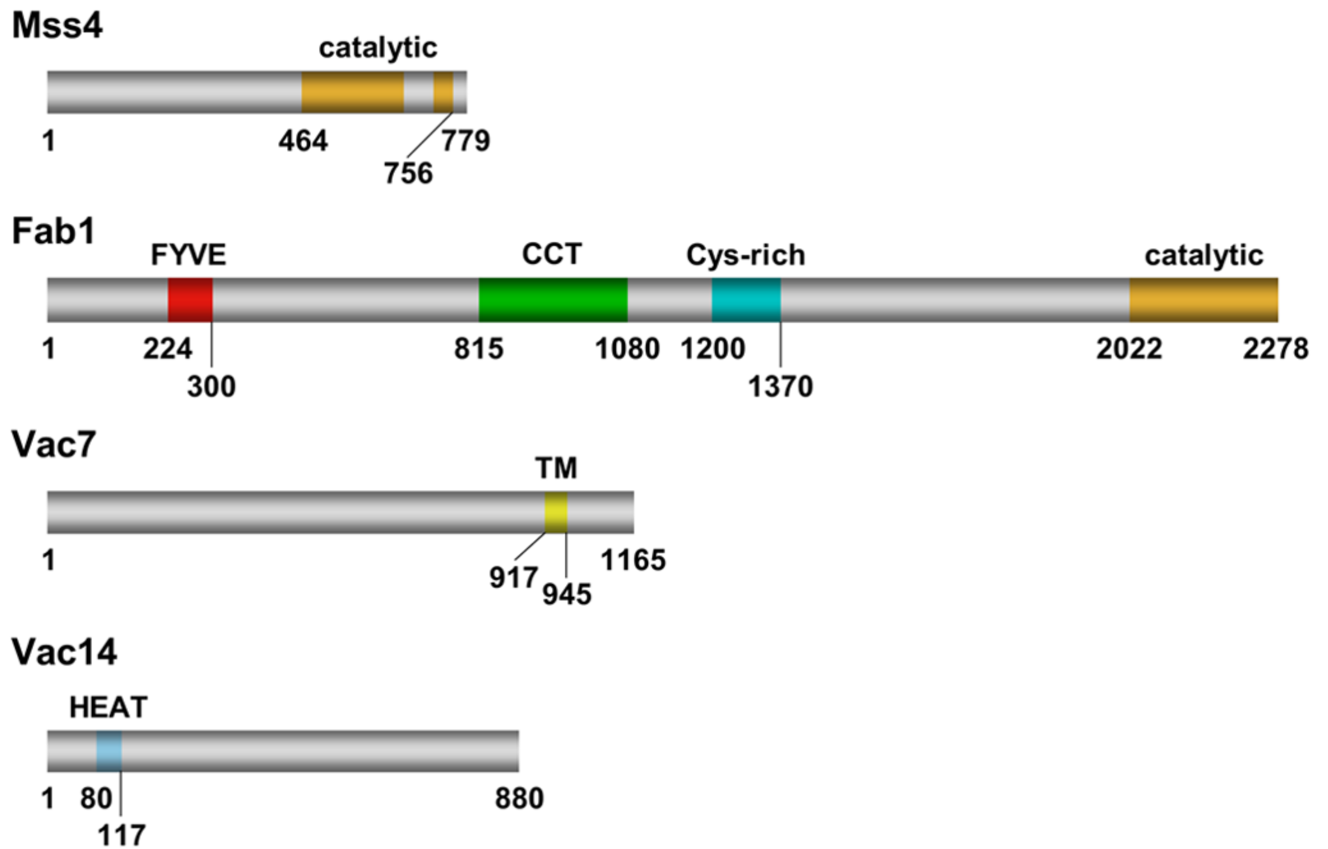

Figure 9. Domain structure of the PtdInsP-kinases and their accessory proteins in yeast
Conserved regions are shown as colored boxes and homologous regions are depicted in the same color. CCT, chaperonin containing T-complex homology region; FYVE, Fab1, YGL023, Vps27, and EEA1 domain; HEAT, HEAT repeat motif; TM, transmembrane domain.

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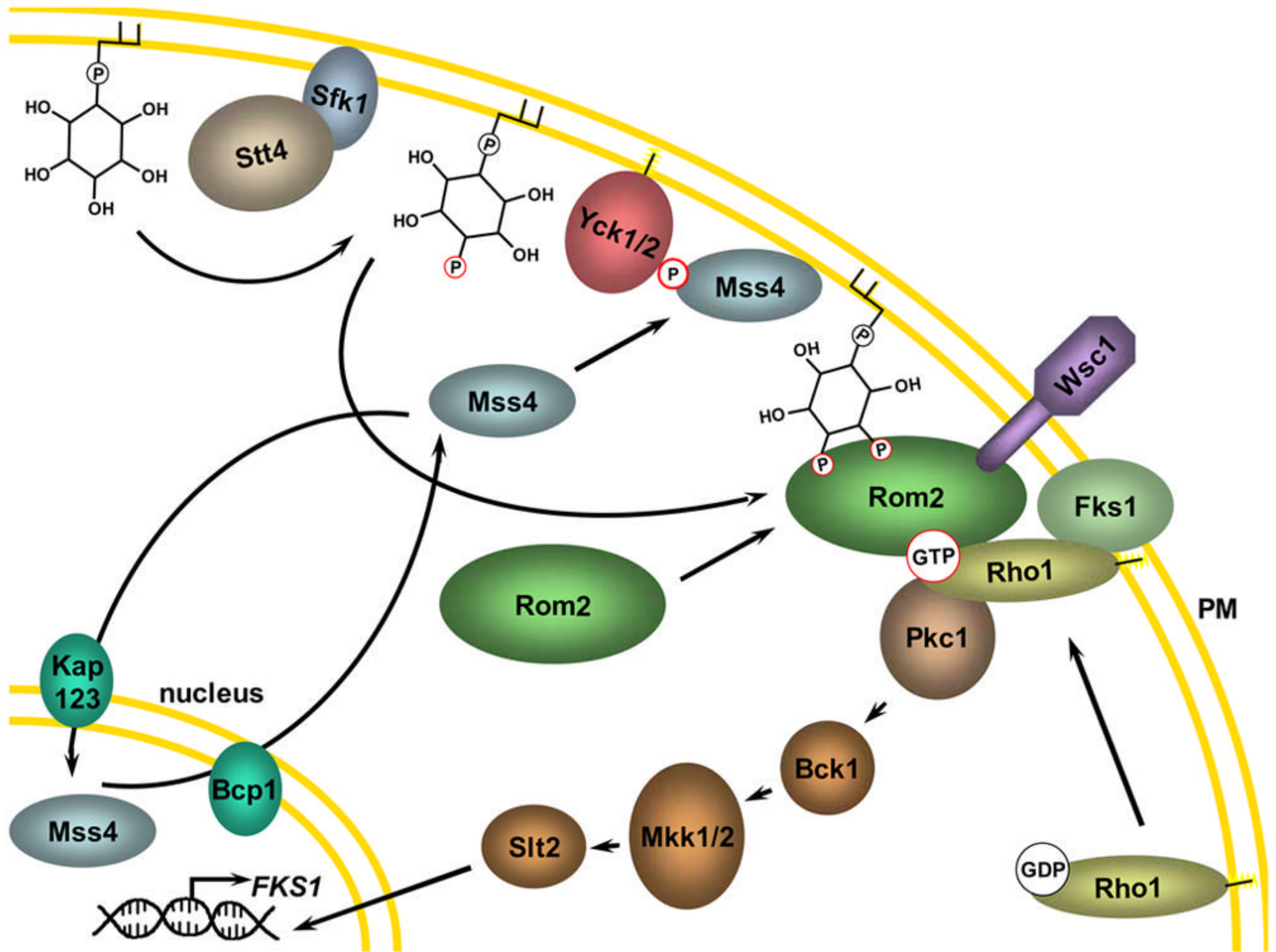

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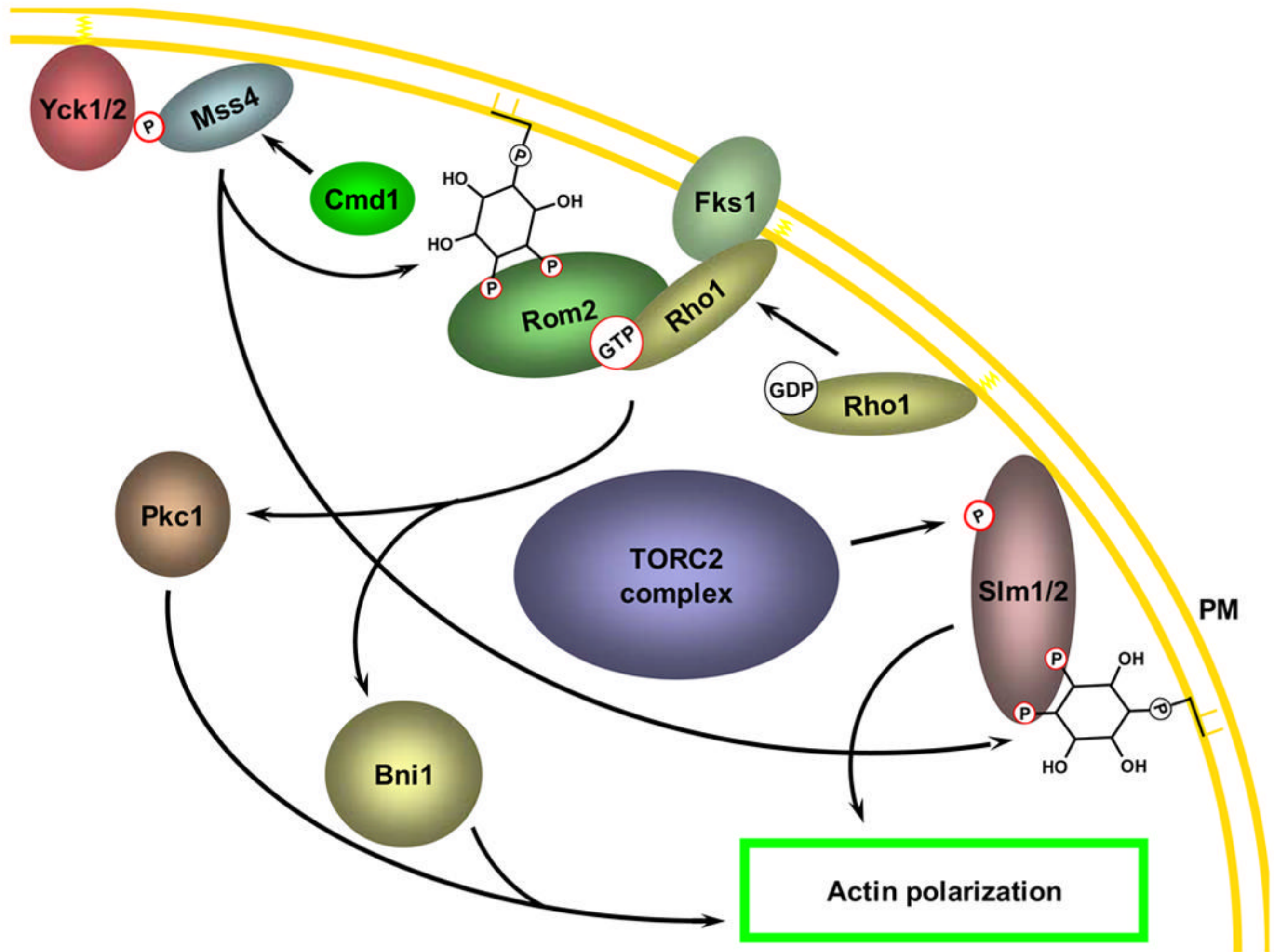

Figure 10. Schematic representation of the cellular functions of Mss4

(A) Regulation of the subcellular localization and activity of Mss4 and its role in the cell wall integrity pathway. Nucleocytoplasmic shuttling of Mss4 depends on Kap123 and Bcp1, whereas its recruitment to the plasma membrane involves phosphorylation by the casein kinase I isoforms Yck1 and Yck2. At the plasma membrane Mss4 acts downstream of the PtdIns 4-kinase Stt4 and upstream of the Rho GEF Rom2, which directly binds to PtdIns[4,5]P_2 generated by Mss4. In concert with one of the cell surface sensors that function in cell wall integrity sensing such as Wsc1, Rom2 stimulates nucleotide exchange on Rho1, which in turn activates the β-1,3-glucan synthase Fks1. Another effector of activated Rho1-GTP is the serine/threonine kinase Pkc1, which serves as an upstream activator of the Slt2/Mpk1 MAP kinase cascade. Ultimately this signaling cascade leads to changes in the transcriptional output of a number of genes including *FKS1*. (B) Model for the regulation of the actin cytoskeleton by Mss4. Slm1 and Slm2 directly bind PtdIns[4,5]P_2 generated by Mss4 at the plasma membrane, which is itself stimulated by the input of Cmd1. Furthermore, both proteins are subject to phosphorylation by Tor2, a component of the multisubunit TORC2 complex. Both inputs, phosphorylation by TORC2 and PtdIns[4,5]P_2 binding are required for proper localization of Slm1/2 to the plasma membrane and the regulation of the actin cytoskeleton. Other downstream effectors of Mss4 that regulate the polarization of the actin cytoskeleton include the protein kinase Pkc1 and the formin Bni1.

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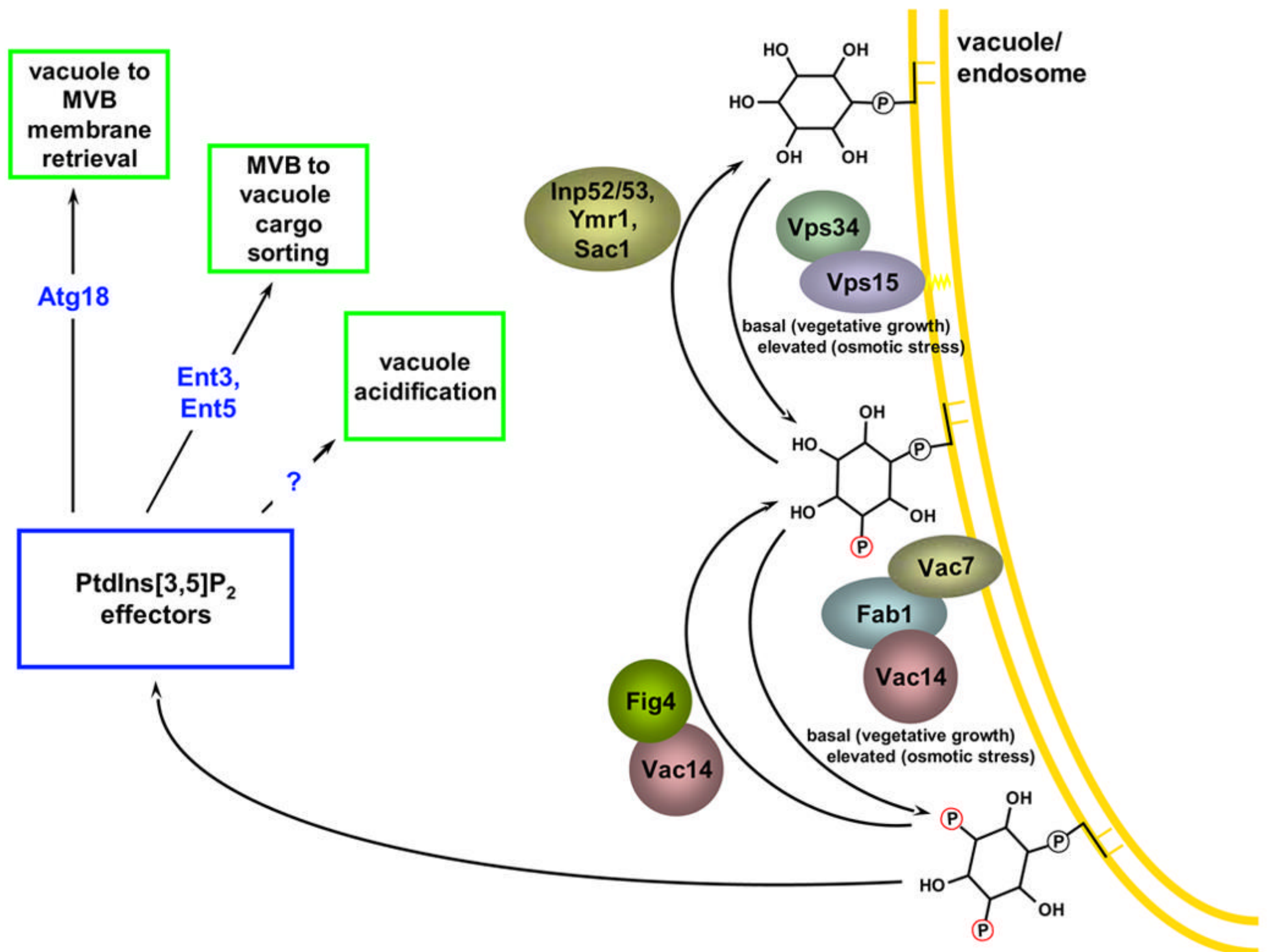

Figure 11. Schematic representation of the cellular functions of Fab1

Fab1 acts in concert with its activators Vac7 and Vac14 at vacuolar/endosomal membranes, where it converts PtdIns[3]P generated by Vps34 to PtdIns[3,5]P_2. This process is temporarily upregulated during conditions of hyperosmotic stress, in a manner that also requires the PtdIns [3,5]P_2-specific phosphatase Fig 4. The downstream effectors of PtdIns[3,5]P_2 include Ent3 and Ent5, as well as Atg18 and function in the regulation of vacuole acidification, the MVB pathway and vacuole to MVB membrane retrieval.

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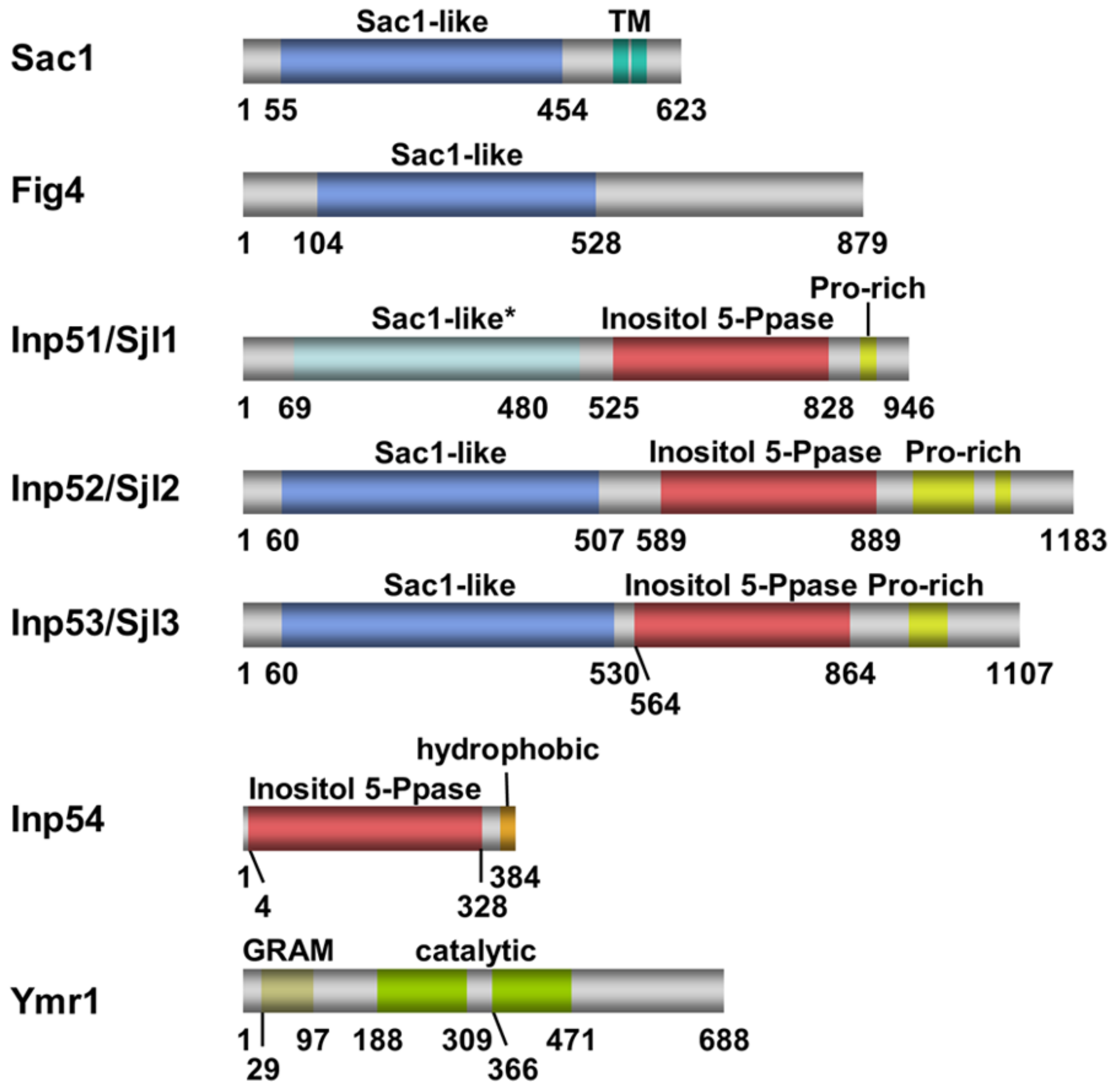

Figure 12. Domain structure of the phosphoinositide phosphatases in yeast

Conserved regions and other functional motifs are shown as colored boxes. GRAM, glucosyltransferase, Rab-like GTPase activator and myotubularin domain. The catalytically non-functional Sac1-like domain in Inp51/Sjl1, which contains substitutions in key active site residues [235], is demarcated with a different color and marked with an asterisk to distinguish it from the catalytically-competent Sac1-like domains present in Sac1, Fig 4, and the other two synaptojanin-like lipid phosphatases, Inp52/Sjl2 and Inp53/Sjl3. Although the Sac1-like domain of Inp51/Sjl1 may not hydrolyze any given phosphoinositide, it may contribute to physiological function of this phosphoinositide 5-phospatase, e.g. by binding to phosphoinositides and thereby assisting in membrane targeting and localization of the enzyme.

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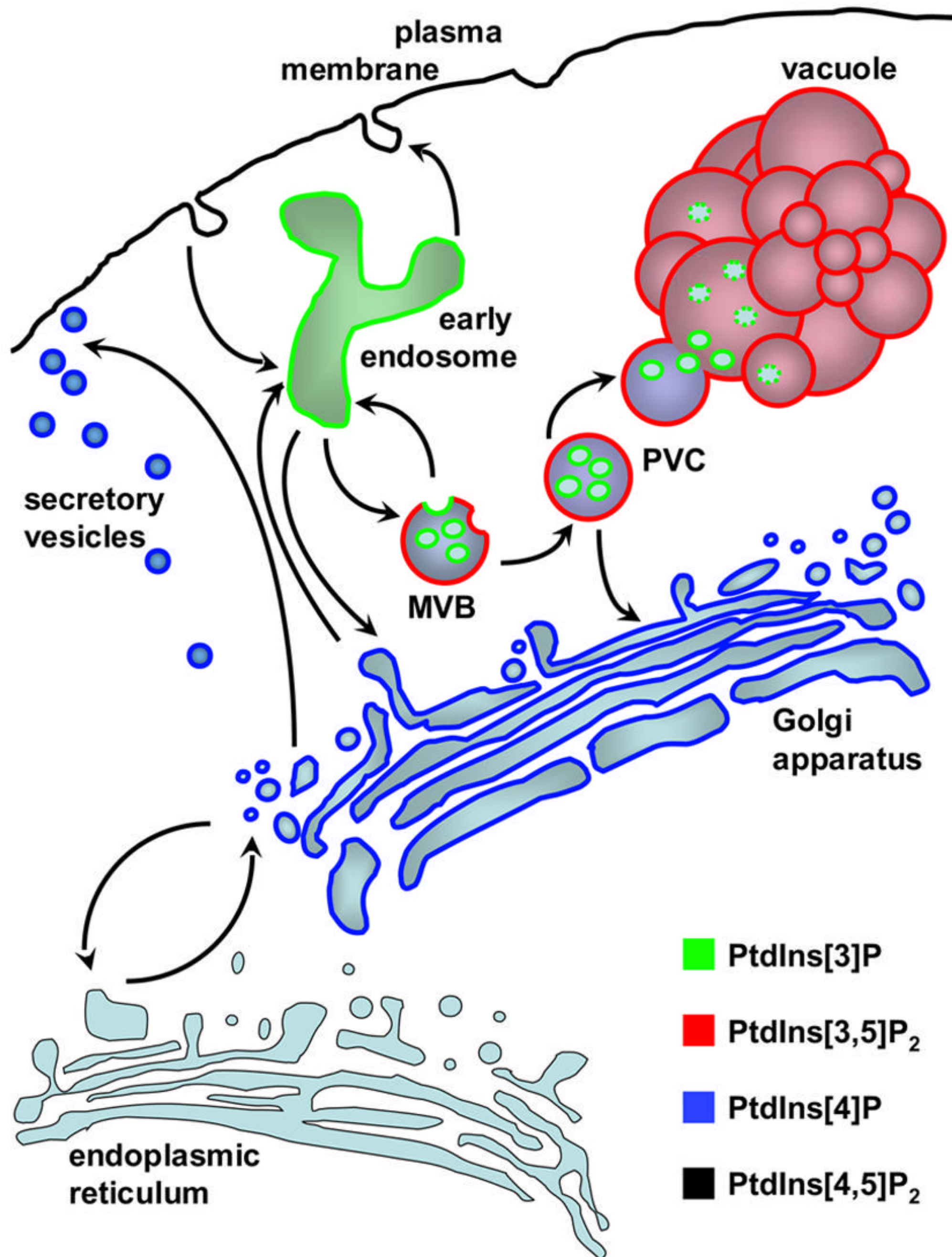

Figure 13. Model for the compartmentalization of phosphoinositides in yeast

The evidence accumulated to date indicates that different phosphoinositide species are generated in a compartment-specific manner and, hence, can be regarded as distinct markers for each organelle. For example, PtdIns[4]P is only detectable at membranes of the Golgi apparatus under normal conditions, and its aberrant accumulation in other locations, such as in the ER when cells carry mutations in genes that affect its turnover, display a number of severe phenotypes directly affecting growth. The pool of PtdIns[4]P generated at the plasma membrane is most likely rapidly and fully converted into PtdIns[4,5]P_2, which is the major phosphoinositide at this location. PtdIns[3]P is found predominantly at membranes of the endosomal system and in multivesicular bodies, where a fraction is converted to PtdIns[3,5]

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P_2, which is also found on vacuolar membranes. The presence and physiological roles of the different phosphoinositides in the nucleus of *S. cerevisiae* still remains unknown.

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Table 1

Properties of PtdIns-specific Phosphoinositide Kinases in *S. cerevisiae*

Yeast Gene	VPS34	LSB6	STT4	PIK1
Category	Class 3	Type II	Type IIIα	Type IIIβ
M_r	95 kDa	55 kDa	215 kDa	125 kDa
ORF	875 aa	607 aa	1900 aa	1066 aa
Phenotype of null allele	ts[a]	viable	lethal[b]	lethal
K_m (ATP)	n. d.	266–650 μM[c]	n. d.	100 μM
Inhibition (IC_{50}) by:				
Wortmannin	3 μM	unaffected	1 nM	unaffected
LY294002	50 μM	n. d.	n. d.	n. d.
Adenosine	unaffected	150 μM	unaffected	unaffected
Localization	particulate	particulate	particulate	soluble
Membrane recruitment	Vps15	S-palmitoylation?	Sfk1	Frq1

[a] Viable, but compromised, at 30° and inviable at 37°C.

[b] In most strain backgrounds tested.

[c] Depending on the particular assay conditions used.

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Table 2

Characteristics and Function of Phosphoinositide Phosphatases in *S. cerevisiae*

Enzyme	Inp51/Sjl1	Inp52/Sjl2	Inp53/Sjl3	Inp54	Sac1	Fig 4	Ymr1
Catalytic Domain	synaptojanin-like, Sac1- like (non functional)	synaptojanin-like, Sac1- like	synaptojanin-like, Sac1- like	synaptojanin- like	Sac1-like	Sac1-like	myotubularin- related
Substrate	PtdIns[4,5]P_2	PtdIns[3]P, PtdIns[4]P, PtdIns[3,5]P_2, PtdIns[4,5]P_2	PtdIns[3]P, PtdIns[4]P, PtdIns[3,5]P_2, PtdIns[4,5]P_2	PtdIns[4,5]P_2	PtdIns[3]P, PtdIns[4]P, PtdIns[3,5]P_2	PtdIns[3,5]P_2	PtdIns[3]P
Subcellular Localization	unknown	cytoplasm, cortical actin patches, Golgi/ endosome (?), nucleus	cytoplasm, cortical actin patches, Golgi/ endosome (?)	endoplasmic Reticulum (hydrophobic tail anchor)	endoplasmic Reticulum (integral), Golgi	vacuolar membrane	endosome/ PVC
Cellular Function Affected in Mutants	actin cyto-skeleton, endocytosis, cell wall in-tegrity, vacuolar and mitochondrial morphology	actin cyto-skeleton, endocytosis, TGN-endosome transport, cell wall maintenance, meiosis, vacuolar morphology	actin cyto - skeleton, endocytosis, Golgi function, TGN-endosome transport, cell wall maintenance, vacuolar morphology	secretion	secretion, actin cyto-skeleton, endocytosis, vacuolar function, cell wall main tenance, ATP uptake into the ER	secretion, hyperosmotic shock response, response to mating pheromone	vacuolar homeostasis, MVB-sorting, maintenance of endosome function, cell wall integrity