Polyphosphoinositide-derived signals in the regulation of vacuole membrane fusion and fission

by

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A thesis submitted to the University of Birmingham

for the degree of

DOCTOR OF PHILOSOPHY

School of Biosciences

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February 2010

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ABSTRACT

The endo-membrane system of eukaryotic cells is a dynamic network of membrane-bound organelles that exists in a constant state of flux as they exchange membrane and protein cargoes. Understanding how these organelles retain their unique protein and lipid compositions in the face of this enormous inward/outward flow is one of the central problems in cell biology and has implications for the fields of biotechnology, virology, immunity and cancer biology.

All protein trafficking can be reduced to a problem of regulated membrane fission events that couple cargo selection with the formation of transport vesicles, followed by selective membrane fusion with the target organelle. The same fusion and fission events are thought to underlie the changes in organelle size and shape that can occur during stress or when organelles divide (e.g. during cytokinesis).

The yeast vacuole is a highly dynamic organelle that receives membrane and cargo from a number of different pathways. This organelle also changes shape in response to both hypo and hyper-osmotic stresses and so has been used to study both membrane fusion and fission. Fusion is relatively well characterized but fission is not well understood.

This work focuses on the molecular events that co-ordinate vacuole fission during hyper-osmotic stress. One polyphosphoinositide pathway was known to regulate vacuole fission before this work began: the Fab1p pathway. Polyphosphoinositides are a group of lipid signals that are rapidly formed in cells and modulate the activity of

protein effectors. The Fab1p pathway is activated during hyper-osmotic stress and causes the vacuole to fragment into many tiny sub-compartments by a process of membrane fission. I characterized a number of the components of this pathway to learn how they work together, including Vac7p, Vac14p, Fig4p and Ymr1p. The specificity and localisation of several of these proteins were characterized and a greater understanding of this protein network emerges from this work.

I then focused on understanding how fission of the vacuole is maintained and fusion inhibited in hyper-osmotic media. It was previously reported that Yck3p, a vacuolar protein casein kinase, was activated by hyper-osmotic stress to phosphorylate Vps41p, a component of the vacuole fusion machinery. The effect of this phosphorylation and the upstream signaling pathway controlling Yck3p were unknown.

I started by looking if any known signalling systems might control Yck3p and ruled out both the Fab1p and Hog1p osmotic stress response pathways as upstream activators. I then found that another polyphosphoinositide signalling system controls Yck3p: namely the Plc1p phospholipase pathway that produces a series of water soluble inositol polyphosphate second messengers. I further found that deletion of any of the inositol polyphosphate kinases in the Plc1p pathway prevented Yck3p signalling, suggesting that the inositol pyrophosphates control Yck3p activity in some fashion.

I also identified the Yck3p phosphorylation sites on Vps41p by FT-ICR mass spectrometry and showed that phosphorylation of these sites was required to block vacuole fusion during hyper-osmotic stress. *vps41* mutants lacking these sites showed

aberrant behavior: their vacuoles underwent normal fission in response to hyper-osmotic stress but then immediately re-fused in an apparent “futile cycle”.

Thus Plc1p controls a novel signalling pathway mediated by Yck3p that prevents vacuole fusion by phosphorylating thus inhibiting a vital component of the fusion machinery: Vps41p.

DEDICATION

I would like to dedicate this thesis to my beloved parents and my wife, who will be proud of me obtaining a Ph.D. degree, and also to my lovely new-born son, I hope that he might one day “follow in my footsteps” and attempt to solve a few “insoluble” lipid problems of his own.

ACKNOWLEDGEMENTS

Throughout the years of Ph.D. study, I have received invaluable support and encouragement from many people. First and foremost, I would like to express my sincere appreciation to Dr. Stephen Dove, my Ph.D supervisor, who has guided me with enthusiasm and patience. I feel that in each lab meeting and personal talk, he helped me to understand much that was beyond me in the early days. Special thanks must also go to Professor Bob Michell: a pioneer in the field of inositol lipids. His insight always brought me out of confusion and into the light. Both in particular, helped me immeasurably during the writing of this thesis, discussing difficulties, reviewing my drafts and gaving me timely feedback.

Next, I would like to express my gratitude to all previous and current members of Dr Dove's lab, and also to all the people on the sixth floor. I am very happy to have known and spent time with you all over the past few years. Many thanks for your help with experiments and bringing me a lot of funs every day. In addition, many thanks must also be given to Dr. Cleidiane Zampronio for her assistance on the mass spectrometry data analysis.

I must acknowledge my beloved family whose emotional support, particularly understanding my need to focus on my studying, was a precious gift.

Finally but importantly, I greatly appreciate the financial support of the Dorothy Hodgkin Postgraduate Award (DHPA) that has funded my studies and my life over last three years in Birmingham, U.K.

ABBREVIATIONS

CK1 & 2	Casein kinase 1 & 2
CORVET	Class C core vacuole/endosome tethering
DAG	Diacylglycerol
DIC	Differential interference contrast
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
HA	Hemagglutinin
HOPS	Homotypic fusion and protein sorting
HPLC	High performance liquid chromatography
IPs	Inositol polyphosphates
IP_3	Inositol 1,4,5-trisphosphate
IP_4	Inositol 1,4,5,6-tetrakisphosphate
IP_5	Inositol 1,3,4,5,6-pentakisphosphate
IP_6	Inositol 1,2,3,4,5,6-hexakisphosphate
IP_7	Diphosphoinositol pentakisphosphate ("PP-IP_5")
IP_8	Bis-diphosphoinositol tetrakisphosphate $(PP)_2$-IP_4
MAPK	Mitogen-activated protein kinase
MTMR	Myotubularin related protein
MVB	Multivesicular body
ORF	Open reading frame
PLC	Phospholipase C
PM	Plasma membrane
PPIn	Polyphosphoinositide
PtdIns	Phosphatidylinositol
PtdIns3*P*	Phosphatidylinositol 3-monophosphate
PtdIns4*P*	Phosphatidylinositol 4-monophosphate
PtdIns5*P*	Phosphatidylinositol 5-monophosphate
PtdIns(3,5)P_2	Phosphatidylinositol 3,5-bisphosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate

PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-trisphosphate
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
S. cerevisiae	*Saccharomyces cerevisiae*
SNARE	Solube N-ethylmaledimide-sensitive factor attachment protein receptor
TGN	Trans-golgi network
ts	Temperature sensitive

CONTENTS

Chapter 1 Introduction

1.1 General introduction and history of PPIns

The inositols are a group of nine isomers of cyclohexanehexol. *Myo*-inositol (referred to simply as inositol in this work) is the most abundant in nature and the isomer most often incorporated into biological molecules. It was first identified as a component of muscle tissue by the German chemist Josef Scherer and named after this original source (*Greek* for muscle) (Scherer, 1850). *Myo*-inositol is the isomer of 1,2,3,4,5,6-hexahydroxycyclohexane that has one axial hydroxyl at the 2-position and the other five hydroxyls equatorial to the six-carbon ring.

The synthesis of inositol is catalyzed by two enzymes: an NAD^+-dependent *myo*-inositol-3-phosphate synthase (MIPS, called Ino1p in yeast) that takes glucose-6-phosphate from glycolysis, and converts it to D-*myo* inositol-3-phosphate (Ins3*P*). Ins3*P* is then the substrate for an inositol monophosphatase (Ins*P*ase) that releases free inositol (see Michell, 2008) (see Fig. 1.1).

The inositides constitute a diverse family of membrane and soluble cell components: phosphatidylinositol (PtdIns), phosphorylated derivatives of PtdIns known as the polyphosphoinositides (PPIns) and a variety of soluble inositol polyphosphates (IPs). Both of the latter groups are interesting as they play roles in the regulation of diverse cellular events. They have been intensively investigated over the past six decades and particularly since the early 1980s (Downes *et al*., 1982; Creva *et al*., 1983; Streb *et al*., 1983; Berridge, 1983). The common structural unit of all PPIn is phosphatidyl-1-D-*myo*-inositol (PtdIns) which is synthesized by the route depicted in Fig. 1.1.

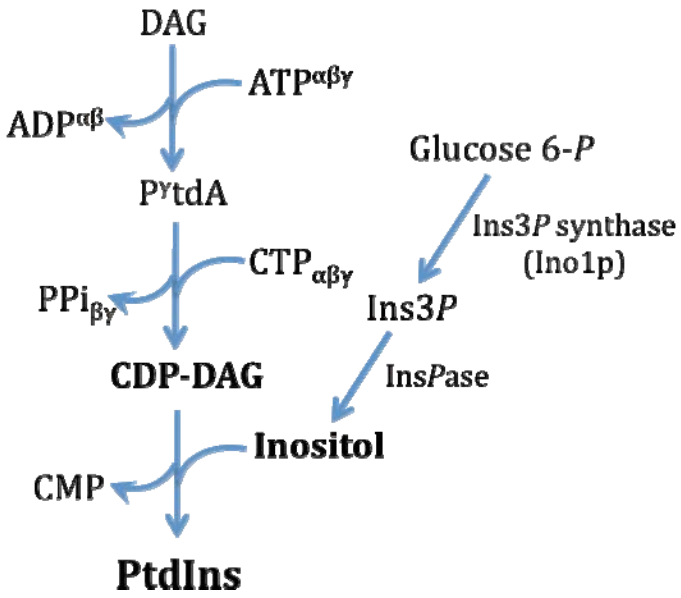


Figure 1.1: Route of synthesis of phosphatidylinositol

DAG, diacylglycerol; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PtdA, phosphatidic acid or phosphatidate; CTP, cytidine triphosphate; CDP, cytidine diphosphate; CMP, cytidine monophosphate; PtdIns, phosphatidylinositol

The history of research on PPIns is complex and first involved pioneering work by a number of chemists in the US. Studies on PPIn can be traced back to 1942 when Jordi Folch-Pi isolated "inositol phosphatide" as a natural component of bovine brain phospholipids in Boston (Folch-Pi and Woolley, 1942; Folch-Pi, 1942; Strahl and Thorner, 2007). More than ten years later, Mabel and Lowell Hokin observed that the ^{32}P labelling of 'RNA' rapidly increased in some tissue preparations in response to external stimuli of carbamylcholine or acetylcholine (with eserine), but then showed – using new lipid analysis techniques developed by Rex Dawson – that the extra ^{32}P was incorporated into PtdIns and phosphatidate rather than RNA (Hokin and Hokin, 1953; Dawson, 1954; Hokin and Hokin, 1955). This phenomenon then became known as 'the Hokin effect'.

Agranoff and Kennedy independently defined the route of PtdIns biosynthesis in the late 1950s (see Agranoff, 2009). Unfortunately, none of these early US-based research groups continued to investigate 'the Hokin effect'. Instead, it captured the imagination of two groups of researchers in the UK: one at the University of Birmingham, led by Tim Hawthorne and later by Bob Michell and the other at The Babraham Institute, headed by Rex Dawson and then Robin Irvine.

The field then moved slowly for a number of years, as researchers investigated the idea that hydrolysis of PtdIns by a phosphoinositide-specific phospholipase C (PLC), was the metabolic event that underlay the Hokin effect. The groups remained divided as to the function of this hydrolysis but a PLC activity had been detected by several groups. It was partially purified and characterized as a "PtdIns-specific PLC" by Dawson and his colleagues in 1959, and by Hawthorne's lab in 1961 and 1968 (Dawson, 1959; Kemp *et al*., 1961; Atherton and Hawthorne, 1968).

A crucial shift in thinking occurred during the 1970s, when Bob Michell and his colleagues published a body of evidence suggesting a direct link between cell surface receptor signalling and phospholipase C-catalyzed PtdIns hydrolysis. Michell proposed in his seminal review of 1975 (Michell, 1975) that PtdIns hydrolysis by PLC might be a step in receptor-controlled Ca^{2+} mobilization. At about the same time, in Babraham, Rex Dawson and Robin Irvine were using methods pioneered by Clinton Ballou's laboratory (Tomlinson and Ballou, 1961) to produce high quality preparations of PtdIns4*P* and PtdIns(4,5)P_2: two minor lipids that were initially thought to occur only in brain and other nervous tissues.

Judith Creba and Peter Downes in Michell's lab then provided another important observation: that receptor-activated PLCs might act on PtdIns(4,5)P_2 and PtdIns4P rather than PtdIns (Creba *et al.*, 1983). Peter Downes also identified an enzyme that could 5-dephosphorylate IP_3, the product of PtdIns(4,5)P_2 cleavage (Downes *et al.*, 1982).

Michell's ideas that PPIn hydrolysis might be linked to Ca^{2+}-signalling and that PtdIns(4,5)P_2 could be the true physiological substrate of PLCs, stimulated a three-way collaboration between the laboratories of Robin Irvine, Mike Berridge and Irene Shultz. They realized that if Michell was right, then IP_3 might be an intracellular second messenger. Using IP_3 made from Irvine's high quality PtdIns(4,5)P_2 and the Shultz lab's rat pancreatic acinar cell preparation, they provided the first direct evidence that IP_3 is indeed a Ca^{2+}-mobilizing agent (Streb *et al.*, 1983).

It had taken 30 years from the discovery of the 'the Hokin effect' to the elucidation of its function: the stimulated release of internal Ca^{2+}. It is now established that IP_3 binds to a family of intracellular calcium channels, known as 'IP_3 receptors', and that this interaction promotes the release of Ca^{2+} from intracellular stores, thus triggering Ca^{2+}-dependent responses such as secretion and smooth muscle contraction (Strahl and Thorner, 2007). The other product of PtdIns(4,5)P_2 breakdown, DAG, was also found to have signalling functions. Yasutomi Nishizuka's laboratory had already shown that it was a potent activator of a novel protein kinase (Takai *et al.*, 1979), and it is now generally accepted that DAG can activate certain isoforms of protein kinase C (PKC), ultimately leading to changes in gene expression (Nishizuka, 1984) (Fig. 1.2).

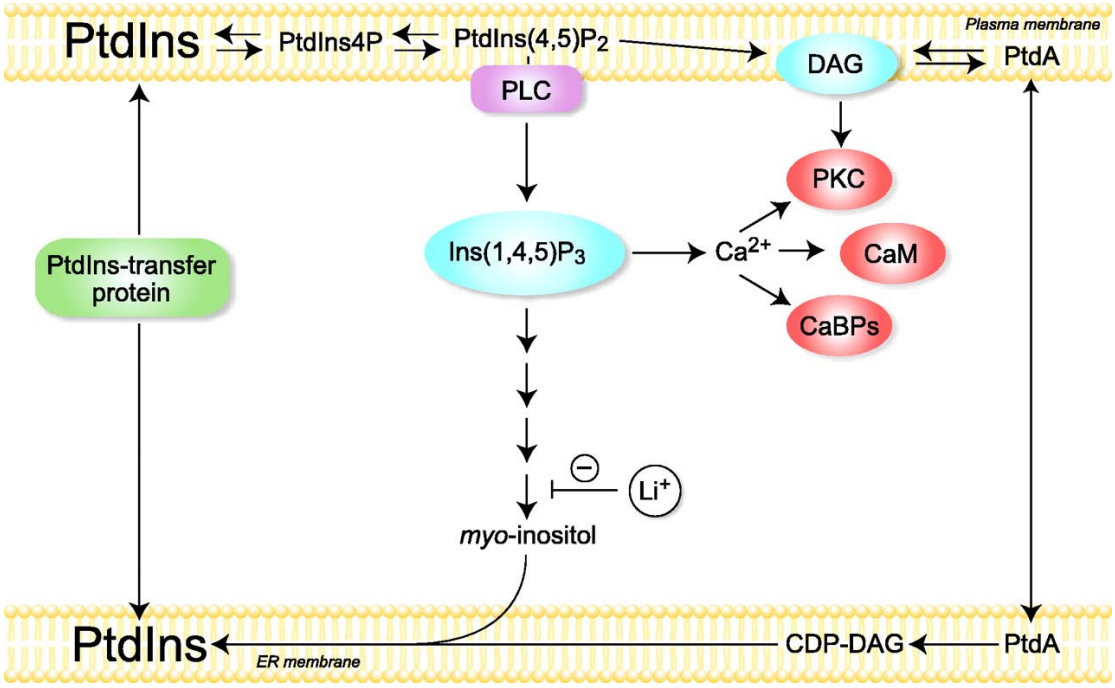


Figure 1.2: The updated version of the classical "Hokin effect" (from Balla, 2006)

Plasma membrane PtdIns(4,5)P_2 is hydrolyzed by PLC enzymes upon receptor stimulation to generate two second messengers: IP_3 and DAG. IP_3 can mobilize intracellular Ca^{2+} from intracellular stores whereas DAG activates PKC enzymes

Research on the 'Hokin effect' dominated work on the inositol lipids from the 1960s until the 1980s. Much of the focus has since shifted, following the discovery of the novel inositol lipids PtdIns3*P* and PtdIns(3,4,5)P_3 in 1988 (Whitman *et al.*, 1988; Traynor-Kaplan *et al.*, 1988), and of PtdIns5*P* and PtdIns(3,5)P_2 in 1997 (Rameh *et al.*, 1997; Dove *et al.*, 1997). Early experiments established that none of the lipids that include a 3-phosphate are substrates for PLCs, so it became clear that any regulatory actions of these lipids would have to involve hitherto unknown mechanisms.

1.2 PPIns: synthesis, degradation and interconversion

PPIns, the phosphorylated derivatives of phosphatidylinositol, include seven distinct species that are distinguished by the presence of phosphate groups on various

permutations of the D3-, 4- and 5-hydroxy positions on the *myo*-inositol headgroup (Fig. 1.3).

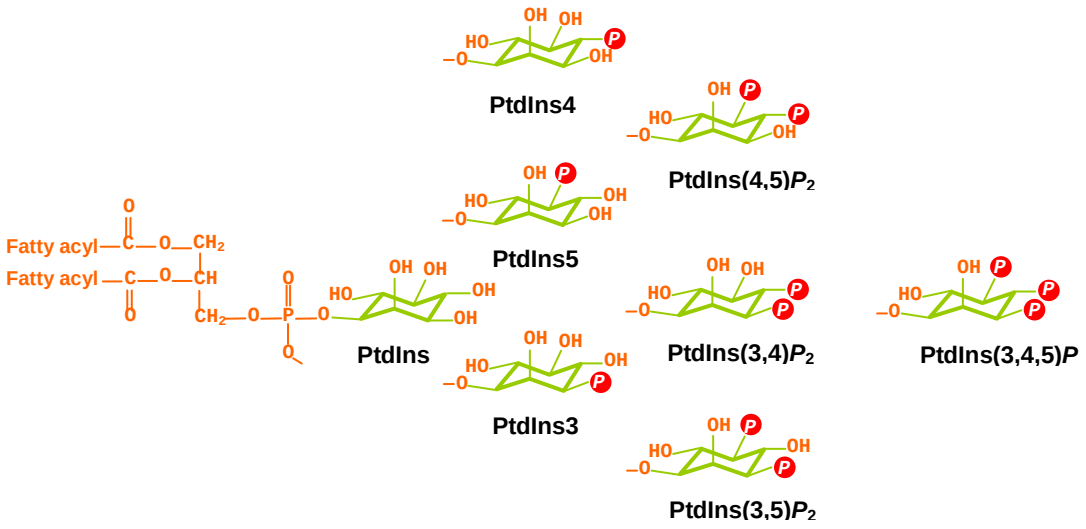


Figure 1.3: The eight 'simple' glycerophosphoinositol lipids of eukaryotic cells

The fatty acid profiles of *S. cerevisiae* are usually dominated by 1-palmitoleoyl, 2-oleoyl lipids

PPIns reside at the cytoplasmic faces of cellular and/or organellar membranes and exert their effects on cell physiology by regulating a suite of downstream effector proteins that gain or lose a function when complexed with their particular lipid(s). Hence, by binding to or recruiting their effectors, PPIns play key regulatory roles in a diverse range of cellular events. For example, PtdIns(3,4,5)P_3 plays important roles in regulating growth, proliferation, differentiation, survival, cancer progression and responses to extracellular environmental changes (Simonsen *et al.*, 2001; Vanhaesebroeck *et al.*, 2001; Katso *et al.*, 2001; Wishart and Dixon, 2002; Deane and Fruman, 2004). Other PPIns have specific biological functions in, for example, cytoskeletal organization, cytokinesis, membrane trafficking, endoplasmic reticulum ATP transport, and sub-cellular compartmentalization (Dove *et al.*, 1997; Maehama *et*

al., 2000; Dove *et al.*, 2002; Yin and Janmey, 2003; Roth, 2004; Raftopoulou *et al.*, 2004; Di Paolo and De Camilli, 2006; Coronas *et al*., 2007).

PPIns are interconverted by the action of a suite of exquisitely controlled lipid kinases and lipid phosphatases (Fig. 1.4). Usually, each of these enzymes will only phosphorylate or dephosphorylate a PPIn at a particular hydroxyl or phosphomonoester at the D-3-, 4-, or 5-position of the inositol ring. As alluded to earlier, PLC can only hydrolyze PPIns that lack a D3 phosphate. Given the importance of the various PPIns to multiple aspects of cell regulation, it was to be expected that these lipid kinases and phosphatases would be controlled by upstream regulators, with the activity state of some of these linked to cell surface receptors.

The control of levels of PPIns in a cell is critical for regulation of their physiological functions. For instance, the levels of PtdIns(3,4,5)P_3 regulate cell survival in mammalian cells via activation of PKB/Akt (Hawkins *et al.*, 2006). If too much PtdIns(3,4,5)P_3 is persistently present in a cell, it will not respond to pro-apoptotic stimuli such as Fas ligand. This can contribute to inappropriate cell survival, e.g. in cancer (Stephens *et al*., 2005; Engelman *et al*., 2006).

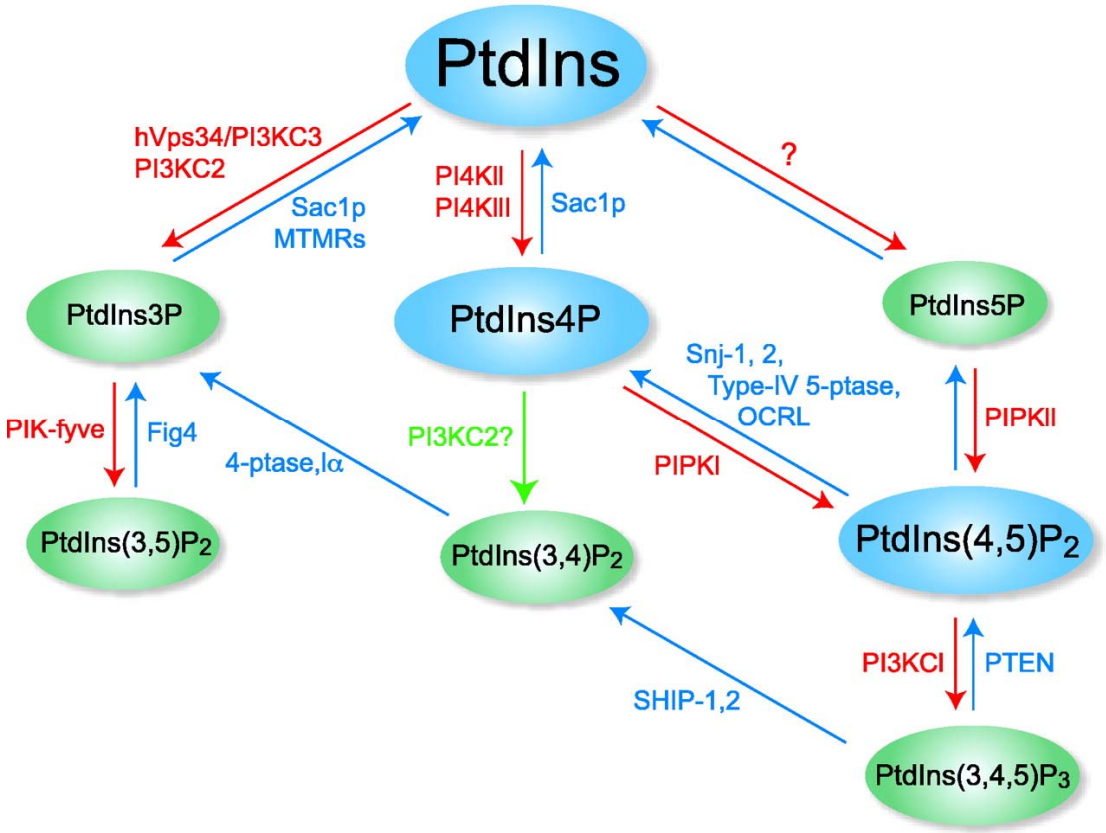


Figure 1.4: The network of interconversion between PPIn (from Balla, 2006)

PPIns are phosphorylated (red arrows) by PPIn kinases and are dephosphorylated (blue arrows) by PPIn phosphatases. Most (but not all) of the kinases are quite specific whereas the substrate specificity of most (but not all) phosphatases is less stringent *in vitro*

1.3 PPIns in budding yeast

The budding yeast *Saccharomyces cerevisiae* possesses the essential elements of most inositol lipid signalling pathways which, with the exception of the PtdIns(3,4,5)P_3 pathway, appear to be conserved throughout eukaryotic phylogeny (Michell, 2008). The functions of these lipids can therefore be more readily elucidated by a combination of biochemistry, genetics and cell biology in yeast. Four PPIns (PtdIns3*P*, PtdIns4*P*, PtdIns(3,5)P_2 and PtdIns(4,5)P_2) have been identified and investigated in *S. cerevisiae*, showing that they are involved in regulating numerous processes, including membrane trafficking, cytoskeletal rearrangements and responses

to extra-cellular environmental changes (see review of Strahl and Thorner, 2007).

All PPIn kinases that have been identified in yeast exhibit specificity for a single substrate (Fig. 1.5A). Three different enzymes, namely Lsb6p, Pik1p and Stt4p, can catalyse the phosphorylation of PtdIns to PtdIns4*P*, and each of the other reactions is catalysed by one kinase (see Section 1.4). In contrast to the PPIn kinases, the PPIn phosphatases of *S. cerevisiae* show a high degree of redundancy, as several of them can dephosphorylate a number of substrates with relatively little specificity (Fig. 1.5B and Section 1.5). Fig4p, Inp51p/Sjl1p and Inp54p are highly selective for a single substrate, whereas Inp52p/Sjl2p and Inp53p/Sjl3p can convert most yeast PPIns to PtdIns.

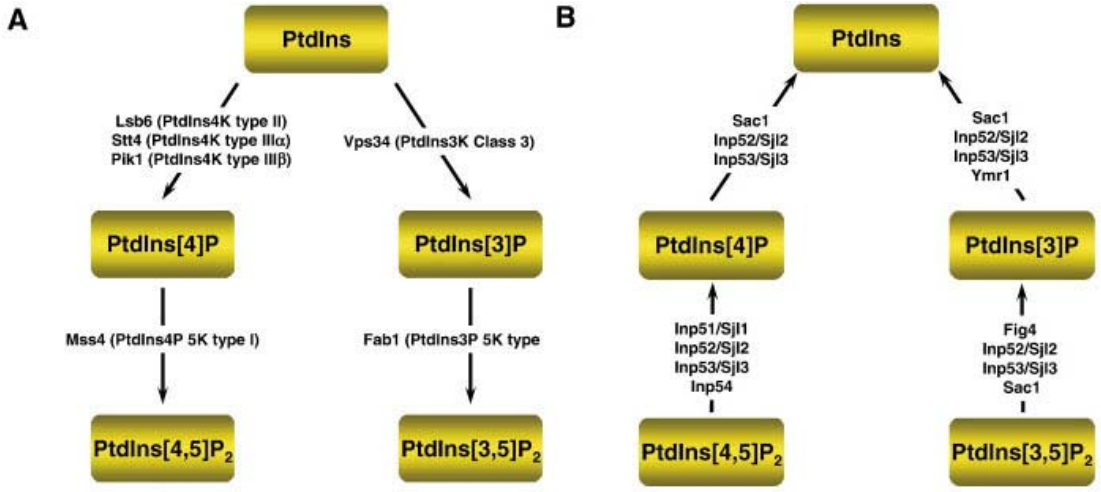


Figure 1.5: Interconversion of PPIns in yeast (from Strahl and Thorner, 2007)

A: PPIn kinase reactions in *S. cerevisiae*; B: PPIn phosphatase reactions *S. cerevisiae*

1.4 PPIn kinases (PIKs)

Most PPIn kinases are conserved throughout evolution, so understanding the properties of PPIn kinases in unicellular model organisms such as yeast can inform investigations in more complex mammalian cells, and vice-versa. I will focus primarily on yeast, digressing to compare the situation in higher organisms (Fig. 1.6).

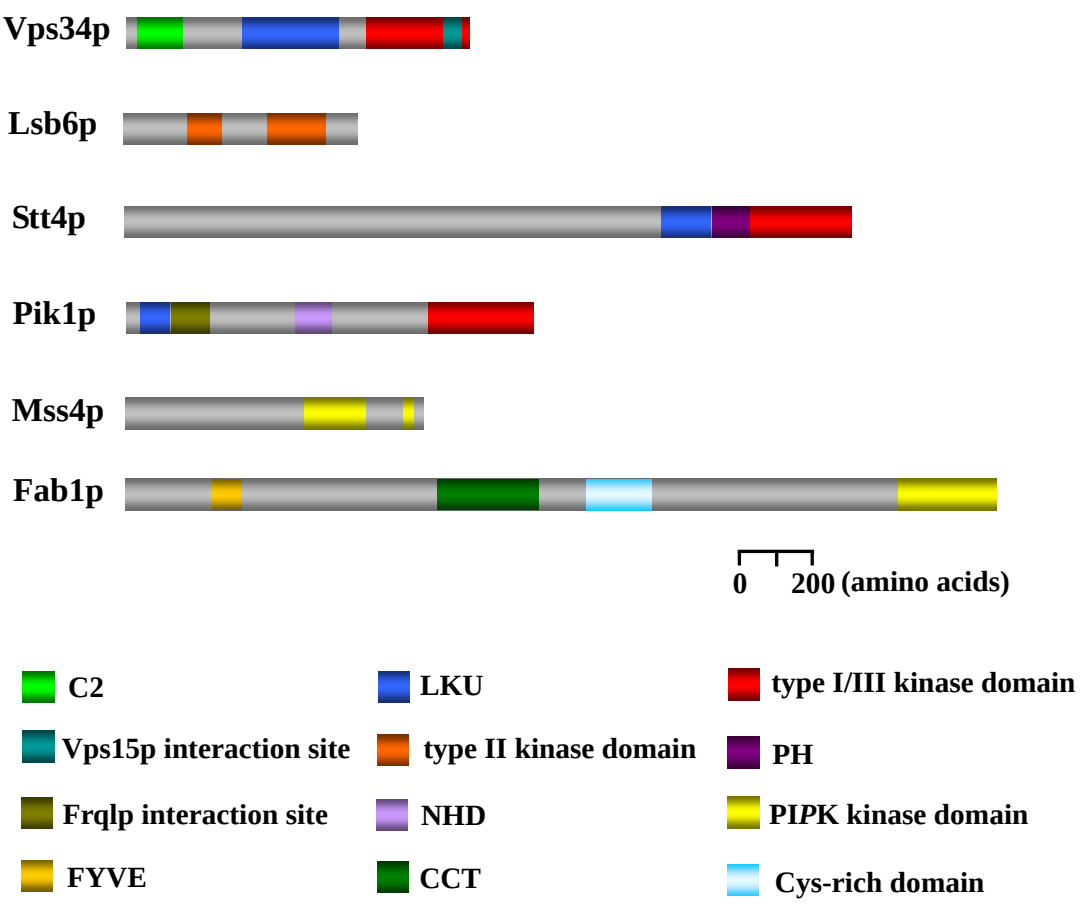


Figure 1.6: The schematic domain structures of six PPIn kinases in S. cerevisiae (adapted from Strahl and Thorner, 2007)

C2, C2 domain; LKU, lipid kinase unique domain; PH, pleckstrin homology domain; NHD, novel homology domain; FYVE, Fab1, YGL023, Vps27, and EEA1; CCT, chaperonin containing T-complex homology region

Inositol lipid kinases comprise three basic families: the Type I/III "PtdIns-kinases", the type II "PtdIns-kinases" that share no sequence homology with the Type I/III enzymes and the PtdIns*P* kinases. The PPIn kinases were originally classified into three classes: I, II and III (Fruman *et al.*, 1998) based upon the properties of impure enzymes: for example, such as K_M for ATP, sensitivity to inhibitors (e.g. adenosine), activation by detergents (e.g. Triton X-100), inhibition by wortmannin and inhibition by a monoclonal antibody to the Type II "PtdIns-kinases". Later it was found that these properties did separate these enzymes into meaningful groups. For example, the Type I and Type III 'PtdIns-kinases' share significant sequence homology, domain structure, and modes of regulation, and Type II 'PtdIns-kinases' have a disparate amino-acid sequence. Most of the inositol lipid kinases are specific, phosphorylating only one substrate lipid at one position of the inositol ring (Balla, 2006). In this context, they have alternatively – and more appropriately – been classified according to the reactions they catalyse: PtdIns 3-kinases (PI3Ks), PtdIns 4-kinases (PI4Ks) and three types of PtdIns*P* kinases (PIPKs) (Strahl and Thorner, 2007). I will mainly use this classification system.

1.4.1 PtdIns 3-kinases (PI3Ks)

Originally known as "Type I PtdIns-kinases", these kinases are all specific for the D3 position of the inositol ring. They have been reclassified based on their domain structures and their different substrate selectivities (Domin and Waterfield, 1997; Vanhaesebroeck *et al.*, 2001).

1.4.1.1 Class I PI3Ks (PI3KC1)

Class I PI3Ks are heterodimers comprising of a ~110-kilodalton catalytic subunit

(p110) and an adaptor/regulatory subunit. PI3KC1s can phosphorylate PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 *in vitro*, but PtdIns(4,5)P_2 is their preferred substrate *in vivo*. In cells they synthesise PtdIns(3,4,5)P_3, the most heavily phosphorylated PPIn (Fruman, *et al.*, 1998). PI3KC1s can further subdivided into class IA and class IB enzymes, which signal downstream of tyrosine kinases and heterotrimeric G protein-coupled receptors (GPCRs), respectively (Vanhaesebroeck *et al.*, 2001). A major signalling pathway downstream of PI3KC1s is through the sequential protein kinases PDK1 and Akt/PKB (Franke *et al.*, 1997; Alessi *et al*., 1997). These two kinases regulate a wide variety of cellular events including glucose metabolism, protein synthesis and cell division, and also a major anti-apoptotic pathway in animal cells (Balla, 2006).

The class IA PI3Ks are composed of a catalytic p110 subunit and a regulatory subunit. In mammalian cells, three Class IA p110 isoforms (p110α, β and δ) and at least seven regulatory subunits have been identified (Vanhaesebroeck *et al.*, 1997). Class IA PI3Ks are mainly cytosolic but can be recruited to the membrane by their regulatory subunit binding to phosphorylated tyrosine residues that are generated by activated tyrosine kinase receptors. The class IB PI3K includes a p110γ catalytic subunit and a p101 regulatory subunit. The p110γ/p101 heterodimers are activated, downstream of activated G-protein-coupled receptors, by βγ-complexes released from heterotrimeric G proteins. No PI3KC1s have been found in yeast or plants, consistent with the lack of PtdIns(3,4,5)P_3 detected in either group of organisms. This is likely to be because of the loss of these machineries from plants and yeast somewhere during the course of evolution since slime moulds still have PI3KC1s despite being thought to be evolutionary more distant from animals than fungi. This assertion is strengthened by

the finding that both plants and fungi have PTEN homologues (Mitra *et al.*, 2004).

1.4.1.2 Class II PI3Ks (PI3KC2)

Class II PI3Ks are larger than PI3KC1s, at ~170 kilodaltons. A C-terminal C2 domain is a defining feature, through which they interact with their lipid substrates *in vitro* in a Ca^{2+}-independent manner. Deletion of the C2 domain does not compromise their predominant membrane localisation (Arcaro *et al.*, 1998), which suggests that there must be other mechanism(s) targeting class II PI3Ks to membranes. The Class II PI3K-C2α isoform has been found in clathrin-coated pits at the plasma membrane and on the Golgi (Domin *et al.*, 2000), so it may play a role in membrane traffic (Gaidarov *et al.*, 2001). No class II PI3Ks are found in yeast or plants.

Like Class I PI3Ks, Class II PI3Ks can phosphorylate PtdIns, PtdIns4*P* or PtdIns(4,5)P_2) *in vitro*, with the order of preference PtdIns>PtdIns4*P*>PtdIns(4,5)P_2, but their substrate specificity *in vivo* remains uncertain. *In vitro*, the class II PI3K-C2α isoform showed an elevated lipid kinase activity towards phosphorylated PPIns upon binding clathrin (Gaidarov *et al.*, 2001), suggesting that the substrate preferences of class II PI3Ks *in vivo* may be modulated by its binding partners.

1.4.1.3 Class III PI3Ks (PI3KC3) and Vps34p

Class III PI3Ks, typified by the yeast protein Vps34p, are responsible for most PtdIns3*P* synthesis in cells. Both *in vitro* and *in vivo*, Class III PI3Ks only phosphorylate PtdIns to PtdIns3*P*, so they should be more properly called "PtdIns3Ks". The activity of the catalytic subunit of PI3KC3 is regulated by a Ser/Thr protein kinase: Vps15p in yeast and p150 in mammals. Vps15p and p150 are post-

translationally modified to contain an N-terminal myristoyl moiety that helps to tether the Vps34p/Vps15p complex to membranes. All eukaryotes seem to possess Vps34p and Vps15p homologues. Since the cellular PtdIns3*P* levels change little during extracellular stimulation, it seems that PI3KC3 enzymes play little or no role in signal transduction. Instead, they have more constitutive cellular functions in a number of membrane trafficking pathways which are nicely exemplified by considering their roles in yeast.

Vps34p was first identified as a protein required for the sorting of vacuolar enzymes from the Golgi to the vacuole in *S. cerevisiae* (Herman and Emr, 1990) and was then shown to be the sole PI3K responsible for PtdIns3*P* synthesis in budding yeast (Hiles *et al.*, 1992; Schu *et al.*, 1993). Vps34p is cytosolic but can be recruited to endosomal membranes by activated membrane-bound Vps15p (Stack *et al.*, 1993). Mutating the kinase domain of *vps15* resulted in a substantial decrease of PtdIns3*P* level and vacuolar protein sorting defects that phenocopy those of a *vps34* mutant (Stack *et al.*, 1993), indicating that this interaction requires the kinase activity of Vps15p.

A *vps34*^{ts} mutant also displays very acute and pronounced defects in protein sorting to the vacuole when grown at the restrictive temperature (Stack *et al.*, 1995). In parallel, Vps34p^{ts} loses lipid kinase activity when incubated at 37ºC and levels of PtdIns3*P* in the cell plummet upon temperature upshift, suggesting that Vps34p and/or PtdIns3*P* have a direct role in vesicular transport from the Golgi to the vacuole. The key to the role of Vps34p in TGN-to-Golgi trafficking is almost certainly that proteins of the Sortin Nexin (SNX) family (see Section 1.6.1) require PtdIns3*P* for membrane localisation. The SNXs Vps5p and Vps17p are required for recycling of Vps10p, a

protein needed for the transport of soluble vacuolar cargoes from the TGN to the vacuole. In the absence of active Vps34p, vacuolar proteins are secreted instead of being correctly targeted (Horazdovsky *et al*., 1997; Burda *et al*., 2002).

In budding yeast, the Vps34p/Vps15p heterodimer can form at least two distinct multi-subunit complexes that modulate different pathways (Kihara *et al.*, 2001). Both complexes share three proteins, Vps34p, Vps15p and Vps30p, but are distinguished by their fourth component, with Atg14p specific for Complex I and Vps38p diagnostic for Complex II (see Fig. 1.7). The N-terminal myristoyl moiety of Vps15p is necessary to direct Complex I to the pre-autophagosomal structure (PAS) and Complex II to the prevacuolar endosomal compartment (PVC). Atg14p is required for Complex I to interact with the PAS, but Vps38p is dispensable for the residency of Complex II on the PVC. Complex I regulates autophagy, a mechanism for degradation and subsequent recycling of a cell's own components/organelles that allows starving cells to weather periods of nutrient limitation. In unstarved cells, there is a related process termed the Cvt pathway (Cytoplasm-to-vacuole targeting) that functions constitutively even under nutrient-rich conditions and delivers a vacuolar protease (API) to the vacuole, in which Complex I also participates. The more abundant complex II regulates the classical CPY sorting pathway (Vps pathway) described earlier in which PtdIns3*P* mediates recycling of Vps10p.

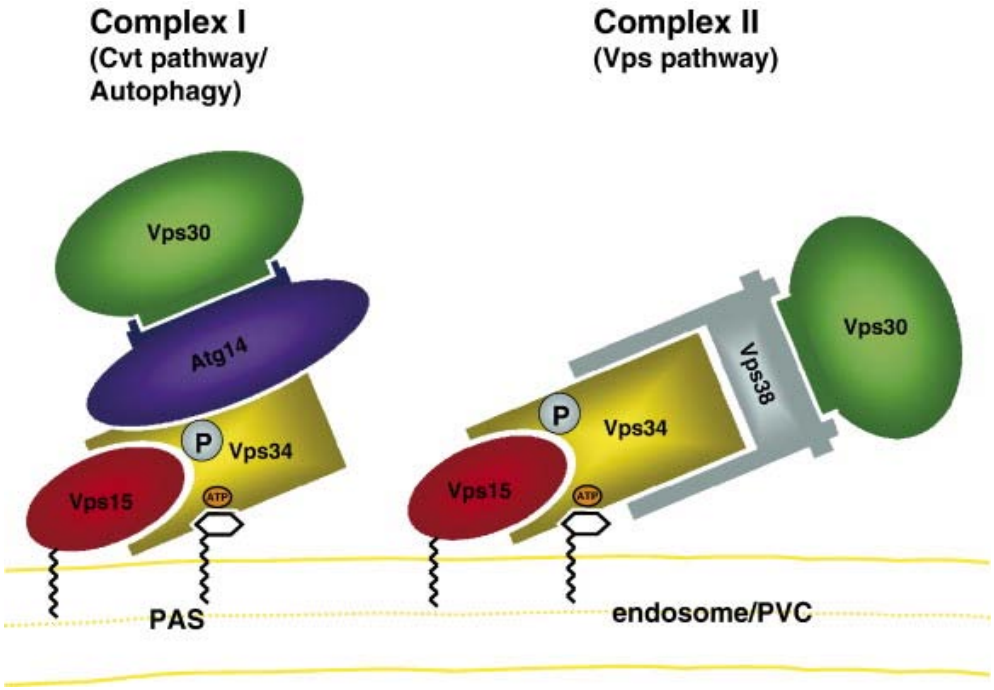


Figure 1.7: Two Vps34-containing PtdIns 3-kinase complexes regulating two distinct pathways (from Strahl and Thorner, 2007)

The yeast Vps34p forms two different multi-subunit complexes: Complex I and Complex II, which function in distinct biological processes: the autophagy/cytoplasm-to-vacuole transport (Cvt) and vacuolar protein sorting (Vps) pathways respectively

1.4.2 PtdIns 4-kinases (PI4Ks)

PI4Ks synthesise cellular PtdIns4*P* by phosphorylating the D-4 position of the inositol ring. The Type II and Type III PIKs discussed earlier are both PtdIns 4-kinases. Lsb6p is the yeast ortholog of Type II PI4Ks, and the two yeast PI4Ks (Stt4p and Pik1p) are homologous to mammalian Types IIIα and IIIβ, respectively.

1.4.2.1 Type II PI4Ks (PI4KIIs) and Lsb6p

The catalytic kinase domains of PI4KIIs exhibit very little sequence homology to PI4KIIIs or PI3Ks, and form a separate lipid kinase family (See Fig. 1.6 for Lsb6p). Lsb6p, the yeast PI4KII, is not essential for normal vegetative growth.

In mammals, PI4KIIs exist as two isoforms: PI4KIIα and PI4KIIβ (Barylko *et al.*, 2001; Minogue *et al.*, 2001). PI4KIIα is mainly found on Golgi membranes where it plays a role in membrane trafficking out of the Golgi (Wang *et al.*, 2003). PI4KIIβ is a cytosolic protein that is recruited to the plasma membrane and activated by Rac1 upon growth factor stimulation (Wei *et al.*, 2002). In mammals, PI4KIIs account for the majority of cellular PtdIns4*P* synthesis, but Lsb6p only produces a minor fraction of yeast PtdIns4*P* (< ~10%) (Audhya *et al.*, 2000). Like its mammalian PI4KIIs homologue, Lsb6p behaves like an integral membrane protein as a result of its C-terminal S-palmitoylation (Han *et al.*, 2002). This post-translational modification sets Lsb6p apart from the other yeast PIKs, which usually require accessory proteins for their membrane loading (Strahl and Thorner, 2007).

In addition to its PM localisation, Lsb6p is the only yeast PI4K found on the vacuole. If it is assumed that Lsb6p supplies the PtdIns4*P* substrate for the PtdIns4*P* 5-kinase Mss4p in the vacuole, it might partially account for the PtdIns(4,5)P_2 requirement for homotypic vacuole fusion (Mayer, *et al.*, 2000),

Lsb6p had been identified earlier in a two-hybrid screen as a binding partner of Las17p (Lsb6p is named Las17-binding). Las17p is the yeast ortholog of the human Wiskott-Aldrich Syndrome Protein (WASP), a promoter of actin filament polymerization (Madania *et al.*, 1999). Lsb6p also has a kinase activity-independent function in endosome motility that involves its interaction with Las17p. The average speed of endosome movement was dramatically impeded in *lsb6*Δ cells, but was normal in *lsb6*Δ expressing a catalytically-inactive form of Lsb6p (Chang *et al.*, 2005).

1.4.2.2 Type III PI4Ks (PI4KIIIs)

PI4KIIIs are larger than PI4KIIs, and their catalytic domains show sequence homology with PI3Ks. Mammals and yeast both express two PI4KIIIs: PI4KIIIα and PI4KIIIβ in mammals, and Stt4p and Pik1p in yeast (Gehrmann and Heilmeyer, 1998). In yeast, both enzymes are essential, suggesting distinct physiological roles. Stt4p principally functions at the plasma membrane to support regulation of the actin cytoskeleton and to maintain the integrity of the cell wall, whereas Pik1p has a major role in Golgi membrane trafficking, and the mammalian homologues fulfil generally similar functions.

1.4.2.2.1 PI4KIIIα and Stt4p

Mammalian PI4KIIIα, homologous to yeast Stt4p, was chiefly detected on the Endoplasmic reticulum (ER) but also contributes to the control of a PtdIns4*P* pool at the plasma membrane (Wong *et al.*, 1997). This apparent paradox has been partially resolved by assuming that the PI4KIIIα-dependent generation of PtdIns4*P* may occur at ER-PM contact sites where there is close apposition between the ER and the PM (D'Angelo *et al.*, 2008).

Genetically, Stt4p (Staurosporine- and temperature-sensitive-4) was first recognized when *stt4* mutants that were resistant to the protein-kinase inhibitor staurosporine were isolated (Yoshida *et al.*, 1994a). Stt4p, localised on the plasma membrane, produces the PtdIns4*P* that is further converted to PtdIns(4,5)P_2 by Mss4p, the sole yeast PtdIns4*P* 5-kinase (see Section 1.4.3.1). Their combined activity is necessary for activation of the Pkc1p-mediated MAP kinase cascade, a major cell wall integrity pathway (Audhya and Emr, 2002): both *stt4* and *mss4* mutants show synthetic defects

when combined with other mutants that impair the Pkc1p pathway. In addition, most PtdIns(4,5)P_2 generation on the PM requires Stt4p rather than Pik1p (Levine and Munro, 2002; Perera *et al.*, 2004).

It appears that the isolated PH-like domain of Stt4p alone is not sufficient for plasma membrane targeting (Levine and Munro, 1998), and another protein Sfk1p (Suppressor of four kinase), a multicopy suppressor of the *stt4-4*^{ts} allele, seems to be required for Stt4p to associate with the PM membrane (Audhya and Emr, 2002). In addition to its function, with Mss4p, in producing PtdIns(4,5)P_2, some studies suggest that Stt4p-synthesised PtdIns4*P* may have independent roles in, for example, the intracellular aminophospholipid transport needed for phosphatidylethanolamine synthesis (Trotter *et al.*, 1998), regulation of the actin cytoskeleton (Audhya *et al.*, 2000) and the polarized growth of budding cells (Wild *et al.*, 2004).

1.4.2.2.2 PI4KIIIβ and Pik1p

Mammalian PI4KIIIβ is a ~110KDa protein and its yeast homologue is designated Pik1p. It largely resides on the Golgi complex but some can be found in the nucleus (Wong *et al.*, 1997; De Graaf *et al.*, 2002). Maximal kinase activity of PI4KIIIβ requires two upstream activators, the GTP-bound form of Arf1p on the Golgi membrane (Godi *et al.*, 1999), and neuronal calcium sensor-1 (NCS-1). Consistent with its location, the main roles of PI4KIIIβ are to support the TGN-to-PM transport of newly synthesized protein and to maintain the structural architecture of the Golgi complex (Godi *et al.*, 1999; D'Angelo *et al.*, 2008).

Pik1p was first characterized biochemically in yeast cell extracts, and the

corresponding gene was cloned from a genomic library by using oligonucleotides deduced from the amino-acid sequence of the purified enzyme (Flanagan and Thorner, 1992; Flanagan *et al.*, 1993). Pik1p is an essential 125-KDa enzyme and behaves as a soluble protein. It is targeted to the TGN by binding to Frq1p, an essential 22-KDa EF-hand-type Ca^{2+}-binding protein with a N-terminal myristoyl modification that is homologous to mammalian NCS-1 (Hendricks *et al.*, 1999; Weisz *et al.*, 2000). Both Ca^{2+}-binding and N-terminal myristoylation are indispensable for the membrane association of Frq1p and for the targeting of Pik1p to the Golgi complex. Pik1p also displays genetic interactions with the small GTPase Arf1p, with the loss of Arf1p leading to a striking decrease of PtdIns4*P* levels (Audhya *et al.*, 2000).

In Golgi, the main function of Pik1p, through its product PtdIns4*P*, is to regulate the transit of secretory proteins from the Golgi to plasma membrane via the exocyst complex. This observation was first suggested by a study in the *sec14-3*^{ts} mutant, which manifested a substantially reduced PtdIns4*P* level and a severe defect in protein secretion at the non-permissive temperature (Hama *et al.*, 1999). Sec14p has PtdIns-Transfer Protein (PITP) activity, is localised to the Golgi and seems to be responsible for PtdIns transport from ER to the Golgi complex (Bankaitis *et al.*, 1990), where it can be phosphorylated to PtdIns4*P* by Pik1p. The link between PtdIns4*P* and the secretory pathway was further strengthened when it was found that over-expression of Pik1p – but not of Stt4p or Vps34p – or functional loss of the PPIn phosphatase Sac1p could reverse the growth and exocytosis defects in a *sec14-3*^{ts} mutant. Both rescue methods partially restored cellular PtdIns4*P* levels.

Pik1p has also been found in the nucleus (Garcia-Bustos *et al.*, 1994) and undergoes

nucleocytoplasmic shuttling (Strahl *et al.*, 2005). Although the nuclear function of Pik1p remains elusive, there are some hints that it may be involved in cytokinesis and in the production of nuclear PPIn and IPs (Walch-Solimena and Novick, 1999; Sciorra *et al.*, 2005).

Yeast requires active Pik1p to be present both at the Golgi and in the nucleus for viability. This was shown by the use of two catalytic active Pik1p variants, Pik1p-CCAAX (which is restricted to the Golgi) and Pik1p$^{\Delta 10\sim 192}$ (which is confined within the nucleus). Neither restored viability to *pik1*Δ cells, but co-expression of both did (Strahl *et al.*, 2005).

1.4.3 PtdIns*P* kinases (PI*P*Ks)

PI*P*Ks, initially purified as PtdIns4*P* 5-kinases, were classified as Type I and Type II based on sequence relationships and sensitivities to phosphatidic acid (PA) (Moritz *et al.*, 1992; Boronenkov and Anderson, 1995). However, it was later realized that Type II PI*P*Ks phosphorylate the D-4 position of the inositol headgroup, and so convert PtdIns5*P* to PtdIns(4,5)P_2 *in vivo* – and also PtdIns3*P* to PtdIns(3,4)P_2 *in vitro* (Rameh *et al.*, 1997; Lecompte *et al.*, 2008) – so they should be named PI*P*4Ks. And then the putative yeast PI*P*K Fab1p was identified as a PtdIns3*P* 5-kinase (Cooke *et al.*, 1998; Gary *et al.*, 1998), followed by its mammalian orthologue, PIKfyve (Sbrissa *et al.*, 1999). Both were classified as prototypic members of the type III PI*P*K family, responsible for conversion of PtdIns3*P* to PtdIns(3,5)P_2 *in vivo*. In summary, therefore, there are three types of mammalian PI*P*Ks: Type I are PtdIns4*P* 5-kinases, with the yeast homologue Mss4p; Type II are PtdIns5*P*/PtdIns3*P* 4-kinases, with no known yeast or plant examples; and Type III are PtdIns3*P* 5-kinases.

1.4.3.1 Type I PI*P*5K and Mss4p

The three mammalian isoforms of Type I PI*P*5K (α, β and γ) are encoded by the same gene as a result of alternative splicing (Ishihara *et al.*, 1998). The PI*P*5Ks are found at the PM and in the nucleus (Fruman *et al.*, 1998), and their kinase activity can increase ~10 fold upon stimulation with phosphatidic acid (Ishihara *et al.*, 1998). Small G proteins of the Arf family are also activators of PI*P*5Ks and so influence the actin cytoskeleton at the PM (Roth, 2004). All mammalian PI*P*5Ks are stimulated by PtdIns to undergo auto-phosphorylation *in vitro*, and this auto-phosphorylation suppresses their PI*P*5K kinase activity (Itoh *et al.*, 2000).

In budding yeast, active Mss4p is essential for viability. It was first defined as a multicopy suppressor of the temperature-sensitive growth of the *stt4-1*^{ts} mutant (MSS4) (Yoshida *et al.*, 1994b), consistent with one of its main functions being to produce PtdIns(4,5)P_2 from the PtdIns4*P* synthesized by Stt4p. PtdIns(4,5)P_2 is very important for numerous cellular functions, including endocytosis, cytoskeletal re-organization, polarized cell growth, secretory vesicle fusion with the PM and sphingolipid turnover (see review of Strahl and Thorner, 2007). All of these functions of Mss4p require its association with the PM. This is partially regulated by the phosphorylation of Mss4p by casein kinase I (CK1) (a plasma membrane-bound activity encoded by the *YCK1* and *YCK2* genes): newly synthesized Mss4p does not tether to the PM in cells lacking functional CK1 (Robinson *et al.*, 1993). A minor fraction of Mss4p was also found in the nucleus (Audhya and Emr, 2003). Given the simultaneous presence of Pik1p in the nucleus, it is reasonable to assume that Pik1p and Mss4p are responsible for the supply of a pool of PtdIns(4,5)P_2 in the nucleus. In this respect, it is interesting that Plc1p, the only phospholipase C in budding yeast,

also undergoes nucleo-cytoplasmic shuttling (Huynh, 1998). Plc1p can hydrolyse PtdIns(4,5)P_2 to produce IP_3 and thence a variety of inositol polyphosphates, and these are becoming a focus of interest because of their regulatory roles in various nuclear activities (York, 2006). One of the main functions of Pik1p/Mss4p-generated PtdIns(4,5)P_2 in the nucleus may therefore be to provide the substrate of Plc1p.

1.4.3.2 Type III PI*P*5Ks and Fab1p

Mammalian cells express a single Type III PI*P*5K, PIKfyve, which synthesises PtdIns(3,5)P_2 from PtdIns3*P* *in vivo*. It can also convert PtdIns to PtdIns5*P* *in vitro* (Sbrissa *et al*., 2000). PIKfyve is localised on the endosome by interacting with its PtdIns3*P* substrate via its FYVE domain, a diagnostic domain for the type III PI*P*5K (Sbrissa *et al*., 2002). PIKfyve has intrinsic protein kinase activity but the physiological function of this remains unknown (Sbrissa *et al*., 2000). The characterized functions of PIKfyve so far are related to the lysosome and late endosome: the orthologs of the yeast vacuole. Fab1p, the yeast PtdIns3*P* 5-kinase, was a major focus at the beginning of the current project, and it will be introduced in more detail later (see Section 1.9).

1.5 PPIn phosphatases

Inositol lipid phosphatases have been less well characterized than lipid kinases. However, several human genetic diseases have been traced to mutations in PPIn phosphatase genes (Wishart and Dixon, 2002; Pendaries *et al.*, 2003**;** Decker and Saltiel, 2005; Sleeman *et al.*, 2005; Hughes *et al.*, 2000a; Srivastava *et al.*, 2005), and the extra attention this has brought has accelerated progress in their identification and characterization (see Fig. 1.8). In addition, the virulence factors injected into cells by

some bacterial pathogens are sometimes inositol lipid phosphatases. The PPIn phosphatases can be divided into four families based on their catalytic site sequence homologies and substrate specificities: Type II 5-phosphatases, 3-phosphatases, 4-phosphatases and Sac domain phosphatases.

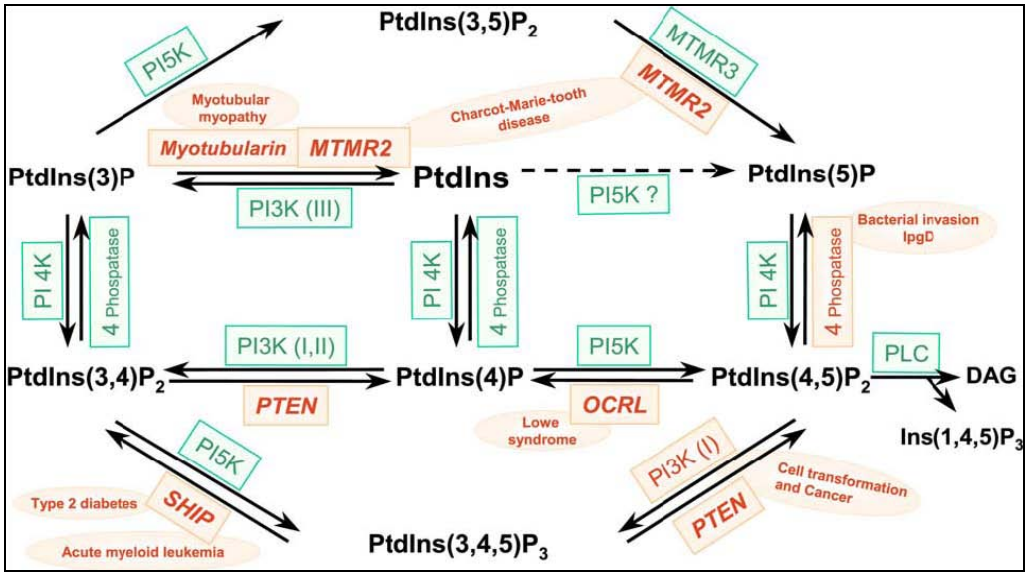


Figure 1.8: PPIns metabolism and its implication in human diseases (from Pendaries et al., 2003)

PTEN, phosphatase and tensin homologue deleted on chromosome ten; *MTMR*, myotubularin related protein; *SHIP*, Src homology 2-containing inositol-5-phosphatase; *OCRL*, phosphatase implicated in the oculocerebrorenal syndrome of Lowe; *IpgD*, entry-mediating invasion of *S. flexneri*

1.5.1 Type II PPIn 5-phosphatases

Type II 5-phosphatases share a highly conserved catalytic domain with type I phosphatases (type I only hydrolyze the water-soluble substrates IP_3 and IP_4), but contain an additional "Type II domain" located N-terminal to the catalytic domain (Majerus *et al.*, 1999). The large Type II 5-phosphatase family can be further divided into five subgroups based on their domain organisation (Fig. 1.9).

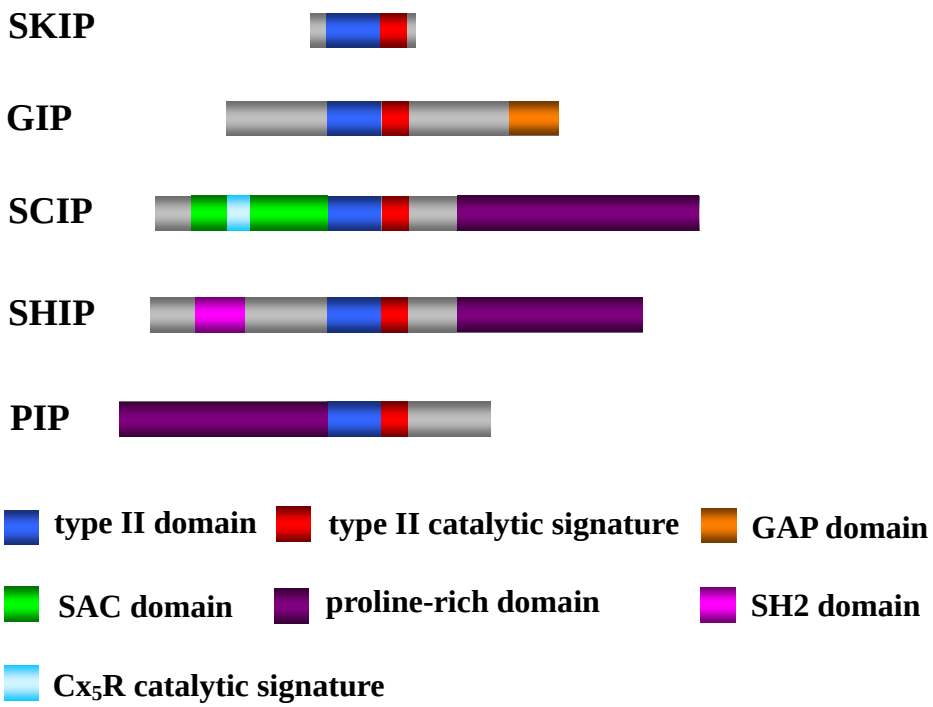


Figure 1.9: Modular structure of the Type II 5-phosphatase subtypes (adapted from Vanhaesebroeck et al., 2001)

The simplest are enzymes of the *SKIP* family (Skeletal Muscle and kidney-Enriched inositol 5-phosphatase). These largely comprise the canonical catalytic domain and Type II domain and are abundantly expressed in skeletal muscle and kidney. PtdIns(4,5)P_2 appears to be their preferred substrate (Ijuin *et al.*, 2000).

The second subgroup, the *GIPs* (GAP domain-Containing inositol 5-phosphatase), possess a C-terminal partial GAP (GTPase-activating protein) domain: these are OCRL and 5-phosphatase II. They can dephosphorylate PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3, and the corresponding inositol polyphosphates (Jefferson and Majerus, 1995; Jackson *et al.*, 1995; Suchy *et al.*, 1995).

The SCIPs (SAC domain-containing inositol 5-phosphatases) have an additional

catalytic SAC domain which confers dual 3-phosphatase and 4-phosphatase activities upon them (Guo *et al.*, 1999). In principle, therefore, SCIPs could hydrolyze most PPIns all the way to PtdIns (Hughes *et al.*, 2000b; Cremona *et al.*, 1999). There are three putative *S. cerevisiae* SCIPs (Inp51p, Inp52p, Inp53p) and at least two mammalian enzymes: Synaptojanin I (Synapto I) and Synaptojanin II (Synapto II) (Hughes *et al.*, 2000a) (Fig. 1.10). Except for Inp51p because its SAC domain is inactive due to mutations in the putative catalytic motif (Guo *et al.*, 1999), the other four enzymes can all hydrolyze PtdIns(4,5)P_2 to PtdIns. Synapto I is located in nerve terminals and associated with membranes coated with clathrin, and Synapto II seems to be present in all tissues. Both of these 5-phosphatase enzymes have important roles in regulated exocytosis (Bauerfeind *et al.*, 1997; Ramjaun *et al.*, 1999; Nemoto and De Camilli, 1999)

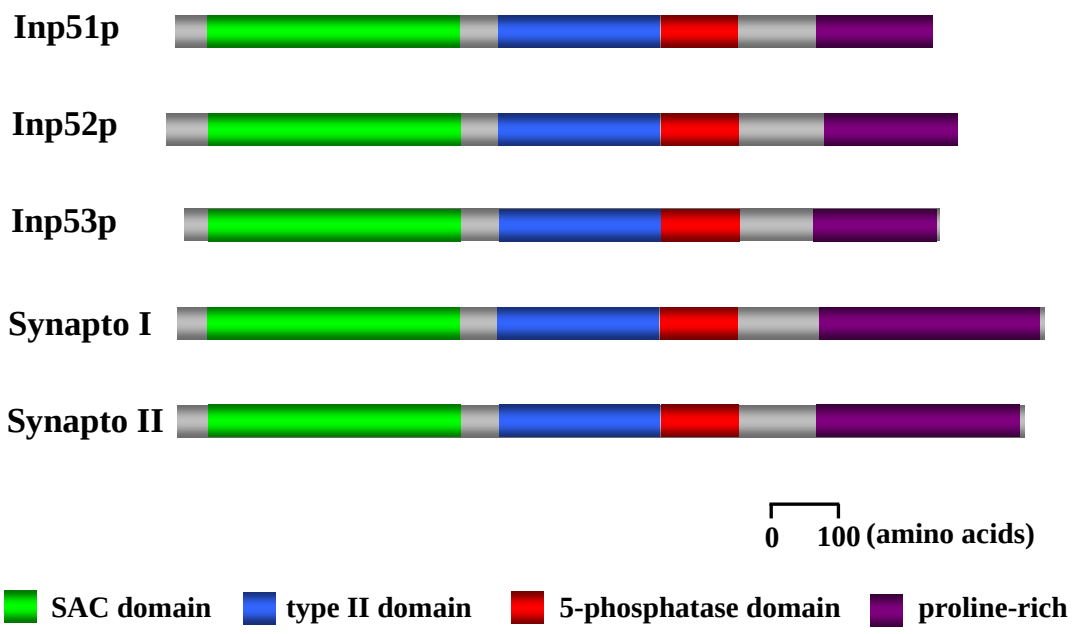


Figure 1.10: Modular structure of SCIP family PPIn phosphatases (adapted from Hughes et al., 2000a)

Enzymes of the *SHIP* (SH2 Domain-containing Inositol 5-phosphatases) subgroup include an N-terminal SH2 domain and a C-terminal proline-rich domain (Krystal *et al.*, 1999; Rohrschneider *et al.*, 2000). PtdIns(3,4,5)P_3 appears to be the substrate of *SHIP in vivo*, and the 5-phosphatase function of *SHIP* complements the 3-phosphatase activity of PTEN on PtdIns(3,4,5)P_3 (Taylor *et al.*, 2000b). PTEN appears to keep basal levels of PtdIns(3,4,5)P_3 very low, and *SHIP* is thought to be responsible for the degradation of receptor-stimulated PtdIns(3,4,5)P_3. The final subgroup, enzymes of the PIP (Proline-rich domain-containing Inositol 5-phosphatases) subgroup of the type II 5-phosphatases, has an N-terminal proline-rich domain.

1.5.2. PPIn 3-phosphatases

The PPIn 3-phosphatases, an expanding inositol lipid phosphatase family, includes PTEN, Myotubularin (MTM1) and the myotubularin-related proteins (MTMR) of mammals and other organisms. The PTEN gene was originally identified as a candidate tumour suppressor gene and was regarded as belonging to the family of dual specificity protein tyrosine phosphatases (PTPs) (Steck *et al.*, 1997; Myers *et al.*, 1997). However, it was proved later that PTEN utilizes PtdIns(3,4,5)P_3 as its physiological substrate and so functions as a negative regulator of the downstream signalling pathways mediated by PtdIns(3,4,5)P_3 (Maehama and Dixon, 1999). PTEN is inactivated in up to 50% of human tumours (Li *et al.*, 1997; Liaw *et al.*, 1997; Engelman *et al.*, 2006) indicating that its disruption and the concomitant rise in PtdIns(3,4,5)P_3 levels may be the important contributors to carcinogenesis.

MTM1 was identified as the defective gene in X-linked myotubular myopathy, a human genetic disease characterized by severe muscle degeneration (Sarnat, 1990). In

addition, thirteen human genes have been identified that encode MTMRs (Fig.1.11) (Laporte *et al*., 2003). MTMR2 inactivation is the cause of another human genetic disease, Charcot-Marie Tooth syndrome, a neuropathy characterized by improper nerve formation (Bolino *et al.*, 2000).

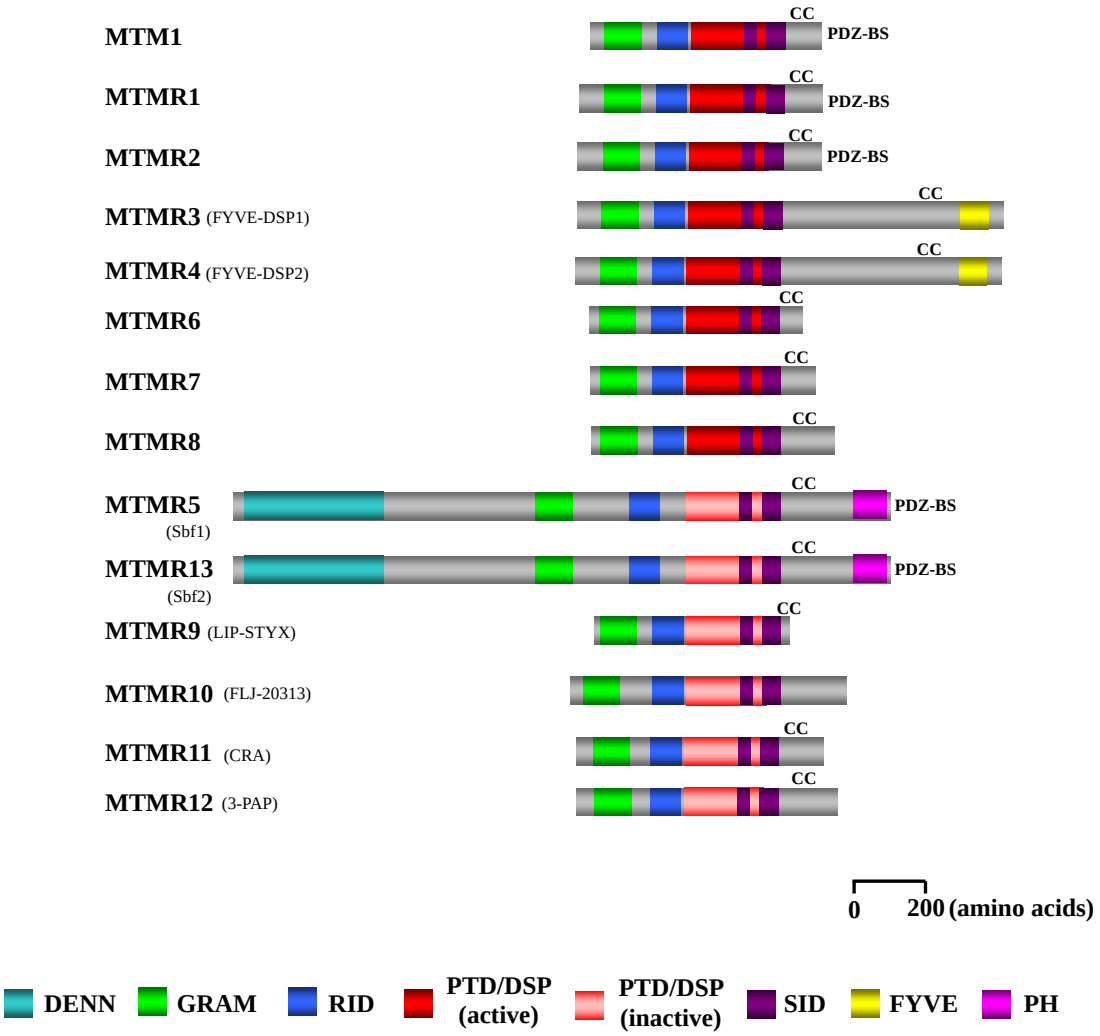


Figure 1.11: **scaled representation of the protein domains of human myotubularins *(adapted from Laporte et al., 2003)***

DENN, differentially expressed in neoplastic versus normal cells; *GRAM*, Glucosyltransferase, Rab-like GTPase Activator and Myotubularins; *RID*, Rac-Induced recruitment Domain; *PTP/DSP*, tyrosine/dual-specificity phosphatase; *SID*, SET-interacting domain; *PDZ*, PSD-95/Dlg/ZO-1

Several of the myotubularin family of enzymes can dephosphorylate PtdIns3*P* and/or PtdIns(3,5)P_2 but not other PPIns that contain a 3-phosphate, and some contain a PtdIns3*P*-binding FYVE domain, so it is likely that they are involved in regulating membrane trafficking. Ymr1p (YJR110w) is the yeast myotubularin homologue, and its catalytic activity had not been intensively investigated when my work began although it may attack PtdIns3*P* in yeast (Parrish *et al.*, 2004). Our *in vitro* colorimetric phosphatase assay showed that Ymr1p can utilize PtdIns3*P* and PtdIns(3,5)P_2 but not PtdIns4*P* or PtdIns(4,5)P_2 as substrates (see Section 3.2.5 for more detail).

1.5.3. PPIn 4-phosphatases

Knowledge about PPIn 4-phosphatases is currently less complete. The two lipid 4-phosphatases that have been cloned appear to prefer PtdIns(3,4)P_2 as substrate (Munday *et al.*, 1999; Norris and Majerus, 1994). There are no identified yeast homologues, which is not surprising since yeast does not produce much PtdIns(3,4)P_2.

1.5.4 Sac domain phosphatases

The Sac domain contains approximately 400 residues and is composed of seven highly conserved motifs that seem necessary for their phosphatase activity. The sequence RXNCLDCLDRTN within the sixth motif may well represent the catalytic core (Hughes *et al.*, 2000a). Currently, the Sac domain phosphatase family includes the mammalian proteins KIAA0274, KIAA0966 and KIAA0851 and the yeast enzymes Sac1p and Fig4p (Fig. 1.12).

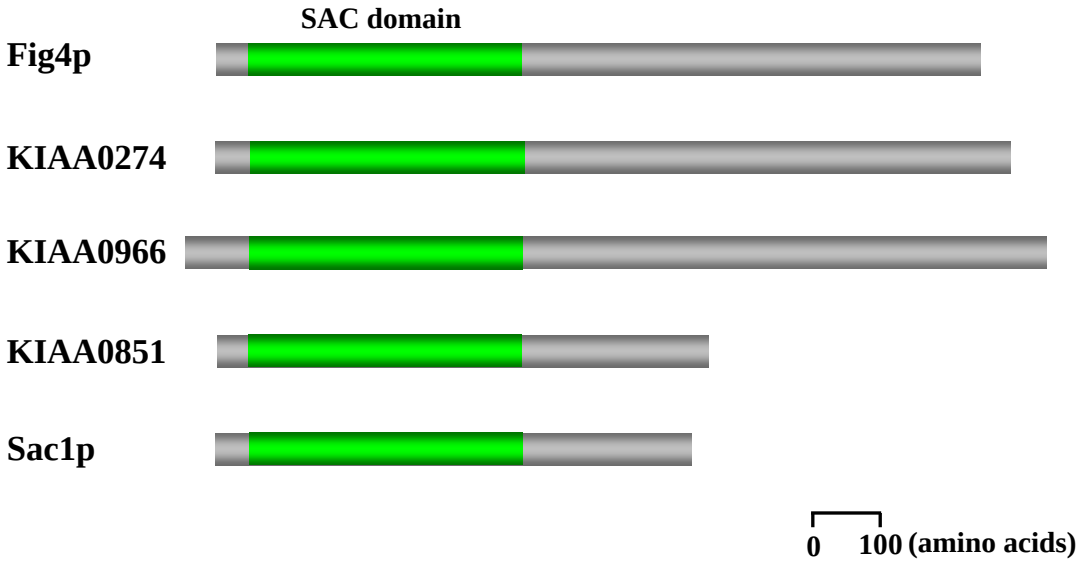


Figure 1.12: Modular structures of Sac domain phosphatase (adapted from Hughes et al., 2000a).

Despite the identification of so many PPIn phosphatases, many of their physiological functions and the details of their regulation are still less than clear. Indeed, there is not even a general consensus as to the individual functions of each of these enzymes. For example, some argue that the myotubularins act as effectors of inositol lipids and that they also happen to include an active site that switches off the signals (Schaletzky *et al*., 2003). One part of this thesis considers brief studies of a number of the enzymes involved in the metabolism of PtdIns(3,5)P_2 in yeast in order to characterize these proteins enzymatically (see Chapter 3). My preliminary data suggests that yeast myotubularin-like Ymr1p can convert PtdIns(3,5)P_2 to PtdIns5*P*. If this is true, then Ymr1p can be regarded as the off switch for the Fab1p pathway.

1.6 General introduction of individual PPIn

1.6.1 PtdIns3*P*, a key component in endosomal membrane trafficking

PtdIns3*P* was discovered by Lew Cantley and his colleagues in 1988, as the product of a PtdIns kinase that was specifically activated in cells transformed by tyrosine kinases such as Src or by the Middle T antigen of polyoma virus. Unexpectedly, this kinase produced the novel inositol phospholipid PtdIns3*P* when given PtdIns as a substrate (Whitman *et al,* 1988). It was later realized that PtdIns3*P* is constitutively generated from PtdIns by the class III PtdIns 3-kinase/Vps34p in mammalian cells. It makes up approximately 1.8% of the total PPIns in yeast (Hughes *et al*., 2000a).

PtdIns3*P* is highly enriched in early endosomal membranes and the internal vesicles of the multivesicular body (MVB), and is also found on vacuolar and endosomal membranes in yeast (Gillooly *et al*., 2000). Numerous lines of evidence have shown that PtdIns3*P* plays a key role in regulating the membrane trafficking within the endosomal compartment (Corvera, 2001), and in Golgi-to-lysosome/vacuole sorting (Odorizzi *et al*., 2000). Specific depletion of PtdIns3*P* in endosomes results in an aberrant morphology of these organelles (Lemmon, 2003).

Several protein effectors of PtdIns3*P* have been identified, most of which possess either a FYVE or Phox (PX) domain that binds PtdIns3*P*. The crystal structures of FYVE and PX domains have revealed how they can selectively recognize PtdIns3*P* in membranes (Bravo *et al*., 2001; Dumas *et al*., 2001, Misra *et al*., 2001) (Fig. 1.13).

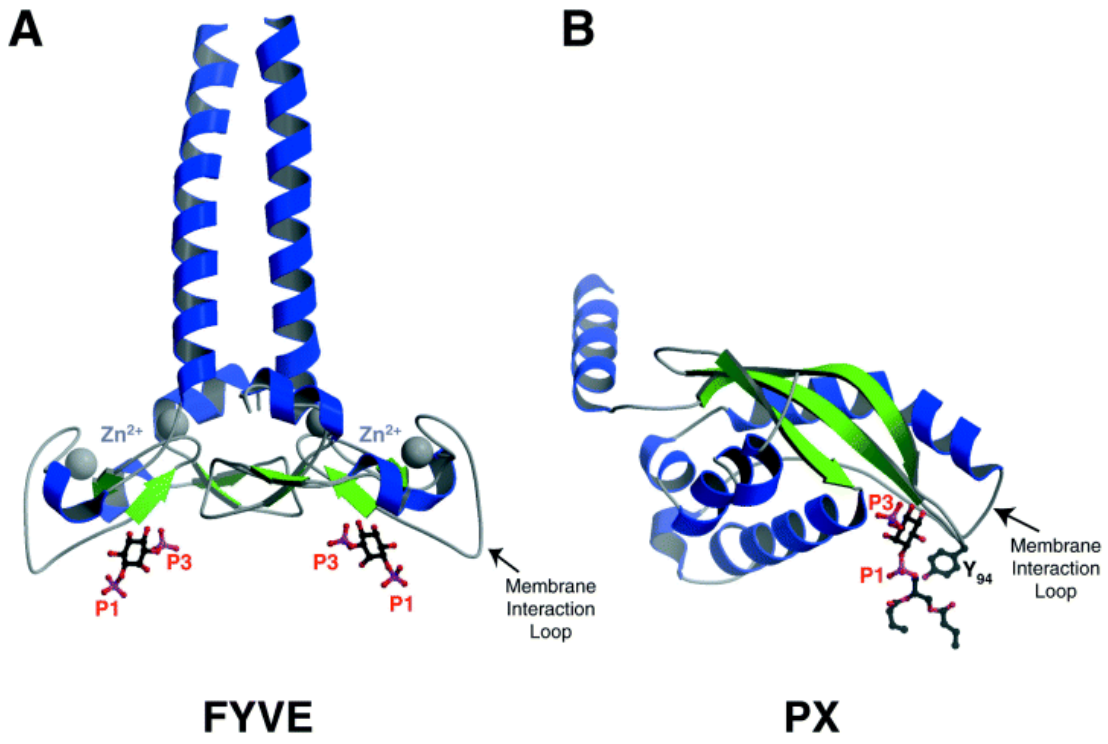


Figure 1.13: Structures of: (A) the EEA1 FYVE domain dimer bound to Ins(1,3)P_2; and (B) the p40^{phox} PX domain bound to dibutanoyl-PtdIns3P (from Lemmon, 2003)

The FYVE domain comprises a ~60-70 amino acid sequence and was named for the first four proteins in which it was found: Fab1, YOTB, Vac1p and EEA1 (Stenmark *et al.*, 2002). At least 38 mammalian FYVE domain-containing proteins have been identified so far, most of which have functional roles in endocytic membrane trafficking (Corvera, 2001). EEA1 (Early Endosome Autoantigen-1) and Hrs are two of the best-understood FYVE domain proteins. EEA1 is targeted to the early endosomal membrane by binding to PtdIns3*P* via its FYVE domain, but this membrane association also requires the GTPase Rab5 (Stenmark *et al.*, 1996). EEA1 functions as a Rab5 effector to promote vesicle fusion in concert with SNARE proteins (Lawe *et al.*, 2000). Hrs is a mammalian homologue of yeast Vps27p protein

and interacts with clathrin as well as the ubiquitin-interacting molecules STAM1 and STAM2. The resulting Hrs-clathrin-STAM complex then recruits the ESCRT-I complex (Endosomal Sorting Complex Required for Transport-I) to the endosomal membrane, facilitating cargo concentration and MVB vesicle formation (Sasaki *et al.*, 2007). Consistent with their distinct functional roles in the endosomal sorting system, EEA1 and Hrs are located on different microdomains of the limiting membrane of endosomes (Gillooly *et al*., 2000). Clearly, PtdIns3*P* is not the only determinant for their association with membrane, other requirements include Rab5-binding domain of EEA1 and the coiled-coil domain of Hrs (Stenmark *et al.*, 1996; Raiborg *et al*., 2001).

The PX domain-containing proteins make up another large group of PtdIns3*P* effectors. The PX domain, a 130 amino-acid fold, was originally identified in two subunits of the phagocyte NADPH oxidase (phox) complex – $p40^{phox}$ and $p47^{phox}$. (Ponting, 1996). Most PX domain-containing proteins are involved in vesicular trafficking, protein sorting or lipid modification (Lemmon, 2003).

SNXs comprise a large sub-family of this group of PtdIns3*P* effectors and function in the Golgi/endosomal axis (Roth, 2004). For example, SNX1, SNX2, SNX5 and SNX6 have been suggested to complex with cargo selection components such as Vps35p to form the retromer complex, and this governs the recycling of cargo receptors such as CI-M6PR from endosomes to the trans-Golgi network (Clague *et al*., 2009). The full function of SNX1 requires not only its FYVE domain but also its carboxyl terminal BAR (Bin-Amphiphysin-Rvs) domain that facilitates SNX1 dimerization, senses membrane curvature and drives membrane tubulation (De matteis *et al*., 2005).

1.6.2 PtdIns4*P*, the main PPIn player in Golgi complex

PtdIns4*P* is the most abundant monophosphorylated PPIn in mammalian cells (Lemmon, 2008). It constitutes ~2% of cellular PPIns in *S. cerevisiae* (Hughes *et al*., 2000a).

Most of the cell's PtdIns4*P* is usually phosphorylated to PtdIns(4,5)P_2, by PtdIns4*P* 5-kinase, so PtdIns4*P* was until recently considered just as the precursor of PtdIns(4,5)P_2. However, PtdIns4*P* has begun to capture more attention over the last decade as a PPIn that has its own functions in cellular processes. PtdIns4*P* is found in several cell compartments, but is particularly concentrated in the Golgi apparatus, especially in the TGN. Indeed, several putative PtdIns4*P* effectors are localised in the Golgi complex, strengthening the conclusion that PtdIns4*P* has important functions there (D'Angelo *et al*., 2008). These PtdIns4*P* effectors can be categorized into two classes: first is adaptor and coat components, such as AP-1 (adaptor protein-1), GGA protein (Golgi-localised, gamma adaptin ear-containing, ARF-binding protein) and Epsin R; second is lipid transfer proteins i.e. OSBP (oxysterol-binding protein), CERT (ceramide transfer) protein and the FAPP **(**four-phosphate-adaptor protein) (D'Angelo *et al*., 2008). AP-1, GGA and Epsin R participate in TGN-to-endosomal trafficking (De Matteis *et al*., 2005), FAPP1 and FAPP2 are involved in the TGN-to-plasma membrane transport, FAPP2 uses its glycolipid-transfer domain to facilitate sphingolipid transfer across the Golgi (De Matteis and D'Angelo, 2007), CERT operates for the transfer of ceramide from the ER to the Golgi (Hanada *et al*., 2003).

At the TGN, PtdIns4*P* is recognized by the γ-adaptin subunit of the AP-1 complex and promotes AP-1-dependent formation of clathrin-coated vesicles. Decreasing the

PtdIns4*P* content of the Golgi by siRNA suppression of PtdIns4KIIα displaces AP-1 clathrin coats from Golgi membrane, and results in the Golgi fragmented (Mills *et al*., 2003). Overexpression of a catalytically inactive mutant of PI4KIIIβ also perturbs the structure of the Golgi complex and suppresses vesicular transport from the Golgi to the plasma membrane (Godi *et al*., 1999). Similarly, the loss of the PtdIns kinase activity of Pik1p also results in strong defects in the secretory pathway in yeast (D'Angelo *et al*., 2008).

Epsin R is an epsin that binds to PtdIns4*P* through its ENTH (Epsin N-terminal homology) domain. It also interacts with the 'ear' domain of the APs and with clathrin to induce cargo clustering and membrane curvature (Ford *et al*., 2002). Its association with the Golgi membrane is dependent on both PtdIns4*P* and a small GTPase Arf, but independent of AP-1 (Hirst *et al*., 2003).

PtdIns4*P*'s role in the non-vesicular transport of membrane lipids is best exemplified by the characterization of CERT, one of its effectors. CERT is a glycosphingolipid transfer protein which mediates the transit of ceramide from ER to the Golgi for its conversion to sphingomyelin by sphingomyelin synthase-1 (Hanada *et al*., 2003). PtdIns4*P* functions as a Golgi membrane anchor to recruit the CERT through its PH domain, and the FFAT motif targets CERT to the ER membrane by interacting with the ER membrane protein VAP-A (D'Angelo *et al*., 2008).

It is intriguing that association of most PtdIns4*P* effectors (AP-1, GGAs, Epsin R, FAPP and OSBP1) with Golgi membrane involves interactions with both PtdIns4*P* and Arf1, in a way reminiscent of EEA1 binding to PtdIns3*P* and Rab5 at endosomes.

1.6.3 PtdIns5*P*, the orphan in the PPIns family

Of the seven PPIns, PtdIns5*P* is the least characterized. It was identified in mammalian cells in 1997 and in plants in 2001 (Rameh *et al*., 1997; Meijer *et al*., 2001). PtdIns5*P* had not so far been detected in yeast when my work started. PtdIns5*P* levels are regulated by agonist stimulation (see below), but its sub-cellular distribution and the mechanisms controlling its turnover are only beginning to be understood.

Several routes of synthesis and metabolism have been proposed. Rather than being synthesized from PtdIns by a phosphorylation, cellular PtdIns5*P* seems mainly to be formed by the action of PPIn phosphatases on PtdIns(3,5)P_2 and/or PtdIns(4,5)P_2 (Roberts *et al*., 2005). No *bona fide* PI5K active *in vivo* has been identified, even though the Type I and Type III PIP kinases can phosphorylate PtdIns to PtdIns5*P* *in vitro* (Sbrissa *et al*., 1999; Tronchere *et al*., 2004).

However, there is evidence that PtdIns5*P* may be a regulator in some cellular processes. The concentration of PtdIns5*P* is stable in resting or actively growing cells but can increase in thrombin-stimulated platelets (Morris *et al*., 2000) or insulin-stimulated adipocytes (Sbrissa *et al*., 2004), and within the nucleus at G1 in the cell cycle (Roberts *et al*., 2005).

The virulence factor IpgD of the bacterium *Shigella flexneri* is a PPIn 4-phosphatase that can make PtdIns5*P* by dephosphorylating PtdIns(4,5)P_2. During the first step of infection, IpgD is injected into the target cell and the resulting PtdIns5*P* is concentrated at the entry foci of the bacteria, somehow activating the host cell PI3K/Akt pathway (Niebuhr *et al*., 2002; Pendaries *et al*., 2006). In mammalian cells,

the PPIn 3-phosphatase myotubularin (MTM1) can influence PtdIns5*P*. In L6 myotubes overexpressing MTM1, hyperosmotic shock triggered an elevation of PtdIns5*P* levels, and this increase was 50% smaller in the inactive MTM1^{D278A} mutant, suggesting slower PtdIns(3,5)P_2 conversion to PtdIns5*P* might be a factor in the etiology of myotubular myopathy (Tronchere *et al*., 2004).

There is also a possibility that PtdIns5*P* might be implicated in tumorigenesis. It can bind to the PHD domain of the tumour suppressor ING2 (inhibitor of growth protein-2) to mediate apoptosis and cell death in response to DNA damage (Gozani *et al*., 2003; Sasaki *et al*., 2007).

1.6.4 PtdIns(3,4)P_2: lipid second messenger or downstream metabolite?

PtdIns(3,4)P_2 is poorly understood so far and remains a target for future research. It accumulates transiently in the plasma membrane upon stimulation, but a substantial fraction is likely to be formed by Type II 5-phosphatase action on PtdIns(3,4,5)P_3 (Stephens *et al*., 1993a). However, a PtdIns(3,4,5)P_3-independent route to PtdIns(3,4)P_2 has been observed after induction of oxidative stress by H_2O_2 in mammalian cells or after integrin ligation on platelets, possibly as a result of PtdIns3*P* or PtdIns4*P* phosphorylation (Vanhaesebroeck *et al*., 2001). Some PH domain-containing PtdIns(3,4,5)P_3 effectors like PKB (prorein kinase B) and DAPP1 (dual adapter of phosphotyrosine and 3-phosphoinositide protein) can also recognize PtdIns(3,4)P_2 (Lemmon, 2003). The only proteins so far claimed to bind PtdIns(3,4)P_2 selectively are the TAPP1 (tandem PH domain-containing protein-1) and TAPP2 (Dowler *et al*., 2000; Kimber *et al*., 2002), but the physiological functions of these lipid-protein specific interactions remain less than clear.

1.6.5 PtdIns(4,5)P_2, has roles in a myriad of cellular activities

PtdIns(4,5)P_2 is normally the most abundant of the doubly phosphorylated PPIns (Vanhaesebroeck *et al*., 2001). It represents approximately ~1% of total cellular PPIns if PtdIns is included (Hughes *et al*., 2000b). PtdIns(4,5)P_2 is predominantly concentrated at the plasma membrane, possibly enriched in raft-like structures (Di Paolo and De Camilli, 2006), but is also present in the Golgi and the nucleus (Halstead *et al*., 2005). The canonical pathway for PtdIns(4,5)P_2 synthesis requires two consecutive phosphorylations of PtdIns by Type III PtdIns 4-kinase and Type I PtdIns4*P* 5-kinase (Hinchliffe *et al*., 1998), but a small fraction of PtdIns(4,5)P_2 can also be made from the small cell pool of PtdIns5*P* by Type II PtdIns5*P* 4-kinase (Santarius *et al*., 2006).

PtdIns(4,5)P_2 is a substrate for signalling by the PLC family and by PI3KC1s, and can be metabolized by PtdIns(4,5)P_2 phosphatases (Halstead *et al*., 2005). Although its overall concentration remains nearly invariant in many stimulated cells, local changes in PtdIns(4,5)P_2 concentration are likely to occur within cells (Pendaries *et al*., 2003). In addition, there is now abundant evidence that, by serving as a binding or anchoring site for participating proteins, PtdIns(4,5)P_2 regulates or is otherwise involved in a broad spectrum of cellular activities: these include actin cytoskeletal dynamics, ion channel gating, clathrin-mediated endocytosis, exocytosis, vesicle trafficking, focal adhesion formation (Downes *et al*., 2005; van Rheenen *et al*., 2005; Di Paolo and De Camilli, 2006; Santarius *et al*., 2006).

There is clear evidence that PtdIns(4,5)P_2 is required for vacuole fusion both *in vitro* and *in vivo* (Mayer *et al*., 2000; Wiradjaja *et al*., 2007). The *in vitro* homotypic

vacuole fusion reaction is crippled following addition of antibodies to PtdIns(4,5)P_2 or addition of PLC. In this system, PtdIns(4,5)P_2 modulates two steps of vacuole fusion: first is the priming step during which SNARE protein complexes are dissociated; The second is after docking of the vesicle but prior to fusion (Mayer *et al*., 2000). However, an excess of PtdIns(4,5)P_2 *in vivo* can limit vacuole fusion. In intact cells, probes detect PtdIns(4,5)P_2 mainly at the plasma membrane, with little at the vacuole. When the two PtdIns(4,5)P_2 phosphatases, Inp54p and Sac1p were inactivated, PtdIns(4,5)P_2 bioprobes exhibited loss of plasma membrane localisation and instead decorated a subset of fragmented vacuoles. Inactivation of the phosphatases also causes vacuole fusion defects that were rescued by inactivating the PtdIns4*P* 5-kinase Mss4p. This result was interpreted as evidence that the vacuolar fragmentation/fusion equilibrium depends on proper maintenance of the traffic of plasma membrane-synthesised PtdIns(4,5)P_2 between vacuolar and plasma membranes (Wiradjaja *et al*., 2007).

A genetic analysis of deletion mutants showed that a Plc1p is required for vacuolar fusion (Seeley *et al*., 2002), and vacuole fusion *in vitro* was blocked by sequestration of DAG by the C1b domain from a mammalian PKCβII or by PLC inhibitors (Jun *et al*., 2004). These results suggest that PtdIns(4,5)P_2 metabolites – DAG, IPs or both – might regulate vacuole fusion. In this thesis, I offer some evidence in support of this idea.

1.6.6 PtdIns(3,5)P_2, functions in the endo-lysosomal system

PtdIns(3,5)P_2 is one focus of this project and will be introduced in detail in Section 1.9 and chapter 3.

1.6.7 PtdIns(3,4,5)P_3, the most phosphorylated and fundamentally important PPIn

Unlike its precursor, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 exists in very low concentrations in quiescent cells but its levels can be transiently and dramatically enhanced in response to the activation of Type I PI3Ks. The local generation of elevated levels of PtdIns(3,4,5)P_3 results in the recruitment of its interacting proteins, whose activation mediates synergistic signalling cascades. Accordingly, PtdIns(3,4,5)P_3 plays multi-faceted roles in a repertoire of fundamental cellular activities: cell proliferation, differentiation, survival, motility, migration, chemotaxis, metabolism and immune responses (Di Paolo and De Camilli, 2006). PtdIns(3,4,5)P_3 metabolic balance is thus the focus of intense research into novel drugs for the treatment of cancer, type II diabetes, cardiac failure, inflammatory diseases and problems involving auto/hyper-immune response (Downes *et al*., 2005).

One of the best-defined PtdIns(3,4,5)P_3-mediated pathways is the activation of Akt/PKB, the cellular homologue of the retroviral oncogene v-Akt (Vivanco and Sawyers, 2002). Following PI 3-kinase activation, PtdIns(3,4,5)P_3 recruits PDK (Phosphoinositide dependent kinase) and Akt at the plasma membrane, where Akt is phosphorylated and activated by PDK to integrate with important signalling pathways such as the Tor pathway (Nave *et al*., 1999). Because PTEN and SHIP-1 and 2 can negatively regulate the level of PtdIns(3,4,5)P_3, both phosphatases are therefore tumour suppressors and have a potential therapeutic function for cancer and type II diabetes respectively (Cantley, 2002; Czech, 2003).

1.7 Inositol Polyphosphates (IPs)

The growth of interest in the PPIns has been accompanied by an increased focus on inositol polyphosphates (IPs), which are their soluble counterparts and metabolites thereof. In addition to IP_3 functioning as a second messenger in intracellular Ca^{2+} release, as mentioned earlier, many IPs are now emerging as agents that regulate other cellular functions, including several nuclear processes, e.g. transcription, chromatin remodelling and mRNA editing and export (York, 2006; Alcazar-Roman and Wente, 2008).

IPs contain only an inositol core and various numbers of phosphomonoester groups attached to one or more of the six hydroxyl moieties. In theory, there could be 63 simple *myo*-inositol phosphates isoforms (including isomers or stereoisomers), of which more than half have been found in nature (Menniti *et al*., 1993; Hughes and Michell, 1993). The discovery of inositol pyrophosphates – in which one more additional phosphate groups is added to an existing phosphomonoester group – expands the possible number of naturally occurring IPs indefinitely (Stephens *et al*., 1993b; Shears, 1996, 2007; Burton *et al*., 2009).

The synthesis of the various IPs and inositol pyrophosphates is achieved by a variety of IP kinases (IPK), IP phosphatases and inositol pyrophosphate synthases (IPS), almost always using as their starting material the IP_3 liberated during hydrolysis of PtdIns(4,5)P_2. The pathway for the synthesis of IPs from IP_3 was built on a genetic screen aimed at finding yeast genes that are involved in mRNA export (York *et al*., 1999). The genes that were identified encoded Plc1p and the kinases necessary to catalyse a previously suggested pathway to synthesize IP_6 from IP_3 (Ongusaha *et al*.,

1998). One was an Ins(1,3,4,5,6)P_5 2-kinase named Ipk1p, and the second was the kinase: Arg82p/Ipk2p that can transform IP_3 to Ins(1,3,4,5,6)P_5 by sequentially adding phosphate groups on the 6- and 3- hydroxyls (Odom *et al*., 2000; Saiardi *et al*., 1999, 2000). This screen was also the first to indicate that production of IPs was linked to a nuclear function (mRNA export).

The fully phosphorylated IP_6 is not the end point of this synthetic route. The discovery of an IP_6 5-kinase Kcs1p (Saiardi *et al.*, 1999) and the recently identified IP_6 1/3-kinase Vip1p (Mulugu *et al.*, 2007; Lin *et al*., 2009) has extended this pathway to the conversion of IP_6 to several inositol pyrophosphates that are loosely designated IP_7 and IP_8.

The involvement of inositol pyrophosphates in nuclear regulation is well illustrated by the function of IP_7 in the phosphate-responsive signalling (Pho) pathway. In budding yeast, inorganic phosphate limitation inactivates the Pho80p-Pho85p cyclin-cyclin dependant kinase (CDK) complex via the CDK inhibitor Pho81p, leading to expression of genes involved in nutrient homeostasis (Schneider *et al*., 1994). IP_7 is required for maintaining the Pho81p-dependent inactivation of this complex. The genetic studies showed that this IP_7 is made by Vip1p but not Kcs1p. *In vitro* assays strengthened this result because 4/6 PP-IP_5 (the IP_7 made by Vip1p) is more effective than 5PP-IP_5 (made by Kcs1p) in inhibiting the Pho80p-Pho85p complex (Lee *et al*, 2007). This is the first isomer-specific molecular action of an IP_7 linked to a physiological response (Irvine, 2007). The story became more complete when it was found that IP_7 interacts non-covalently with the Pho80p-Pho85p-Pho81p ternary complex and induces additional interactions between Pho81p and Pho80p-Pho85p

that prevent substrates from accessing the kinase active site (Lee *et al*., 2008). Alcazar-Roman and Wente (2008) suggest that IPs might modulate protein functions in a variety of ways: for example, by serving essential structural cofactors, by triggering allosteric or induced-fit structural changes in enzymes, or by direct competition with substrates.

1.8 Vacuole fusion and its regulation

Eukaryotic cells include multiple membrane-bound compartments, including ER, Golgi complex, endosomes and lysosomes, and their proteins must be targeted to the correct organelles. This protein trafficking is achieved by the movement of small membrane-bound vesicular transport intermediates between organelles. It involves: membrane vesicle budding from a “donor” compartment by a process that allows selective incorporation of cargo into the forming vesicles; vesicle transport between the “donor” compartment and the “acceptor” compartment; vesicle fusion with an appropriate “acceptor” compartment whereby the vesicle unloads its cargo into the surrounding membrane or lumen of the acceptor organelle. Such selective membrane fusion and fission events underlie both subcellular compartmentation and secretory processes (e.g. neurotransmission and hormone secretion). Fig. 1.14 summarises major trafficking routes in eukaryote cells. It should be noted that progress on the molecular mechanisms of vesicle trafficking relied on two sets of seminal studies: the selection and characterization of secretion (*sec*) mutants of yeast by Schekman’s laboratory (Novick and Schekman, 1979); and the development of cell-free assays of compartment mixing by Rothman and colleagues (Balch *et al*., 1984).

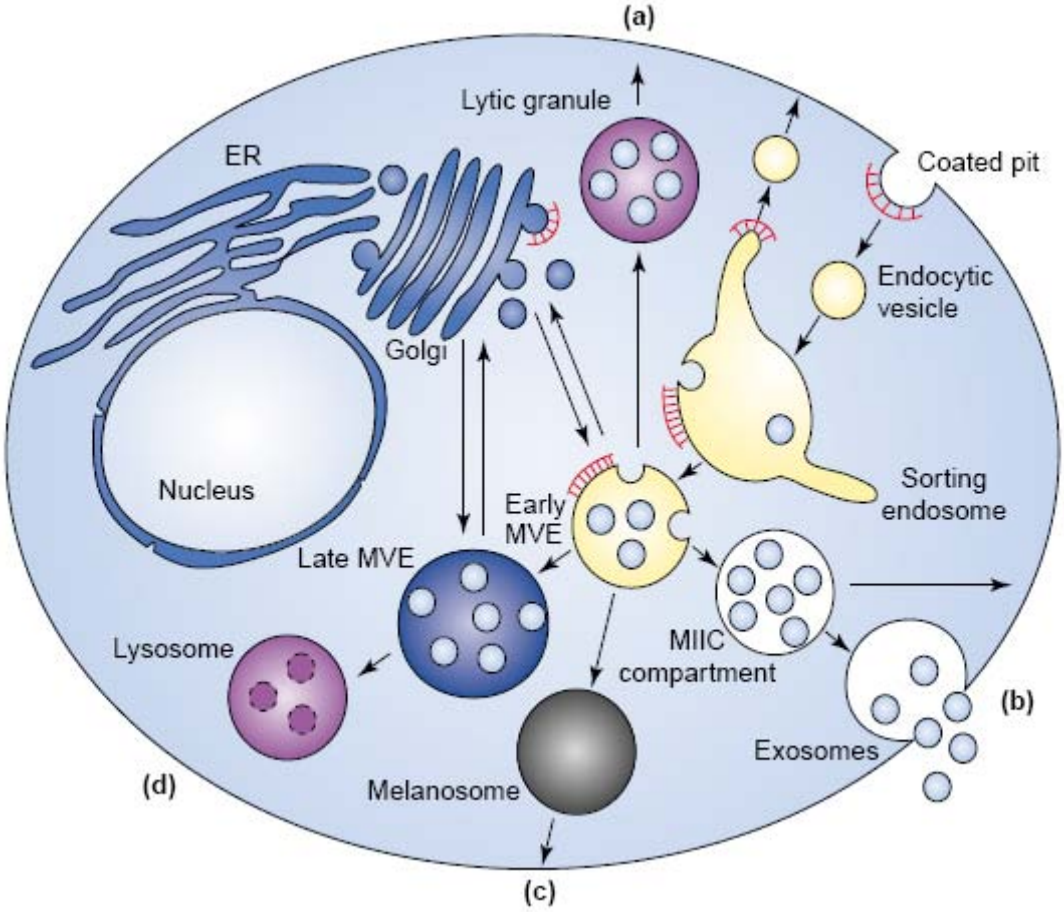


Figure 1.14: Membrane-bound protein trafficking in Eukaryotic cells (from Raiborg et al., 2003)

(a) lytic granules in T lymphocytes, (b) MHC class II compartments and exosomes in antigen-presenting cells, (c) melanosomes in melanocytes, (d) late MVEs/lysosomes in most nucleated cells

The vacuole of budding yeast is homologous to the mammalian lysosome or to a late endosome/lysosome hybrid structure. Several factors make the yeast vacuole an excellent model organelle with which to study membrane fusion and fission. In particular, a variety of genetic and biochemical tools are available for yeast, vacuoles can be easily isolated, and they can be visualised *in vivo* and *in vitro* by fluorescent techniques. Rapid and quantitative *in vitro* assays for homotypic vacuole fusion have been established recently (Haas *et al*., 1994). *In vivo*, the fusion of multiple vacuole

subcompartments and fission of vacuoles into multiple subcompartments occur in response, respectively, to hypo- and hyper-osmotic stress, and analysis of these processes allows genetic screens to identify novel components of fusion/fission pathways.

The yeast vacuole is a highly dynamic organelle, and its biogenesis and functions are regulated by more than 70 proteins identified by several elegant mutant screens for defects in endocytosis (End), delivery of vacuolar peptidase (Pep), sorting of proteins to the vacuole (Vps), vacuole inheritance during budding (Vac) and vacuole morphology (Vam) in budding yeast (reviewed by Bowers and Stevens, 2005). Disruption of many of these genes leads to perturbed vacuole morphology. These aberrant morphologies have been classified into six classes (Raymond *et al*., 1992):

- Class A have wild-type vacuoles that comprise 3-5 clustered spherical compartments;
- Class B mutants have partly fragmented vacuoles, often with >10 small compartments;
- Class C mutants have highly fragmented vacuole, so that no true vacuole can be recognized;
- Class D mutants have a single large unlobed vacuole;
- Class E vacuoles are enlarged and have an aberrant late endosome/MVB (the class E compartment) adjacent to the vacuoles; and
- Class F mutants have one large vacuole surrounded by a number of small vacuolar subcompartments.

To give rise to this multiplicity of vacuolar morphologies, vacuole membranes must be able to undergo homotypic fission and fusion *in vivo* (Wickner and Haas, 2000).

Experimental analysis of the fusion process has been facilitated by the establishment of an *in vitro* assay (Haas *et al*., 1994). For this assay, vacuoles are isolated from two deletion strains: one lacks the *PHO8* gene that encodes the proenzyme form of the vacuole alkaline phosphatase (pro-Pho8p); and the other lacks the *PEP4* gene that encodes a major intravacuolar protease that catalyses the maturation of vacuolar pro-proteins (including pro-Pho8p). Neither of two purified vacuole preparations harbors active Pho8p, but upon fusion of the vacuoles from the two mutants their lumenal contents mixing and Pep4p gains access to and activates pro-Pho8p, the activity of which is measured by the liberation of yellow *p*-nitrophenyl from *p*-nitrophenylphosphate. The activity and rate of fusion can therefore be followed colorimetrically.

Yeast vacuole fusion requires on the cooperative action of many proteins and lipids, including ATPases, GTPases, SNARE complexes, the HOPS (homotypic vacuole fusion and vacuole protein sorting) complex, PPIns, ergosterol, diacylglycerol, cholesterol and Ca^{2+} (reviewed by Wickner and Haas, 2000; Wickner, 2002; Ostrowicz *et al*., 2008). The SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex is the engine of fusion, with its functions regulated by other fusion factors. SNARE proteins are defined by their heptad-repeat "SNARE" motifs that assemble them into four-helical "coiled-coil" bundles *in cis* (anchored to same membrane) or *in trans* (anchored to apposed membranes) (Sutton *et al*., 1998; Fasshauer *et al*., 1998; Poirier *et al*., 1998). Most SNARE proteins are bound to membranes by a C-terminal apolar region or a prenylated tail. Budding yeast vacuoles have at least five abundant SNAREs: three Q-SNAREs (Vam3p, Vam7p, Vti1p) and two R-SNAREs (Nyv1p and Ykt6p) where the Q or R residues at the core

of the “SNARE” motif interact in helical bundles. Regulation of vacuole fusion can be seen primarily as the regulation of SNARE complex assembly and disassembly by distinct SNARE chaperones. Vacuole fusion can be experimentally separated into four sub-reactions termed priming, tethering, docking and fusion (Fig. 1.15).

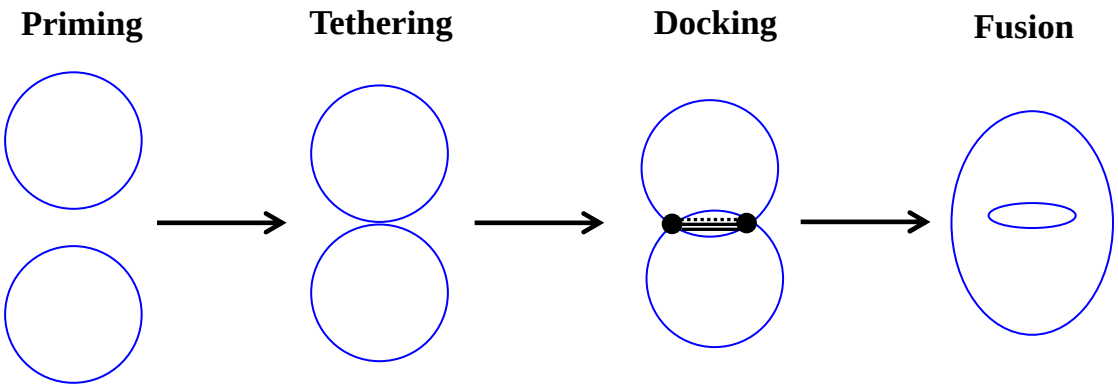


Figure 1.15: The four stages of yeast homotypic vacuole fusion (adapted from Wickner, 2002)

The priming stage that starts a new round of fusion is characterized by disassembly – by the AAA-ATPase Sec18p/NSF (N-ethylmaleimide-sensitive factor) – of the *cis*-SNARE complexes that catalysed the previous round of fusion. This leads to the release from vacuoles of Sec17p/αSNAP (soluble N-ethylmaleimide-sensitive factor attachment protein) and of Vam7p, establishing a pool of unpaired SNAREs that are in an active, fusion-competent state ready for subsequent pairing *in trans* (Söllner *et al.*, 1993; Mayer *et al.*, 1996; Ungermann *et al.*, 1998). Upon priming by Sec18p and ATP, a hexameric complex, termed the HOPS complex, is also freed from pairing with the *cis*-SNAREs so that it can bind to Ypt7p and initiate the docking stage (Seals *et al.*, 2000). The close association of primed vacuoles prior to fusion proceeds through two steps: first the reversible tethering of SNAREs between vacuoles *in trans* and

then their irreversible pairing or docking (Ungermann *et al*., 1998).

Tethering is the first contact between vesicular and target vacuoles, in which the HOPS complex, SNAREs complex and Ypt7p become concentrated in a ring – the vertex ring – that encircles the apposed patches of membrane on the paired vacuoles. After the vertex ring has formed, one R-SNARE from the vesicle membrane (vesicle- or v-SNARE) and three Q-SNAREs from the target membrane (target- or t-SNARESs) bind each other to form a zipper-like 3Q:1R *trans*-SNARE complex (Stroupe *et al.*, 2006) – but this formal vesicle/target distinction is, of course, irrelevant in homotypic vacuole fusion between identical membranes. Ypt7p is thought to orchestrate the association between the Q-SNAREs and the HOPS complex, facilitating the transition from transient tethering to formation of the irreversible *trans*-SNAREs complex. Docking triggers a brief release of calcium from the vacuole lumen (Peters and Mayer, 1998) and leads to the bilayer/bilayer fusion and to the mixing of lumenal contents (Collins *et al*., 2005).

In addition to the SNAREs complex, the HOPS complex is central to the fusion process. HOPS is a hexameric complex consisting of four Class C Vps proteins (Vps11p, Vps16p, Vps18p, Vps33p) and two Class B Vps proteins (Vps39p and Vps41p) (Fig. 1.16). The Class C proteins were first characterized as parts of a 65S SNARE-containing complex existing before priming (Rieder and Emr, 1997), and Vps39p and Vps41p were later found associated with the four SNARE proteins. Vps39p is a guanyl-nucleotide exchange factor (GEF) that regulates the activity of Ypt7p, the yeast Rab7 homologue (Wurmser *et al*., 2000). Vps33p, one of the HOPS complex subunit, belongs to the Sec1/Munc18 (SM) family of SNARE-binding

proteins (Banta *et al.*, 1990), suggesting a potential role for the HOPS in regulating the assembly of SNAREs complexes.

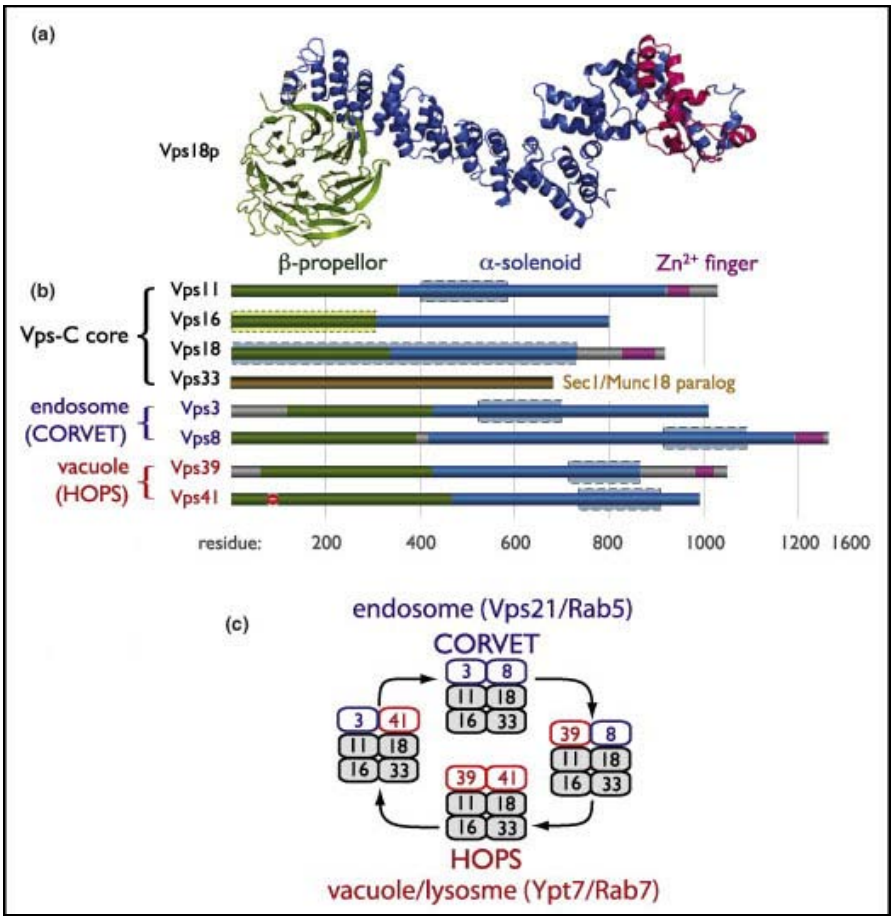


Figure 1.16: Composition and domain structure of the HOPS and CORVET complexes (from Nickerson et al., 2009)

a. Ribbon diagram depicting a folding model for Vps18; **b**. Summary of tertiary structure predictions indicated by color coding for Vps-C components; **c**. Working scheme for Vps-C complex function, in which interactions of CORVET and HOPS with endosomal and lysosomal Rabs regulate membrane fusion events at late endosomes and lysosomes, respectively. Hybrid complexes contain both HOPS and CORVET subunits

Upon priming, the HOPS complex dissociates from the SNARE complex as a 38S sub-complex which then "hops" to Ypt7p, activating this GTP-dependent Ypt/Rab switch to initiate docking by promoting and proofreading the formation of the *trans*-

SNAREs complex at the docking step (Starai *et al.*, 2008). Thus the HOPS complex facilitates the transition from ATP-dependent disassembly of the *cis*-SNARE complex active in priming to the assembly of the Ypt7p-dependent *trans*-SNARE complex for docking.

Purification of the active HOPS complex later revealed its affinity for PPIn and the recognition of the PX domain of the SNARE Vam7p (Stroupe *et al*., 2006), leading to the proposal that HOPS is enriched at the vertex ring by directly interacting with Ypt7p, Vam7p and PtdIns(4,5)P_2. *In vitro* fusion assays then demonstrated that vacuolar SNARE complexes bind either Sec17p or the HOPS complex, but not both. Thus, Sec17p may displace HOPS from SNAREs to allow the subsequent round of fusion (Collins *et al.*, 2005).

Functions have also been defined for other HOPS complex subunits. Vps11p and Vps18p contain C-terminal RING (Really interesting new gene) domains that are believed to mediate protein-protein interactions (Schultz *et al.*, 1998) and so they may have a role in HOPS complex assembly. Indeed, the C-terminal 148 amino acids of Vps39p were found to be necessary for the interaction between Vps39p and Vps11p (Wurmser *et al.*, 2000). Vps16p remains the least well-defined HOPS subunit. The role of Vps41p in vacuole fusion has been characterized in some detail recently. The phosphorylation of Vps41p by the vacuole casein kinase Yck3p reduces association of the the HOPS complex with vacuole contact sites and so may negatively regulate vacuole fusion, and a *YCK3* deletion mutant displays a strong accumulation of Vps41p at vacuole membrane micro-domains that are believed to be fusion docking sites (LaGrassa and Ungermann , 2005).

Regulation of Vps41p by Yck3p is a focus of one chapter of this thesis, so it will be discussed in more detail later.

1.9 PtdIns(3,5)P_2 and vacuole fission

PtdIns(3,5)P_2 is one of the seven PPIns that is almost ubiquitous in eukaryotes (Michell *et al.*, 2006). The existence of PtdIns(3,5)P_2 in animal cells was first suggested in 1989 (Auger *et al.*, 1989), but it was not until 1997 that it was identified almost simultaneously in the budding yeast *S. cerevisiae* and in resting mouse fibroblasts (Dove *et al.*, 1997; Whiteford *et al.*, 1997). Dove *at el*. (1997) also showed that its cellular levels are strikingly elevated upon hyper-osmotic treatment, suggesting that this lipid might have a physiological function in yeast's adaptation to hyper-osmotic stress.

As mentioned earlier, PtdIns(3,5)P_2 is synthesized from PtdIns by two consecutive phosphorylations of PtdIns, by the PtdIns 3-kinase Vps34p and the PtdIns3*P* 5-kinase Fab1p (Herman and Emr, 1990; Schu *et al.*, 1993; Gary *et al.*, 1998; Cooke *et al.*, 1998) (Fig. 1.17). Correct regulation of the kinase activity of Fab1p requires three additional proteins: Vac14p, Vac7p and Fig4p. Cells that lack any of these three proteins display deficient PtdIns(3,5)P_2 synthesis and recapitulate most or all of the defects present in *fab1Δ* cells (Bonangelino *et al.*, 1997; Dove *et al.*, 2002; Duex *et al.*, 2006a; Duex *et al.*, 2006b), and Atg18p, one of the effectors of PtdIns(3,5)P_2, modulates Fab1p activity in a negative fashion (Dove *et al.*, 2004).

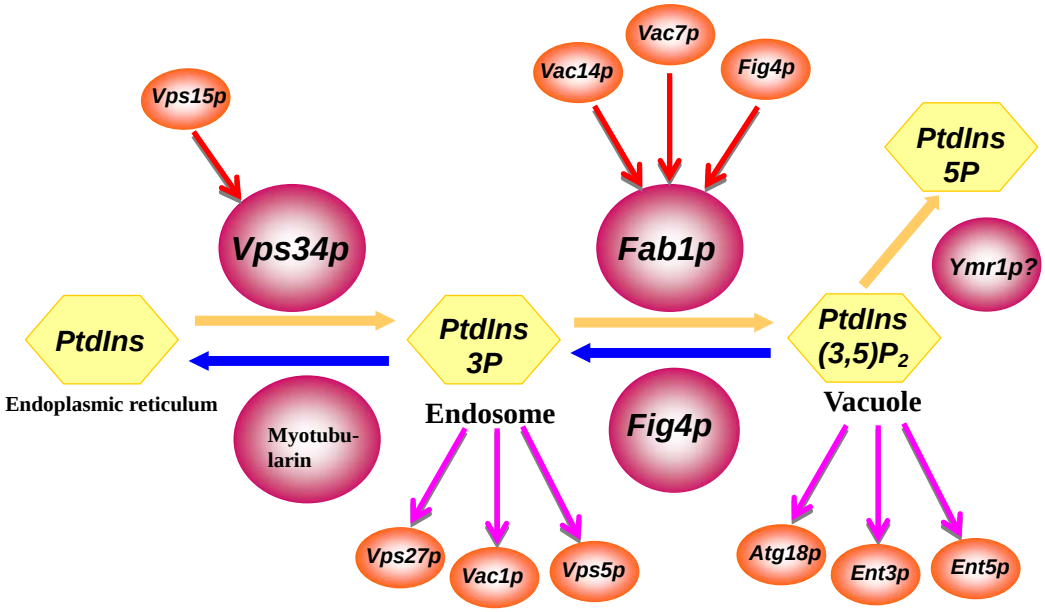


Figure 1.17: PtdIns(3,5)P_2 synthesis and degradation in S. cerevisiae

Fig4p is a Sac1p-like PPIn phosphatase that 5-dephosphorylates PtdIns(3,5)P_2 to PtdIns3*P* (Gary *et al.*, 2002). In the one published study of recombinant Fig4p, PtdIns(3,5)P_2 was the only PPIn that it attacked (Rudge *et al.*, 2004), even though its catalytic site is very similar to that of the relatively non-specific PPIn phosphatase Sac1p (Hughes *et al.*, 2000a). Fig4p includes a canonical CX_5R catalytic motif and has an extensive C-terminal coiled-coil domain that mediates its interaction with Vac14p: deletion or inactivation of Vac14p or removal of the C-terminal segment of Fig4p renders it completely cytosolic, as does deletion of *FAB1* (Rudge *et al.*, 2004). Surprisingly, inactivation of this PtdIns(3,5)P_2 5-phosphatase, in yeast or in animal cells, leads to a profound defect in PtdIns(3,5)P_2 synthesis (Rudge *et al.*, 2004; Chow *et al.*, 2007). Recent work has unravelled this apparent conundrum by demonstrating that Fab1p, Vac14p and Fig4p form a large complex, with Vac14p acting as its central scaffold, that co-ordinately regulates the metabolism of PtdIns(3,5)P_2 (Botelho *et al.*, 2008; Jin *et al.*, 2008).

The multiple defects displayed by mutants deficient in PtdIns(3,5)P_2 synthesis give clues to the function of this lipid. These include (Michell *et al.*, 2006; Dove and Johnson, 2007):

- Gross enlargement of the vacuole;
- A defect in the ubiquitin-dependent sorting of proteins into the internal vesicles of MVB that are subsequently trafficked to the vacuole lumen;
- Abnormal sensitivity of the cells to multiple stresses, including heating or alkalinization of the external medium;
- Failure to acidify the vacuole normally.

These phenotypes seem to originate from independent dysfunctions in that they are partially separable. All of the underlying functions depend on the lipid kinase function of Fab1p, as they are also displayed by cells in which wild-type Fab1p is replaced by a kinase-dead variant of Fab1p (*fab1*^{D2134R}) (Gary *et al.*, 1998).

PPIns exert their effects on cellular activities primarily by serving as membrane binding sites for specific effector proteins, and there seems no reason for PtdIns(3,5)P_2 to be exceptional. Proposed effectors for PtdIns(3,5)P_2 include the PROPPIN (β-propeller(s) that binds PPIn) family of seven-bladed β-propellers (Svp1p/Atg18p, Hsv1p/Atg21p and Hsv2p in yeast) (Michell *et al*., 2006). Atg18p is necessary for PtdIns(3,5)P_2-dependent retrieval of membranes from the vacuole to the late endosome (Dove *et al.*, 2004; Michell *et al.*, 2006). The PtdIns(3,5)P_2-dependent functions of Hsv1p and Hsv2p remain less well defined. Proteins that may be implicated in PtdIns(3,5)P_2-dependent MVB sorting include the epsin-like proteins Ent3p and Ent5p (Friant *et al.*, 2003). No PtdIns(3,5)P_2 effectors that are required for vacuole acidification have yet been identified.

One of the principal function of PtdIns(3,5)P_2 is to regulate the fission/fragmentation of lysosome/vacuoles both under normal conditions and in stressed cells. Changes in vacuole morphology – by fusion or fission – are easy to induce by hypo- or hyper-osmotic stress and are readily seen by optical microscopy, so investigation of PtdIns(3,5)P_2-dependent control of vacuole morphology may help to advance our understanding of the molecular mechanisms of this type of membrane fission. The concentration of PtdIns(3,5)P_2 in yeast, and in some plant and some animal cells, increases in response to hyper-osmotic stress, concomitant with fragmentation of the budding yeast vacuole (Dove *et al.*, 1997; Rudge *et al.*, 2004). The rise is transient, with the degree of hypertonicity regulating the duration and magnitude of the PtdIns(3,5)P_2 accumulation. Modest stresses (e.g. 0.4 M NaCl) cause slight vacuole fragmentation and provoke a modest accumulation of PtdIns(3,5)P_2 that reverses within ~10 min, whereas more intensive stresses (e.g. 0.9M NaCl) lead to highly fragmented vacuoles and a large PtdIns(3,5)P_2 accumulation that takes up to 60 min to revert to pre-stress values (Duex, *et al.*, 2006a; Dove *et al.*, 2009). The transient elevation in PtdIns(3,5)P_2 levels likely provides some osmotic protection for the cell because vacuole fragmentation dramatically decreases the total vacuole volume. It is also possible that the increase in vacuole membrane PtdIns(3,5)P_2 might regulate transmembrane channels to release ions, other osmolytes and water from the vacuole into the cytoplasm.

The correlation of vacuole fragmentation and PtdIns(3,5)P_2 synthesis was nicely demonstrated by three related *fab1* mutants. A kinase-dead *fab1* mutant (D2134R) displayed an enlarged vacuole all the time (Gary *et al.*, 1998; Odorizzi *et al.*, 1998); a *fab1* mutant (G2042V/G2045V) that only maintains 10% of the normal levels of

PtdIns(3,5)P_2 manifests some vacuole fragmentation (Gary *et al*., 1998; Odorizzi *et al*., 1998); and *FAB1*-5 – a *FAB1* mutant that makes supra-normal levels of PtdIns(3,5)P_2 – has fragmented or shrunken vacuoles (Gary *et al*., 2002). Any identification of PtdIns(3,5)P_2 effector proteins that influenced this process would contribute to the understanding of the molecular mechanisms of vacuole fragmentation.

The machinery for homotypic vacuole fusion is relatively well characterized (reviewed in Ostrowicz *et al.*, 2008 and discussed above), but that for vacuole fission has only recently begun to be unravelled (Peters *et al.*, 2004; Baars *et al.*, 2007). This has allowed a new recognition that these two processes may use shared components to maintain the equilibrium between vacuole fusion and fission (Fig. 1.18). Vps1p, a dynamin-like protein that had previously been recognised as necessary for vesicle budding at the TGN, was the first protein to be implicated also in vacuole fission (Vater *et al*., 1992; Peters *et al*., 2004). It was found that Vps1p can form polymers and nucleate several t-SNAREs (e.g. Vam3p) together (Peters *et al.*, 2004). At the priming stage of fusion, Vps1p is depolymerised and freed from the vacuole membrane in a process that requires Sec18p/NSF and Vam3p but not Sec17p/α-SNAP and the v-SNARE. This release of Vps1p liberates the t-SNAREs for initiating fusion and at the same time disrupts fission activity which presumably prevents futile cycles of fission and fusion. The idea that vacuole fusion and fission are mechanistically coupled was reinforced when the Mayer group, who had identified the dual roles for Vps1p, showed that the vacuolar V-ATPase is involved in both processes. Fusion requires the physical presence of the V_0 subcomplex – the membrane sector of the V-ATPase complex – but occurs in the absence of a trans-membrane H^+ gradient,

whereas vacuole fission requires the establishment of a proton gradient by the V-ATPase (Baars *et al*., 2007).

The HOPS complex also plays central roles in both vacuole fission and fusion. This is because it can interconvert between two forms: the tethering complex for fusion that was discussed above and a slightly different CORVET (Class C Core vacuole/endosome tethering) complex that catalyses either vacuole fission or vacuole-endosome fusion, or both (Peplowska *et al*., 2007, see Fig. 1.18).

It will be important for future studies to examine whether the CORVET complex might be involved in the link between Fab1p activity, PtdIns(3,5)P_2 concentration and vacuole sub-compartmentation. A link between the function of the HOPS and CORVET machineries and PtdIns(3,5)P_2 availability is suggested by the finding that deletion of some CORVET and/or HOPS subunits can cause mislocalisation of Atg18p, Atg21p and Hsv2p (Kane, 2005). In addition, vacuole fission in yeast cells requires both the V-ATPase and vacuole acidification, and it has long been known that PtdIns(3,5)P_2 is required for vacuolar acidification: *fab1*Δ, *vac7*Δ and *vac14*Δ cells all have neutral vacuoles and this defect and vacuole enlargement seem to occur independently (Rusten *et al*., 2006). How PtdIns(3,5)P_2 regulates vacuole acidification remains enigmatic so far, but is likely to involve undefined effector proteins regulating the V-ATPase either directly or indirectly, and that will exert secondary effects on vacuole fission.

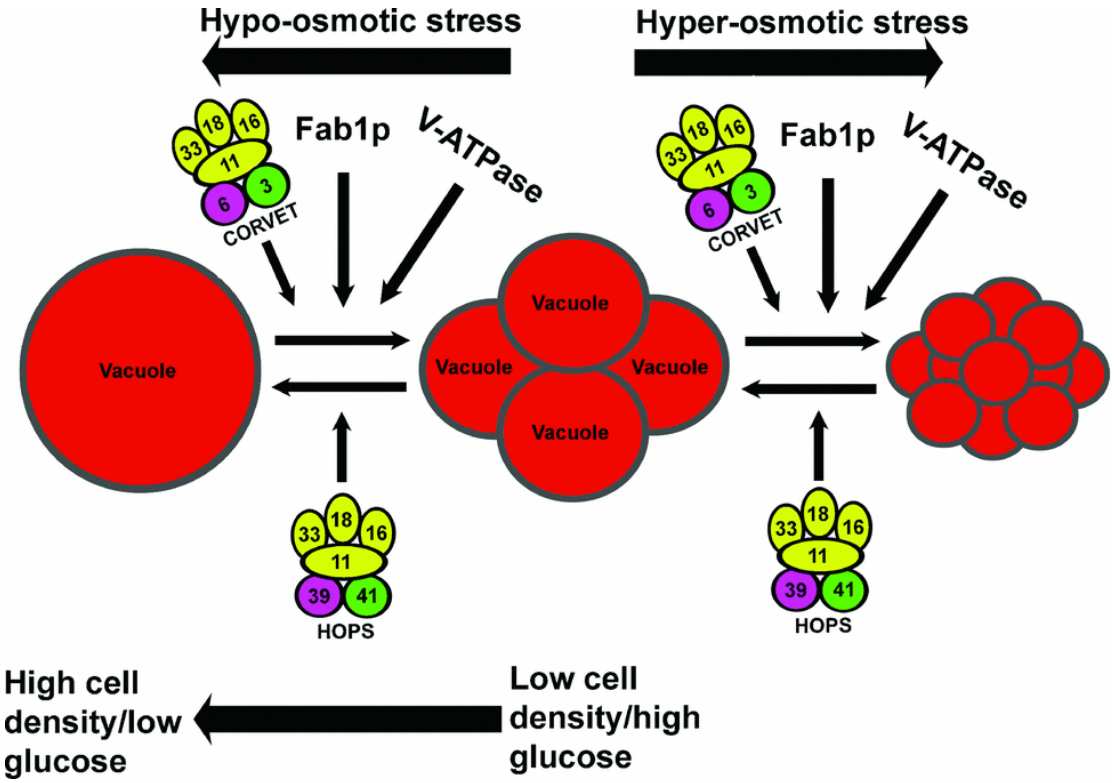


Figure 1.18: CORVET–HOPS, the V-ATPase and the Fab1p pathway co-operate to regulate endo-lysosomal fusion/fission status

The yeast vacuole normally exists as a series of discrete, but tethered, compartments whose size and shape depend upon growth status, osmolarity and activity of the V-ATPase, the HOPS–CORVET equilibrium and the activity of the Fab1p pathway. This diagram illustrates the interplay between these pathways and emphasizes that the interconversion of HOPS into CORVET has profound implications for the fusion–fission equilibrium. In the centre, the vacuole is depicted as it appears in exponentially growing cells. Starvation and/or hypo-osmotic stress can cause vacuolar fusion mediated by HOPS, shifting the vacuole to the single enlarged organelle shown on the left. Alternatively, hyperosmotic stress or an artificial hyperactivation of the CORVET or Fab1p pathways can result in extreme fragmentation of the vacuole, as shown on the right-hand side of the Figure. The casein kinase Yck3p, apparently restrains fusion after fission has occurred by phosphorylating a number of targets; Vps41p, a subunit of HOPS is one of these targets

1.10 Project aims

PtdIns(3,5)P_2 was first identified in budding yeast by Dr. Dove in 1997 (Dove *et al*., 1997). When my Ph.D. work started in 2005, there has been a long history on PtdIns(3,5)P_2 research by Dr. Dove's lab. The study of PtdIns(3,5)P_2 field was mainly targeted on two focuses: its metabolism and its cellular functions. My first project was aimed to the former.

As detailed in Section 1.9, in *S. cerevisiae*, PtdIns(3,5)P_2 is generated by the lipid kinase Fab1p. Fab1p requires the activator proteins, Vac7p and Vac14p for normal activity, based largely on the genetic studies. However there was a dearth of biochemical and cellular evidence to elaborate this regulatory mechanism. PtdIns(3,5)P_2 can also be degraded to PtdIns3*P* or PtdIns5*P* by inositol lipid phosphatases, probably by two enzymes Fig4p and Ymr1p respectively, but again much of the *in vitro* work was lacking. One of the effectors of PtdIns(3,5)P_2, Atg18p, was also thought to have a negative feedback effect on the synthesis of PtdIns(3,5)P_2, but similar to all above propositions, the underlying mechanism remained to be intensively investigated. As far as my project is concerned, I was going to construct endogenous level-expressed, C-terminally GFP-tagged these related proteins to observe their localisation patterns, combined with the standard biochemical techniques, in hope to answer some of above questions.

In addition, PtdIns(3,5)P_2 was well documented to be necessary for the hyper-osmotic induced vacuole fragmentation in budding yeast, but so far this stress signalling was still too slim, calling more components identified. In this case, my second project was launched by a recent discovery that a yeast casein kinase Yck3p was required to

inhibit the vacuole fusion upon hyper-osmotic stress (see Section 1.8 and Chapter 4 for more information). My starting point of second project was that: because PtdIns(3,5)P_2 is required for vacuole fission whereas Yck3p is necessary to inhibit vacuole fusion, therefore it seems both molecules are functioning synergistically to control the hyper-osmotic signalling in budding yeast. The further investigation on Yck3p functional events in the context of regulating vacuole fusion may produce useful information towards the PtdIns(3,5)P_2 knowledge, alternatively at least on Yck3p signalling itself. So, in my second project, I was going to exploit the hyper-osmotic triggered, Yck3p-catalyzed phosphorylation of Vps41p (a subunit of tethering complex regulating the homotypic vacuole fusion) as a phenotype to screen some selective deletion mutants (ideally ~100), expecting to find some novel components which will be part of PtdIns(3,5)P_2-involved stress pathway and Yck3p/Vps41 pathway itself.

Chapter 2 Materials and Methods

2.1 Strains, Media and Reagents

TOP10 competent cells (Invitrogen) were used for all sub-cloning. BL21-CodonPlus(DE3)-RIL cells (Stratagene) were used to express GST-SKIP and GST-Ymr1p fusion proteins. BL21-star (DE3)-RIL cells (made based on the BL21- star (DE3) *E.coli* strains from Invitrogen, see below for the construction strategy) were used to express His6-Fig4p and GST-Fig4p fusion proteins. *E.coli* strains were propagated in LB broth or LB plates (containing appropriate antibiotics for plasmid retention) following routine manipulation.

S. cerevisiae strain FY833 (from ATCC), cells were used to express GST-Fig4p in yeast. Wild type BY4741 and BY4742 as well as deletion strains were purchased from EUROSCARF (Germany). *S. cerevisiae* strains were grown in standard Yeast extract –Peptone-Dextrose (YPD) or Synthetic Complete (SC) minimal media supplemented with essential amino acids. The budding yeast strains used in this study are listed in Table 2.1.

The media for growth of yeast strains and other solutions are described below. Yeast extract, bacto peptone, bacto agar, yeast nitrogen base without amino acids and ammonium sulphate were obtained from Difco Laboratories. All other biochemical reagents were obtained from Sigma-Aldrich except as in indicated, and were of Biotechnological grade or better.

Table 2.1: *S. cerevisiae* strains used in this study

Strain name	Genotype	Source
W303	*MATα; ura3-52; trp1Δ2; leu2-3_112; his3-11; ade2-1; can1-100*	Euroscarf
BY4741	*MAT*a*; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
BY4742	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	Euroscarf
*vps34*Δ	BY4742; *vps34*::*kanMX4*	Euroscarf
*fab1*Δ	BY4742; *fab1*::*kanMX4*	Euroscarf
*vac14*Δ	BY4742; *vac14*::*kanMX4*	Euroscarf
*vac7*Δ	BY4742; *vac7*::*kanMX4*	Euroscarf
*atg18*Δ	BY4742; *atg18*::*kanMX4*	Euroscarf
*ldb19*Δ	BY4742; *ldb19*::*kanMX4*	Euroscarf
*fig4*Δ	BY4742; *fig4*::*kanMX4*	Euroscarf
*vps45*Δ	BY4742; *vps45*::*kanMX4*	Euroscarf
*apm3*Δ	BY4742; *apm3*::*kanMX4*	Euroscarf
*yck3*Δ	BY4742; *yck3*::*kanMX4*	Euroscarf
*hog1*Δ	BY4742; *hog1*::*kanMX4*	Euroscarf
*sho1*Δ	BY4742; *sho1*::*kanMX4*	Euroscarf
*plc1*Δ	BY4742; *plc1*::*kanMX4*	Euroscarf
*arg82*Δ	BY4742; *arg82*::*kanMX4*	Euroscarf

*ipk1*Δ	BY4742; *ipk1*::*kanMX4*	Euroscarf
*kcs1*Δ	BY4742; *kcs1*::*kanMX4*	Euroscarf
*vip1*Δ	BY4742; *vip1*::*kanMX4*	Euroscarf
*apl5*Δ	BY4742; *apl5*::*kanMX4*	Euroscarf
W303 (*ssk2*Δ,*ssk22*Δ)	W303; *ssk2*::*URA3*, *ssk22*::*LEU2*	M.Gustin
W303 (*hog1*Δ, *fab1*Δ)	W303; *hog1*::*TRP1*, *fab1*::*HIS3*	This study
BY4742 (*VAC14-GFP*)	BY4742; *VAC14-GFP*:*HIS3MX6*	This study
*vps34*Δ (*VAC14-GFP*)	*vps34*Δ; *VAC14-GFP*:*HIS3MX6*	This study
*fab1*Δ (*VAC14-GFP*)	*fab1*Δ; *VAC14-GFP*:*HIS3MX6*	This study
*fig4*Δ (*VAC14-GFP*)	*fig4*Δ; *VAC14-GFP*:*HIS3MX6*	This study
*vps45*Δ + pUG36-VAC7	*vps45*Δ	This study
*apm3*Δ + pUG36-VAC7	*apm3*Δ	This study
BY4742 (*VPS41-3HA*)	BY4742; *VPS41-3HA*:*HIS3MX6*	This study
*yck3*Δ (*VPS41-3HA*)	*yck3*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*fab1*Δ (*VPS41-3HA*)	*fab1*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*vac14*Δ (*VPS41-3HA*)	*vac14*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*fig4*Δ (*VPS41-3HA*)	*fig4*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*atg18*Δ (*VPS41-3HA*)	*atg18*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*ldb19*Δ (*VPS41-3HA*)	*ldb19*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*hog1*Δ (*VPS41-3HA*)	*hog1*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*sho1*Δ (*VPS41-3HA*)	*sho1*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*ssk2*Δ *ssk22*Δ (*VPS41-3HA*)	W303 (*ssk2*Δ, *ssk22*Δ); *VPS41-3HA*:*HIS3MX6*	This study
*hog1*Δ *fab1*Δ (*VPS41-kanMX6*)	W303 (*hog1*Δ, *fab1*Δ); *VPS41-3HA*:*kanMX6*	This study
*plc1*Δ (*VPS41-3HA*)	*plc1*Δ; *VPS41-3HA*:*HIS3MX6*	This study

*arg82*Δ (*VPS41-3HA*)	*arg82*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*ipk1*Δ (*VPS41-3HA*)	*ipk1*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*kcs1*Δ (*VPS41-3HA*)	*kcs1*Δ; *VPS41-3HA*:*HIS3MX6*	This study
*vip1*Δ (*VPS41-3HA*)	*vip1*Δ; *VPS41-3HA*:*HIS3MX6*	This study
BY4742 (*VPS41-GFP*)	BY4742; *VPS41-GFP*:*HIS3MX6*	This study
*yck3*Δ (*VPS41-GFP*)	*yck3*Δ; *VPS41-GFP*:*HIS3MX6*	This study
*plc1*Δ (*VPS41-GFP*)	*plc1*Δ; *VPS41-GFP*:*HIS3MX6*	This study
*arg82*Δ (*VPS41-GFP*)	*arg82*Δ; *VPS41-GFP*:*HIS3MX6*	This study
*ipk1*Δ (*VPS41-GFP*)	*ipk1*Δ; *VPS41-GFP*:*HIS3MX6*	This study
*kcs1*Δ (*VPS41-GFP*)	*kcs1*Δ; *VPS41-GFP*:*HIS3MX6*	This study
*vip1*Δ (*VPS41-GFP*)	*vip1*Δ; *VPS41-GFP*:*HIS3MX6*	This study
VPS41^{S24,26,28A}*-3HA*	BY4742; *VPS41* ^{S24,26,28A}*-3HA*:*HIS3MX6*	This study
VPS41^{S44A}*-3HA*	BY4742; *VPS41* ^{S44A}*-3HA*:*HIS3MX6*	This study
VPS41^{S24,26,28,44A}*-3HA*	BY4742; *VPS41* ^{S24,26,28,44A}*-3HA*:*HIS3MX6*	This study
VPS41^{G171R}*-3HA*	BY4742; *VPS41* ^{G171R}*-3HA*:*HIS3MX6*	This study
VPS41$^{Q803stop\ codon}$*-3HA*	BY4742; *VPS41* $^{Q803stop\ codon}$*-3HA*:*HIS3MX6*	This study
VPS41^{S24,26,28,44A}*-GFP*	BY4742; *VPS41* ^{S24,26,28,44A}*-GFP*:*HIS3MX6*	This study
*fab1*Δ*(VAC14-GFP:HIS3)* + pEGKT-FAB1	*fab1*Δ; *VAC14-GFP*:*HIS3MX6*	This study
*fab1*Δ*(VAC14-GFP:HIS3)* + pEGKT-*FAB1*$^{kin-}$	*fab1*Δ; *VAC14-GFP*:*HIS3MX6*	This study
*fab1*Δ*(VAC14-GFP:HIS3)* + pEGKT-*FAB1*$^{fyve-}$	*fab1*Δ; *VAC14-GFP*:*HIS3MX6*	This study
*fab1*Δ*(VAC14-GFP:HIS3)* + pEGKT-*FAB1*$^{CCT\text{-}like-}$	*fab1*Δ; *VAC14-GFP*:*HIS3MX6*	This study
*fab1*Δ*(VAC14-GFP:HIS3)* + pEGKT-*FAB1*$^{PIPKIII\ unique-}$	*fab1*Δ; *VAC14-GFP*:*HIS3MX6*	This study
SEY6210 (FIG4-GFP:HIS3,VAC14-mCHERRY:TRP1)	*MATα; leu2-3,112; ura3-52; his3-Δ200; trp1-Δ901; lys2-801; suc2-Δ9; FIG4-GFP:HIS3; VAC14-mCHERRY:TRP1*	This study

2.2 Media composition and solutions

2.2.1 Luria Broth (LB) (1L)

Yeast Extract (0.5%, w/v), Tryptone (1.0%, w/v), NaCl (1.0%, w/v)

2.2.2 LB + 100 µg/ml Ampicillin or 34 µg/ml chloramphenicol (1L)

LB + Ampicillin (100 µg/ml) or chloramphenicol (34 µg/ml)

2.2.3 LB agar (1L)

LB + Bacto agar (2.0%, w/v)

2.2.4 LB agar + 100 µg/ml Ampicillin or 34 µg/ml chloramphenicol (1L)

LB agar + Ampicillin (100 µg/ml) or chloramphenicol (34 µg/ml)

2.2.5 SOC medium (1L)

Yeast Extract (0.5%, w/v), Tryptone (2.0%, w/v), NaCl (0.05%, w/v), $MgCl_2$ (10 µM), $MgSO_4$ (10 µM), Glucose (20 mM)

2.2.6 Yeast Extract Peptone Dextrose (YPD) (1L)

Yeast Extract (1.0 %, w/v), Bacto Peptone (2.0 %, w/v), Glucose (2.0 %, w/v), Adenine (100mg), Uracil (100mg)

2.2.7 YPD + 0.9 M NaCl or 0.4 M NaCl

YPD + NaCl (0.9 M or 0.4 M)

2.2.8 YPD agar (1L)

YPD + Bacto agar (2.0%, w/v)

2.2.9 Amino-acid stock powder

Isoleucine (2.00g), Serine (2.01g), Adenine (2.03g), Glycine (1.99g), Phenylalanine (2.06g), Proline (2.09g), Valine (2.07g), Arginine (1.99g), Alanine (2.08g), Threonine (2.02g), Aspartic acid (2.11g), Glutamic acid (2.01g), then powder is milled in the A11 basic analytical mill (IKA) for 1 minute and then stored at 4°C.

2.2.10 Synthetic complement (SC) + Glucose (1L)

Yeast nitrogen base (w/o amino acids & w/o ammonium sulfate) (1.7g), Ammonium sulfate (5g), Amino acid stock powder (1g), Tryptophan (100mg), Histidine (100mg), Leucine (100mg), Lysine (100mg), Methionine (100 mg), Uracil (100mg)

The pH of SC was adjusted to 5.7 with 5M NaOH, just prior to the addition of 20 g of Bacto agar (if required). The media was then autoclaved for 15 mins at 121°C, cooled and dextrose added to 2% (from a 40% w/v stock) prior to use.

To prepare selective SC medium, the corresponding amino acid was omitted. For example, to make SC-Ura, 100mg Uracil was left out.

2.2.11 50x Tris-Acetate-EDTA buffer (TAE)

Tris (2.0 M), Glacial acetic acid (0.94 M), 0.05 M EDTA (pH 8.0)

2.2.12 6x DNA gel electrophoresis sample buffer

Tris.HCl (10mM), Glycerol (50%, v/v), Bromophenol blue (0.2%, w/v)

2.2.13 2x SDS-PAGE sample buffer

Tris.HCl (120 mM, pH 6.8), Glycerol (10%, v/v), SDS (4%, w/v), β-mercaptoethanol (5%, v/v), Bromophenol blue (0.004%, w/v)

2.2.14 10x SDS-PAGE running buffer

Tris (250 mM), Glycine (2.5 M), SDS (1%, w/v)

2.2.15 10x blot transferring buffer

Tris (250 mM), Glycine (1.87 M)

2.2.16 Phosphate buffered saline (PBS)

Sterile PBS (8.2 mM Na_2HPO_4, 1.5mM KH_2PO_4, 139mM NaCl, 3mM KCl) was prepared using PBS tablets (Oxoid) and filter sterilized via a 0.22 μm filter or autoclaved.

2.3 Polymerase chain reaction (PCR)

All PCRs were performed with Expand High Fidelity PCR system (Roche), in a TECHNE TC-3000 (Staffordshire, UK) thermal block cycler. Two reaction mixes were prepared based on manufacturers instructions as follows: Deoxyribonucleotide mix (200 μM of each dNTP), upstream primer (300 nM), downstream primer (300 nM), and target DNA were mixed in one "master mix" made up to 50 μl total volume with ice -cold ddH_2O, whilst 10x buffer (5 μl), and Expand enzyme (0.75 μl) were made up to 50 μl total volume with ddH_2O and mixed, before mixing with the master mix 1. All PCRs were then aliquotted to 50 μl, since this was the volume that the thermal cycler was calibrated for. The temperature cycling profile was as is shown in Table 2.2. A two step PCR program was used that used two different Tm values. The T_{m1} was calculated by assuming that all the bases in the primer sequence were complimentary to those on the target DNA whereas the T_{m2} value was obtained by calculating the melting temperature of only that portion of the primer that is homologous to the target DNA in the early rounds of amplification: hence sequences encoding mutations or restriction sites not present in the target DNA were excluded from the calculation of this second melting temperature in order to more accurately reflect the lower affinity of primers for target DNBA during early PCR cycles.I found that this two step PCR program worked much more efficiently than PCR employing a single measurement of Tm for amplification of my target fragments.

Table 2.2: PCR cycling profile

94°C	94°C	T_{m1} - 5°C	68°C	94°C	T_{m2} - 5°C	68°C	68°C	4°C
4min	0.5min	1min	2min/kb	0.5min	1min	2min/kb	10min	12 hours
1 cycle	15 cycles			25 cycles			1 cycle	1 cycle

2.4 DNA sequencing

The DNA sequencing was performed by the Functional Genomics and Proteomics Unit, University of Birmingham (Birmingham, UK). The reaction mix was prepared as follows: template (100 ~ 500 ng), primer (3.2 pmol), then ddH_2O was added to total volume of 10µl. The Genomics facility then added a Terminator Reaction Mix to the target DNA supplied by our lab to give a final volume of 20µl. The reaction mixes were then amplified via PCR and the resulting chain-termination reaction resolved using an ABI sequencing device that separated the dye labelled fragments by capillary electrophoresis. The sequencing data were then analysed in Chromas (version 1.45).

2.5 Plasmid construction and yeast knock-in/out fragment

All DNA restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Inc (Beverly, MA). Standard molecular biology techniques were used for DNA manipulations. All constructs were generated by transforming into *E. coli* TOP10 competent cells and confirmed by DNA sequencing. All yeast knock-in/out fragments were amplified from the knock-in plasmids pFA6a-3HA-HIS3MX6 and/or pFA6a-GFP(S65T)-HIS3MX6 and knock-out plasmids pFA6a-kanMX6 or pFA6a-HIS3MX6 using corresponding oligos (Longtine *et al*., 1998). All oligos were synthesized by Alta Biosciences, University of Birmingham, UK and are listed in Table 2.3.

2.5.1 pET-15b-*FIG4* & pGEX-6P-1-*FIG4*

The FIG4 ORF (open reading frame) was amplified from original pACT2-*FIG4* (a gift of Dr T. Kobayashi from Dr Dove's lab) using oligonucleotides that incorporated unique *Nde*I and *Bam*HI restriction enzyme sites at the 5' and 3', respectively. The PCR product was digested with *Nde* I and *Bam*HI, then sub-cloned into *Nde*I-*Bam*HI-digested pET-15b (Novagen), generating an N-terminally His_6-tagged FIG4 allele for expression in BL21-star (DE3)-RIL cells. *FIG4* was also cloned into vector pGEX-6P-1 for expression of GST-Fig4p in BL21-star (DE3)-RIL cells.

2.5.2 pGEX-6P-1-*YMR1*

The cDNA of *YMR*1 was amplified by PCR from yeast *S. cerevisiae* genomic DNA with 5'- *Xho* I and 3'- *Not* I oligonucleotides and inserted into pGEX-6P-1 vector (Amersham) containing same restriction enzyme sites, generating an N-terminally GST-tagged *YMR*1 for expression in BL21-CodonPlus(DE3)-RIL cells..

2.5.3 pEGKT-*FIG4*

The pEGKT-*FIG*4 construct was generated by ligating PCR-duplicated *FIG*4 containing unique 5'- *Bam*H I and 3'- *Hind* III sites to pEGKT vector digested using same restriction enzymes. The resulting recombinant was expressed in yeast strain FY833.

2.5.4 VAC14-GFP fragment and pUG36-*VAC7*

VAC14-GFP fragment was amplified by PCR from genomic DNA of yeast BY4741 (VAC14-GFP:HIS3) which was previously obtained from Invitrogen. The PCR

product was knocked into wild type and several different *S. cerevisiae* null strains in order to investigate the localisation changes of Vac14p-GFP. pUG36-*VAC7* is from Dove's lab previous work.

2.5.5 pEGKT-*FAB1* and pEGKT-*FAB1* with mutation in four distinct functional domains individually

In order to study which functional domain(s) of Fab1p may influence Vac14p vacuole membrane localisation, four constructs of pEGKT-*FAB1* which have the disrupted functional domains in either the FYVE, PIPKIII unique, CCT-like or PIPKinase domains (constructs originally made by Dr Frank Cooke, UCL) were introduced into *fab1*Δ(VAC14-GFP:HIS3). The conserved domains being considered are defined in Michell *et al*., 2007 and the mutations were made via site-specific mutagenesis by overlap extension (see Section 16 in this chapter).

Table 2.3: Oligos were used in this study

Name	Sequence	comments
pET-15b-*FIG4*	TTTTTT***CATATG***ATGAACAATGATGCAATGGAGCATACCCTT	*NdeI*
	TTTTTT***GGATCC***TTATTGAAAATCAAGTTGTATATCTTTAGAAAAGCACAAATCC	*BamHI*
pGEX-6P-1-*FIG4*	TTTTTT***GGATCC***ATGAACAATGATGCAATGGAGCAT	*BamHI*
	TTTGTT***CTCGAG***TTATTGAAAATCAAGTTGTATATCTTTAGAAAA	*XhoI*
pGEX-6P-1-*YMR1*	TTTTTT***CTCGAG***ATGGAGTACATCAAGATTGCCAAAGTATCT	*XhoI*
	TTTTTT***GCGGCCGC***TCACTTTCTATTAAACATATCAAATCCAAAAATTGAC	*NotI*
pEGKT-*FIG4*	TTTTTT***GGATCC***ATGAACAATGATGCAATGGAGCAT	*BamHI*
	TGGGTT***AAGCTT***TTATTGAAAATCAAGTTGTATATCTTTAGAAAA	*HindIII*
VAC14-GFP	ATTTGATCTTAGCTCCTGCCTCTTTGACCC	
	AAGGGTTAACTTTCAAACACATTACAGGGATGAGTT	
VPS41-GFP or 3HA	AAGCGATATTGAAATTAATGACGACTTAAATGGTGTTTTA***CGGATCCCCGGGTTAATTAA***	*F2 primer*
	AAGTGTACACTTGCCTTGTGTATTAAATGATGATTCGATA***GAATTCGAGCTCGTTTAAAC***	*R1 primer*
YCK3-GFP or 3HA	TAAAATATACAAATATTGTTGTTGCTGTTTTTGTTGCTGT***CGGATCCCCGGGTTAATTAA***	*F2 primer*
	AAAAAAAAAGGAAAAAGAGAAAAGTATAAAAATCGCTTTA***GAATTCGAGCTCGTTTAAAC***	*R1 primer*
PLC1-Knock out	TAAACGTACAACGGTAAGGTCATTCACGCAGTGTATATGA***CGGATCCCCGGGTTAATTAA***	*F1 primer*
	CGTATTTATGAATATGTGTATTTGGCCGGAAAAAGATCGC***GAATTCGAGCTCGTTTAAAC***	*R1 primer*
IPK1-Knock out	AATTGTCAGAGATAAGTTCCTTTTTTGAAAAGAAAGATCG***CGGATCCCCGGGTTAATTAA***	*F1 primer*
	GCATCTGCCAGTACCAAAGGTGGAAAGAAAAGTATACAGT***GAATTCGAGCTCGTTTAAAC***	*R1 primer*
KCS1-Knock out	TTTTTATTTTTTGTATATATAAAACTAAAGCTAAAAGACT***CGGATCCCCGGGTTAATTAA***	*F1 primer*
	GCGCAGCTAAAAGAATATTCATTAGTTCTATCCTTTCTTT***GAATTCGAGCTCGTTTAAAC***	*R1 primer*
VIP1-Knock out	CAAAAGCATCTCGTAGCATATTAATATATTGCAGAAGGTC***CGGATCCCCGGGTTAATTAA***	*F1 primer*
	TTTAGTTTTGGGTTACTAAATTAAAAATTGGGTGTGATCA***GAATTCGAGCTCGTTTAAAC***	*R1 primer*
VPS41$^{S\text{-}A}$-A (FW)	GTCTGACCTGGTTATGCAGGTCATGCTCTA	no mutation
VPS41$^{S\text{-}A}$-B (RV)	CGTC***GGC***GAT***GGC***ATT***GGC***CTCATCAATTGTTCGTTCGCCT	*S*- 24,26,28 -*A*
VPS41$^{S\text{-}A}$-C (FW)	G***GCC***AAT***GCC***ATC***GCC***GACGAGAATAATGTTGATAATAAGAG	*S*- 24,26,28 -*A*
VPS41$^{S\text{-}A}$-D (RV)	TTAGCCTGTACCGATCACTGGAAACTGGAT	no mutation
VPS41$^{S\text{-}A}$-E (RV)	TTTGTTGG***TGC***AGTTACATTAACGTCTTCCCTC	*S*- 44 -*A*
VPS41$^{S\text{-}A}$-F (FW)	AATGTAACT***GCA***CCAACAAAAAGTGTTAGTTGCATATCAC	*S*- 44 -*A*
VPS41^{G171R} (RV)	CTGTCCCGTCAATAGA***TCG***CGTAGCAAAATATTTTCCGTCAGTG	*G*-171-*R*
VPS41^{G171R} (FW)	GCTACG***CGA***TCTATTGACGGGACAGTGATTATCGGA	*G*-171-*R*
VPS41$^{Q803stop\ codon}$	AGATACGGACGACTCGTTCATGATAAGTCCTTATGAAAAT***CGGATCCCCGGGTTAATTAA***	*F2 primer*
	GAAGGCTTTGTCGGTCGTATTCAGAATAAAGTTCAATTAG***GAATTCGAGCTCGTTTAAAC***	*R1 primer*

2.6 Transformation of *E.coli* (Hanahan 1983)

Plasmid DNA (0.1-10μg) was added to an aliquot of competent *E.coli* cells (100μl), incubated on ice for 30 min and heat shocked at 42°C for 45 seconds. SOC medium (1.0 ml) was added to the cell suspension, mixed gently and incubated at 37°C for 60 min. Cells were collected through centrifugation at 6000rpm for 10 sec, resuspended in 150 μl fresh LB medium and plated onto a LB + 100 μg/ml of Ampicillin agar plate. Incubations were at 37°C for 16-20 h.

2.7 Construction of BL21 star (DE3)-CodonPlus-RIL

The BL21 star (DE3) *E.coli* strain from Invitrogen carries a mutated *rne* gene (*rne131*) which encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA, resulting in an increase in mRNA stability. Whereas BL21-CodonPlus(DE3)-RIL *E.coli* strain from Stratagene harbours a ColE1-compatible, pACYC-based plasmid (~3.5kb) containing extra copies of the *argU*, *ileY*, and *leuW* tRNA genes. These genes encode tRNAs that recognize the arginine codons AGA and AGG, the isoleucine codon AUA, and the leucine codon CUA, respectively. The CodonPlus-RIL strains have the available tRNAs that most frequently restrict translation of heterologous proteins from organisms that have AT-rich genomes. In addition, the pACYC-based plasmid confers the Chloramphenicol resistance which is convenient to select in LB agar plates containing 34μg /ml chloramphenicol.

The benefits of both strains were combined by extracting the pACYC-based plasmid from BL21-CodonPlus(DE3)-RIL, then transforming it to BL21 star (DE3) *E.coli* strains. Theoretically the engineered novel strain should be able to grow in LB-agar medium containing Chloramphenicol but BL21 star (DE3) can not. This novel strain

was designated ‘ BL21 star (DE3)-RIL’.

2.8 Recombinant Protein expression and purification

2.8.1 Expression and purification of GST-Ymr1p fusion protein

Ymr1p was expressed in *E. coli* strain of BL21-CodonPlus (DE3)-RIL as a GST-tagged full-length fusion protein. Protein expression was induced for 2 hours at 30°C by the addition of 1mM isopropyl-β-d-thiogalactopyranoside (IPTG) (Roche). Briefly, bacteria from a 3-litre culture were disrupted with 1% of lysozyme and sonication in 40 ml of 100 mM Hepes (pH 7.5), 150 mM NaCl, 5mM Benzamidine, 10mM EDTA as well as appropriate amount of protease inhibitor cocktail (Sigma, P8465 added immediately prior to use). Triton X-100 was added to 1% (v/v), and the crude lysate was centrifuged at 9,447 xg (8,000 rpm)(Beckman J2-MC, rotor JA18) for 15 min to remove insoluble material. The supernatant was decanted to a fresh high-speed tube for an additional high-speed centrifugation at 64,230 xg (25,000 rpm) (Beckman L-70 ultracentrifuge, rotor Type 70 Ti) to remove the remaining insoluble materials. Proteins were then isolated from the resulting cleared lysates by the addition of 2 ml of equilibrated Glutathione sepharose 4B (Amersham biosciences, prepared in 1:1 slurry with lysis buffer) and incubated for 2 h at 4°C. Resins were then washed twice with lysis buffer with detergent and twice with lysis buffer without detergent. The fusion protein was eluted from resin with 2ml of elution buffer (50mM Tris-HCl, pH 8.5, 150mM NaCl, 5mM Benzamidine, 20mM Glutathione). The eluate was collected in 4 x 0.5 ml fractions. DTT (dithiothreitol) and glycerol were then added to 1 mM and 50% (v/v), respectively, and the protein was stored at -80°C until use.

2.8.2 Expression and purification of GST-Fig4p fusion protein

Expression of GST-Fig4p was accomplished as for GST-Ymr1p with the following modifications: the expression system is *E. coli* strain of BL21-Star (DE3)-RIL. Induction was carried out with 1mM IPTG for 2 hours at 28°C and the lysis buffer was 50 mM Bis-tris (pH 6.5), 150 mM NaCl, 5mM Benzamidine, 1mM EDTA. The rest of the protocol was as above.

2.8.3 Expression and purification of GST-SKIP fusion protein

The protocol was similar to that as described in Section 2.8.1 save for the following modifications: The protein expression was induced at 16°C overnight by the addition of IPTG to a final concentration of 0.1mM. The lysis buffer and washing buffer were 50 mM Tris (pH:7.5), 2 mM EDTA, 10 mM Benzamidine, 10μg/ml Trypsin Inhibitor (Soyabean), and PMSF (phenylmethanesulfonyl fluoride) and DTT were present at final concentration of 1mM (added before immediately use). The Elution buffer was 50mM Tris (pH: 8.0), 500mM NaCl, 20 mM Glutathione.

2.8.4 Expression and purification of His-Fig4p fusion protein

Recombinant plasmid of pET-15b-*FIG4* was transformed into *E. coli* strain BL21-star (DE3)-RIL for generating His6-Fig4p fusion protein. Briefly, protein expression was induced at 18 °C overnight by addition of 0.1mM IPTG. Cell pellets from a one litre culture were resuspended in 30ml of lysis buffer containing: 50mM Tris-HCl (pH: 8.0), 300mM NaCl and 20mM Imidazole, one EDTA-free protease inhibitor cocktail tablet was added (Roche, Cat number: 11873580001) just prior to use. The cell suspension was lysed by addition of 1mg/ml lysozyme and cells were incubated for 30 min at 4°C to give the enzyme time to work. Cells were then sonicated for 5 x 15s,

and then Triton X-100 was added to 1% and crude lysate was centrifuged at 9,447 xg for 10 min at 4°C. The supernatant was decanted into a fresh tube and subjected to additional 64,230 xg centrifugation for 20 min at 4°C. The resulting cleared lysate was incubated with 2ml of pre-equilibriated Ni-NTA agarose (Qiagen, prepared in a 1:1 slurry with lysis buffer) for 2 hour at 4°C with gentle agitation. Resin was then washed twice with lysis buffer with detergent and twice with lysis buffer without detergent. The fusion protein was eluted from Ni-NTA resin with 2ml of elution buffer: 50mM Tris-HCl, pH 7.5, 150mM NaCl, 200mM Imidazole. The eluate was collected in 4 x 0.5 ml fractions. DTT and glycerol were then added to 1mM and 50% (v/v), respectively, and the protein was stored at -80°C until use.

2.8.5 Expression and purification of GST-Fig4p fusion protein from budding yeast *S. cerevisiae*

The yeast strain FY833 was transformed with pEGKT-*FIG4* according to the standard yeast transformation protocol. Cell culture and protein induction were performed as follows: 200 ml of SC-URA$^-$ media, containing 2% dextrose was inoculated with the above strain and grown to late log phase (approximately 1~3 x 10^7 cells/ml). Then this inoculum was added to 4 litres of fresh SC-URA$^-$ containing 2% raffinose and grown to a mid log phase (approximately 0.5~1 x 10^7 cells/ml) at 30°C with good aeration. An additional 40g of yeast extract, and 80g Bacto-peptone (solid) was then added to the culture and the GST-Fig4p fusion protein was induced for 4~6 hours at 30°C by addition of 2% galactose. The cells from 4-litre culture were harvested by centrifugation at 6000 xg and resuspended in 50 ml of lysis buffer: 50mM Tris (pH:7.5), 150mM NaCl, 1mM EDTA, 5mM Benzamidine and appropriate amount of Protease inhibitor cocktail (Sigma P8215) before being disrupted in a bead beater. To

the resulting total lysate was added Triton-X-100 to 1% (v/v) and the lysate incubated on ice for 30 min. Debris was removed by centrifugation at 9,447 xg for 10 min, then the supernatant centrifuged again at 64,230 xg for 15 min. The resulting cleared lysate was filter through a 0.4 μm filter and then directly loaded onto Q Hi-Trap mini column (Pharmacia) and a 50 ml gradient from 150mM to 2M NaCl run to resolve the bound protein into distinct peaks. Fractions were collected at (25 x 2 ml) and analysed for target protein by western blotting with anti-GST antibody. The fractions containing fusion protein GST-Fig4p were combined and purified further using standard Affinity purification of the GST fusion protein as described above.

2.9 SDS-PAGE (Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis)

6x SDS sample buffer (58mM Tris-HCl pH 6.8, 1.6% glycerol, 5% SDS, 0.1M DTT and 1.2mg bromophenol blue) was added to protein samples to a final concentration of 1x. Samples were then vortexed and incubated at 100°C for 5 min. Proteins were separated by SDS-PAGE. Electrophoresis was performed at 120V for approximately 1 hour until the bromophenol blue dye-front reached the end of the gel or until the desired separation of the prestained molecular weight markers was achieved.

2.10 Western blotting

Proteins separated by SDS-PAGE were transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences) for further analysis. The nitrocellulose membrane and two pieces of 3MM filter paper (Whatman) were cut to the size of the separating gel and pre-soaked in the blotting buffer. The gel was removed from the cassette and put into the blotting buffer. A blotting cassette was assembled according

to the manufacturer's instructions, namely in the order of a sponge, two filter papers, gel (rinsed with blotting buffer), a nitrocellulose membrane, two filter papers and sponge, from black (cathode) to the white (anode). The assembled blotting cassette and an ice block for cooling were inserted into the transfer tank with blotting buffer, the transfer was performed at 400 mA for 1 hour.

2.11 Immunochemical protein detection by Enhanced Chemiluminescence (ECL)

After protein transfer had completed, the nitrocellulose membrane was rinsed with distilled water and incubated in blocking buffer (5% skimmed milk powder dissolved in PBST containing 0.1% Tween 20 in PBS) for 1 h at RT or at 4°C overnight on a shaker. Following blocking, the membrane was placed in a heat-sealed bag with 5 ml primary antibody (diluted in blocking buffer), incubated for 1 h at RT with shaking. The membrane was then washed six times (5 min per wash) in PBST washing buffer. The membrane was incubated with an appropriate horseradish peroxidise-conjugated secondary antibody in an appropriate dilution on a shaker for 1 hour at RT. A list of the primary and secondary antibodies with appropriate dilutions used in this study is shown in Table 2.4. The membrane was then washed at least six times (5 min per wash) in washing buffer and bound antibodies were detected by ECL detection reagent (Amersham Biosciences). ECL solution A and B were mixed 1:1 immediately prior to adding to the membrane. Excess reagent was removed and the membrane was wrapped in Saran wrap. Chemiluminescence was detected by Gel Documentation System, Fluor-S^{TM} Multimager (Bio-Rad). Different exposure times were used depending on the intensity of the protein bands.

Table 2.4

Protein detected	Primary antibody	Secondary antibody
Vps41p-3HA	Monoclonal Antibody, HA.11 from mouse, COVΛCE, 1:1000	HRP-Goat Anti-Mouse IgG (H+L), Invitrogen, 1:1000
Vps41p-GFP	GFP (B-2), mouse monoclonal IgG_{2a}, Santa Cruz Biotechnology, 1:500	HRP-Goat Anti-Mouse IgG (H+L), Invitrogen, 1:1000

2.12 High efficiency transformation of budding yeast (Adam *et al.*, 1997)

Cells sub-cultured at 5 x 10^6 cells/ml in YPD medium (2 ml) were grown at 30°C to 2~5 x 10^7 cells/ml. Cells were collected, resuspended in 1ml of 100 mM lithium acetate (LiAc). The cell suspension was centrifuged at 1000g for 30 seconds and the supernatant was removed. Add the following to the cell pellet in the exact order: 240 µl of 50% PEG, 36 µl of 1.0M LiAc, 50 µl of boiled ssDNA, 25 µl of ddH_2O containing the DNA (~ 1ug) to be transformed, and the resulting mixture were mixed by vortexing vigorously until the cells were completely resuspended, then incubated at 30°C for 30 min followed by heat shock at 42°C for 25 min. Cells were collected by centrifugation at 1000 x*g* for 15 seconds, the supernatant was discarded and the cells were resuspended in 200 µl of ddH_2O, plated on selective agar plates and incubated at 30°C for at least 36 hours.

2.13 Genomic DNA purification from budding yeast

The following method is adapted from the Gentra Puregene Handbook, second edition, Qiagen, p47~ 48, 09/2007. Briefly, 2 ml of exponentially growing cells were collected and resuspended in 300μl of Cell Suspension Solution (provided by the kit) with 1.5 μl of Lytic Enzyme Solution. The resulting mixture was incubated at 37°C for 30 min. Cells were then pelleted by centrifugation at 16,000g for 1 min and resuspended in 300 μl of Cell Lysis Solution plus 100 μl of Protein Precipitation Solution, and vortexed vigorously for 20 s at high speed, then centrifuged at 16,000g for 3 min. The supernatant (approx. 400 μl) was decanted into a fresh tube pre-filled with 300 μl of isopropanol. Samples were then mixed well and centrifuged at 16,000g for 1 min. The supernatant was then carefully discarded and the tubes drained by inverting then on a clean piece of absorbent paper. Then DNA pellet was washed once with 300 μl of 70% ethanol and air dried for up to 10 min. Afterwards, the DNA pellet was then resuspended in 50 μl of DNA Hydration Solution plus 1.5 μl of RNase A Solution and incubated at 37°C for 30 min, then incubated at 65°C for 1 hour to dissolve the DNA.

2.14 Disruption (Knock-out) and Epitope tagging (Knock-in) of budding yeast strains

Deletion and tagging of genes in chromosome in budding yeast were done by homologous recombination via transforming corresponding PCR fragments into the desired strains.

For deletion of certain ORFs, the corresponding auxotrophic marker was amplified from the knock-out plasmids pFA6a-kanMX6 or pFA6a-HIS3MX6 (Longtine MS, *et*

al., 1998) by PCR using primers that contained sequences for annealing to the template plasmid, flanked by sequences that encode regions immediately before the start codon and after the stop codon of the corresponding ORFs. PCR products were used to transform yeast strains.

For the chromosomal HA and GFP tagging at 3'-terminal of target gene, the knock-in fragment was amplified from the plasmids pFA6a-3HA-HIS3MX6 and pFA6a-GFP(S65T)-HIS3MX6 (Longtine *et al*., 1998) by PCR using oligonucleotides that included the sequences for annealing to the template plasmid, flanked by sequences complementary to the 40 nucleotides immediately before and after the stop codon of the target gene. PCR products were used to transform yeast strains.

All putative knock out and knock-in strains were confirmed by sequencing the appropriate locus of genomic DNA recovered from transformed cells. All proteins to be over-expressed were N-terminally tagged whereas the epitope tagging in yeast knock-in strains was uniformly C-terminal so as not to affect expression levels from the native promoter.

2.15 Site-specific mutagenesis by overlap extension (Sambrook and Russell, 2001)

Four primers are needed to introduce a site-specific mutation by overlap extension. The first pair of primers (A & B) is used to amplify the DNA that contains the mutation site together with upstream sequences. Of which, the primer A harbours a wild-type sequence whereas primer B contains the mutation(s) to be introduced. Similarly, the second pair of primers (C & D) is used to amplify the DNA that

contains the mutation sites with downstream sequences. Of which, the primer D includes a wild-type sequence whereas primer C harbours the mutation(s) to be introduced.

The two sets of primers are used in two separate amplification reactions to amplify over-lapping DNA fragments (fragment AB and CD). The mutation(s) of interest is located in the region of overlap and therefore in both sets of resulting amplified fragments. The overlapping fragments are mixed, denatured and annealed to generate heteroduplexes that can be extended and, in a third PCR, amplified into a full-length DNA using two primers that bind to the extremes of the two initial fragments (The overall strategy is sketched in Fig. 2.1).

In this study, in order to construct BY4742 (VPS41^{S24,26,28,44A}-3HA:HIS3), firstly, BY4742 (VPS41^{S24,26,28A}-3HA:HIS3) was constructed and the genomic DNA was extracted. Then, the resulting genomic DNA was used as the template DNA to amplify the VPS41^{S24,26,28,44A}-3HA:HIS3 knock-in fragment by using a pair of oligos harbouring the S-44-A mutation. Finally, the resulting VPS41^{S24,26,28,44A}-3HA:HIS3 fragment was transformed into BY4742 wild type strains to obtain BY4742 (VPS41^{S24,26,28,44A}-3HA:HIS3) by growing in SC-HIS3 plate and putative mutants were confirmed by sequencing the target fragment of genomic DNA.

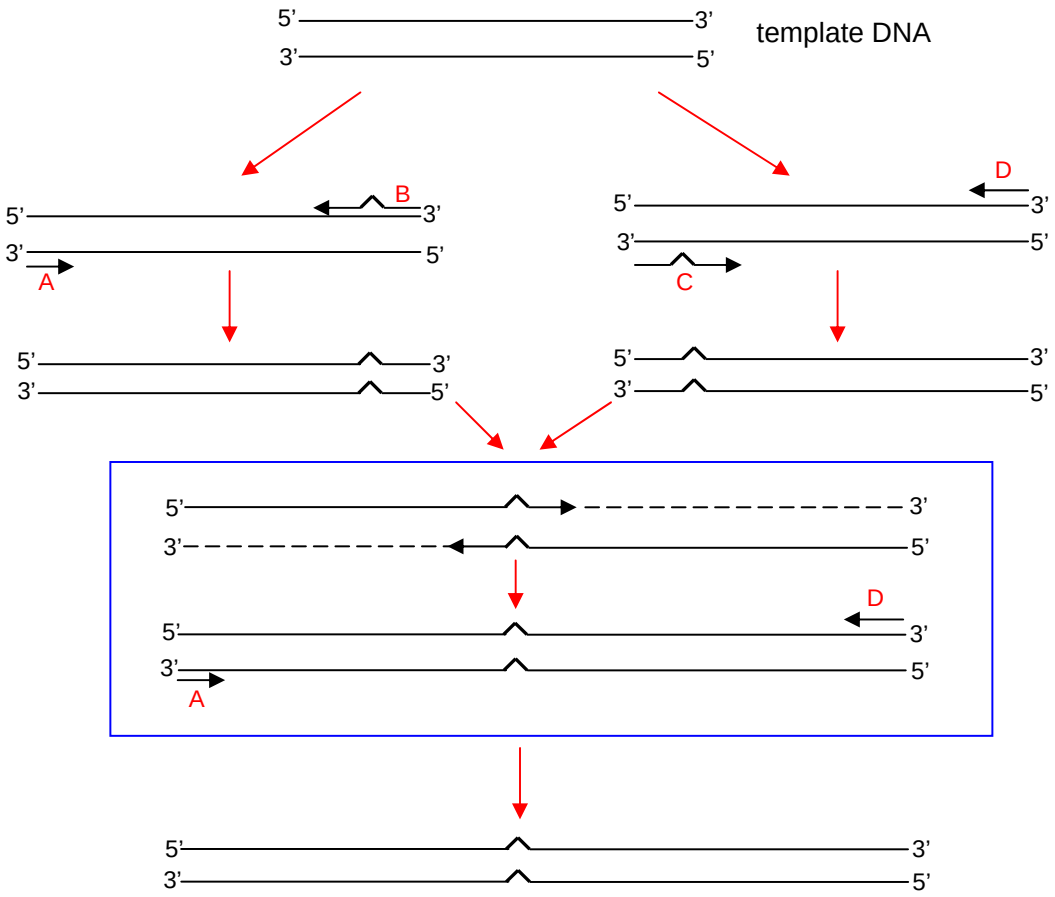


Figure 2.1: Site-directed Mutagenesis by Overlap Extension (from Sambrook and Russell, 2001)

2.16 Detection of phosphorylated Vps41-3HA by immunobloting

3 ml of exponentially growing cells were divided into two 1.5 ml tube and harvested by centrifuging cells at 1000g for 0.5 minutes: one tube of cell pellet was resuspended in 1 ml of fresh YPD medium and the other in 1 ml of fresh YPD containing 0.9M NaCl, then incubated at 30°C for another 15 minutes. These two fractions of cells (hyper-osmotically shocked and non-shocked) were collected again by centrifuging and re-suspended in 200 µl of 0.1M NaOH, and incubated at RT for 5 minutes. The cells were again centrifuged and the supernatant discarded. The cell pellet was washed twice in 300 µl of ddH_2O then resuspended in 50 µl of 1x SDS-PAGE loading

buffer and boiled for 3 minutes. Centrifugation was then carried out at maximum speed (13,000 xg) for 1 minute to remove the debris. The supernatant, containing the extracted total protein, could then be immediately analysed by the routine immunobloting protocol described above except the SDS-PAGE was run at 82V for 6 hours and the Vps41-3HA was probed by anti-HA primary antibody from mouse. For each experiment, equal amount of samples (equivalent to 5 x 10^6 cells) were analysed.

2.17 Quantification of immunoblot bands using ImageJ 1.42

In order to compare the ratio of phosphorylated Vps41p to that of non phosphorylated Vps41p, the intensity of bands on the western blot was measured using the ImageJ 1.42 program (National institutes of Health, Bethesda, MD). In brief, the blot picture was converted to 8-bit grayscale and inverted. Then the phosphorylated and/or nonphosporylated bands were selected by drawing a line around the boundary of the bands, largely based on good judgement about where the edges of the band were located. Although such selection criteria were subjective, particularly when the phosphorylated band and nonphosphorylated band were not sufficiently separated to allow the software resolve the bands as two separate identities, the results from this selection analysis did provide us with some ideas about the trends of quantification change. Once the interested bands were chosen by the freehand selection tool, the integrated intensity of bands was obtained by the software, using the Area and Mean Gray Value columns multiplied together. The resulting values obtained in this way were then divided by one another to determine the ratio of phosphorylated to non-phosphorylated Vps41p.

2.18 Immuno-purification of Vps41p-3HA from budding yeast

300 ml of exponentially growing cells of BY4742 (VPS41-3HA:HIS3) were harvested and subject to the hyper-osmotic stress by incubating in YPD with 0.9M NaCl for 15 minutes at 30°C. Cells were pelleted again and resuspended in 10 ml of 0.1M NaOH and incubated for 5 minutes at RT to weaken the cell-walls. Cells were collected by centrifugation and supernatant was discarded. In order to extract the total protein contents, the cells were lysed in 5 ml of lysis buffer (60mM Tris-HCl pH 6.8, 2% SDS, 2% glycerol, 3% β-mercaptoethanol) by boiling for 3 minutes. The resulting extracted supernatant (approx. 5 ml) contained the total protein content and so also contained the phosphorylated Vps41p-3HA. The total lysates were diluted 10 times using binding buffer (60mM Tris-HCl pH 6.8, 150mM NaCl) and pre-cleared by incubating with Protein G sepharose 4 fast flow (GE Healthcare) for 2 hours to remove the non-specific proteins.

To prepare the anti-HA antibody - conjugated protein G sepharose, 100 µl of protein G sepharose and 30 µl of anti-HA antibody were incubated in 500 µl binding buffer for 2 hours, then washed four times with 10 ml binding buffer: the resulting anti-HA antibody-conjugated protein G sepharose was added into 10 times diluted, pre-cleared 50 ml total lysates and incubated for 2 hours. After incubation, the beads were washed four times with 4 x 10ml binding buffer and the resulting beads (with the Vps41-3HA now bound to the beads) were resuspended in 80 µl of 1 x SDS-PAGE loading buffer, followed boiling for 3 minutes. The resulting supernatants were loaded onto the SDS-PAGE and the immuno-purified phosphorylated Vps41-3HA bands were detected by coomassie staining and excised for analysis by mass spectrometry conducted in the Functional Genomics and Proteomics Unit, University of Birmingham, UK.

2.19 Analysis of Vps41p phosphorylation sites by LC-CID-FT-ICR-MS (Liquid chromatography-Collision induced dissociation-Fourier transform-Ion cyclotron resonance-Mass spectrometry)

Sample preparation

The excised Vps41p bands from the coomassie stained SDS-PAGE gel was first destained with 100 mM ammonium bicarbonate, dehydrated with 100% acetonitrile, reduced with 10 mM DTT at 60°C for 15 minutes and alkylated with 50mM Iodoacetamide for 45 minutes at room temperature in the dark. Proteolytic digestion was performed with Trypsin Gold (Promega, Madison, WI) in 50 mM ammonium bicarbonte at a ratio of 1:50 (enzyme to protein, w/w) at 37°C overnight. The digest was diluted in 0.1% formic acid (Fisher Scientific, Leicestershire, UK) to give a final concentration of 50 fmol/µl.

Liquid chromatography

The resulting digest mixture from above was injected using online Micro AS autosampler and Surveyor MS pump (Thermo Fisher Scientific, Germany) to a 75 µm (internal diameter) Integrafrit (New Objective, USA) C_8 resolving column (length 10 cm). The peptides were separated using a binary solvent system consisting of (A) water (J.T.Baker, Holland) and 0.1% formic acid and (B) acetonitrile (J.T.Baker, Holland) and 0.1% formic acid. A linear 40 min gradient increasing the composition of (B) from 5% to 40% was used. The Triversa Nanospray source (Advion Biosciences, NY) was used to spray the peptide eluted in a flow rate of ~300 nL min^{-1} into the mass spectrometer. The nanoelectrospray voltage was typically +1.7 kV.

Mass spectrometry

All mass spectrometry experiments were performed on a Thermo Finnigan 7-Tesla LTQ FT mass spectrometer (Thermo Fisher Scientific, Germany). The mass spectrometer alternated between a full FT-MS scan (*m/z* 380 – 2000) and subsequent CID MS/MS scans of the three most abundant ions. Survey scans were acquired in the ICR cell with a resolution of 100,000 at *m/z* 400. Precursor ions were isolated and subjected to CID in the linear ion trap, which the product ion was acquired with a resolution of 25 000 at *m/z* 400. Collision activation for the experiment was performed in the linear trap using helium gas at a collision energy normalized to precursor *m/z* of 35% and q_{excite} = 0.25. The width of the precursor isolation window was *m/z* 2 and only multiply charged precursor ions were selected for MS/MS. Data acquisition was controlled by Xcalibur 2.0 software (Thermo Fisher Scientific, Germany). CID MS/MS data were searched against the non-redundant NCBI database and the possible phosphorylation site localisation was assessed by using the SEQUEST algorithm within the Bioworks 3.3.1 (Thermo Electron Corp., Bremen, Germany).

2.20 Staining of cells with the lipophilic dye FM4-64 (Vida and Emr, 1995)

3 ml of yeast cells were grown to mid-logarithmic phase, and harvested by centrifugation at 700g for 1 min, then re-suspend in 150 μl fresh medium with 40 μM FM4-64 (Molecular Probles, Inc), incubated with shaking at 30°C for 15min, washed once with fresh medium, and then resuspended in 1 ml of fresh medium to chase by incubating at 30°C for 1 hour.

Salt treatment of labelled cells (FM4-64) was performed by incubating cells in media containing 0.40M NaCl for 10 minutes or 60 minutes. This concentration of NaCl elicited similar changes in vacuole volumes as 0.9M NaCl but caused less perturbation of the refractive index of the medium, which led to better imaging (Duex *et al.*, 2006a). Cells were viewed at room temperature on a microscope.

2.21 Fluorescence microscopy

Yeast strains were cultured to 0.5 ~ 1 X 10^7 cells/ml in appropriate media and visualized on a Nikon Eclipse E600 microscope with an XF100-3 filter cube (Omega Optical). Images were acquired with an ORCA digital camera (Hamamatsu, Japan) and processed in Adobe Photoshop. Figures showed representative fields from multiple experiments.

2.22 HPLC analysis of inositol lipids

Deacylated inositol lipids or Gro*P*InsP_ns were resolved on a Partisphere 5-μM SAX column (240mm x 4 mm) protected by a SAX guard cartridge. To resolve Gro*P*Ins4*P* from Gro*P*Ins5*P* the following novel HPLC gradient was employed: A= dH_2O, B= 1.25M $(NH_4)_2HPO_4$ pH 4.4 (orthophosphoric acid); 0 min, 0% B; 5 min, 0% B; 10 min, 2% B; 55 min, 4.3% B, 95 min, 40% B; 100 min, 80% B; 105 min, 80% B; 110 min, 0% B. Note that this gradient relies upon a pH shift in order to resolve the two species of glycerophosphoinositols and that the exact elution position of these molecules, and their degree of resolution was variable depending upon the age of the column and the quality of the samples: some adjustments to the gradient were sometimes required to retain optimal separation.

Chapter 3 Regulation of PtdIns(3,5)P_2 metabolism

3.1 Introduction

Chapter 1 introduced the fact that PtdIns(3,5)P_2 is involved in the regulation of a variety of cellular processes, including membrane retrieval by retrograde trafficking, ubiquitin-dependent sorting of proteins from the TGN to vacuole via the MVB pathway, control of vacuole acidity and adaptation to hyperosmotic stress (reviewed by Efe *et al.*, 2005; Michell *et al.*, 2006; Dove and Johnson, 2007). In *S. cerevisiae*, PtdIns(3,5)P_2 is made by the sequential actions of the two lipid kinases: Vps34p and Fab1p, with Fab1p requiring Vac14p and Vac7p to express its PtdIns3*P* 5-kinase activity. PtdIns(3,5)P_2 is dephosphorylated to PtdIns3*P* and PtdIns5*P*, probably by Fig4p and Ymr1p respectively. The domain structures of proteins involved in PtdIns(3,5)P_2 metabolism are delineated in Fig. 3.1. This chapter summarises work designed to determine the relationships between some of these proteins.

To gain a better understanding of the machinery regulating PtdIns(3,5)P_2 synthesis in yeast, I studied the behaviour of Vac14p and Vac7p using GFP constructs. Knock-in strains expressing Vac14p-GFP at endogenous levels were used to examine Vac14p localisation in *fab1Δ*, *fig4Δ* and *vps34Δ* mutants. Vac14p-GFP is normally on the vacuole membrane but fails to localise there in these strains, suggesting that recruitment of Vac14p to the vacuole requires Fab1p, Fig4p and/or Vps34p (or their products). Furthermore, the study of Fig4p localisation revealed that Vac14p and Fig4p are co-residing on some micro-domains of the vacuole membrane, consist with the yeast two hybrid analysis that Vac14p may interact with Fig4p (Dove *et al*., 2002), also strengthening the idea that Vac14p and Fig4p may form a complex to control the

PtdIns(3,5)P_2 synthesis and turnover co-ordinately. Next, when I tried to find what factor(s) is/are affecting the localisation of Vac14p in *fig4Δ cells*, one of the effectors of PtdIns(3,5)P_2, Atg18p, emerged: over-expressed Atg18p partially rescued the mislocalisation of Vac14p in *fig4Δ* cells, suggesting that Atg18p may also regulate PtdIns(3,5)P_2 metabolism by influencing the distribution pattern of the Vac14p-Fig4p complex, and therefore of the Fab1p kinase.

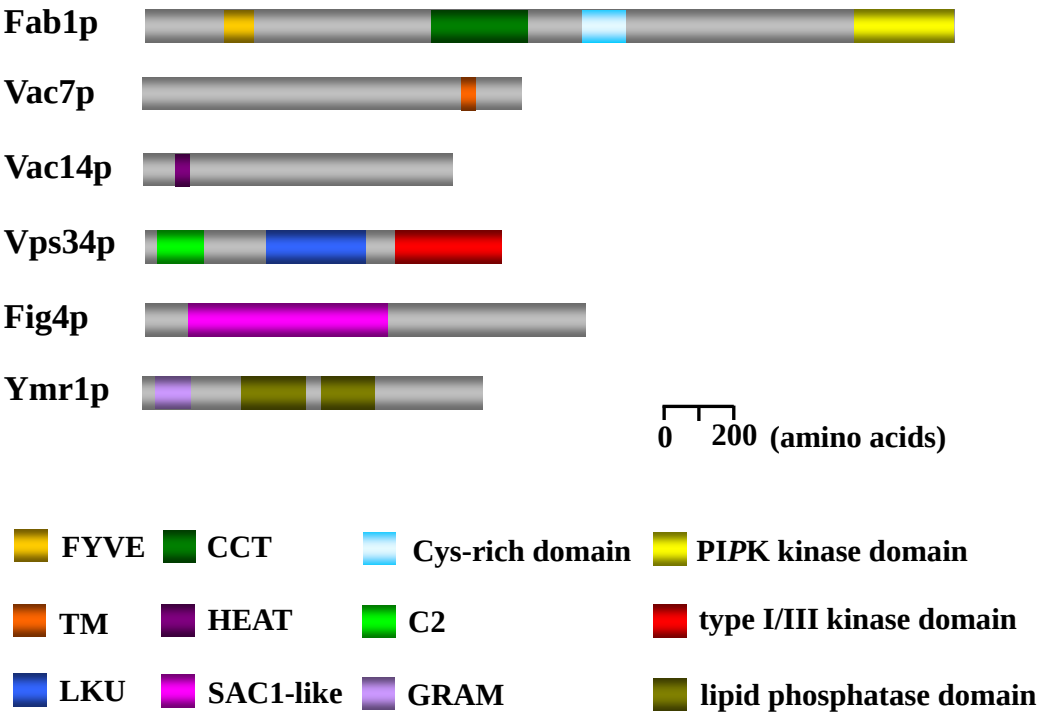


Figure 3.1: Domain structure of proteins in Fab1p pathway (adapted from Strahl and Thorner, 2007)

FYVE, Fab1, YGL023, Vps27, and EEA1; CCT, chaperonin containing T-complex homology region; HEAT, HEAT repeat motif; TM, transmembrane domain; C2, C2 domain; LKU, lipid kinase unique domain; GRAM, glucosyltransferase, Rab-like GTPase activator and myotubularin domain

My studies of Vac7p-GFP suggest that this integral membrane protein traffics from the Golgi via the AP-3 pathway and never passes through the late endosome. This result is important because Fab1p plays critical roles in the late endosome and our

data suggest that Vac7p cannot be involved in this process.

In addition, our modified *in vitro* colorimetric phosphatase assay also demonstrates that the conversion of PtdIns(3,5)P_2 to PtdIns3*P* is likely to be catalyzed by Fig4p whilst Ymr1p may indeed metabolise PtdIns(3,5)P_2.

It still remains unknown whether PtdIns5*P* is present in budding yeast. However, in the work decribed below observed the presence of a peak that co-elutes with authentic [^{14}C]GroPIns5*P* in the HPLC analysis, suggesting the presence of PtdIns5*P* in yeast cells. Combined with the observed ability of Ymr1p to convert PtdIns(3,5)P_2 to PtdIns5*P* *in vitro*, it seems likely that budding yeast contains a very small amount of PtdIns5*P*.

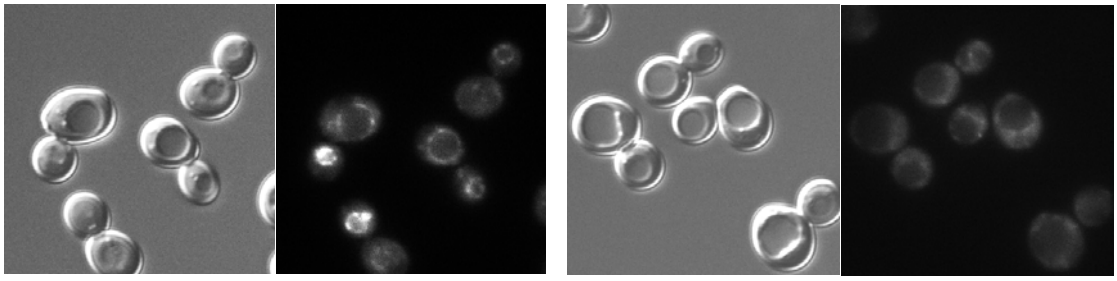
3.2 Results

3.2.1 Vac14p fails to localise on the vacuole membrane in *fab1Δ*, *fig4Δ* and *vps34Δ* cells

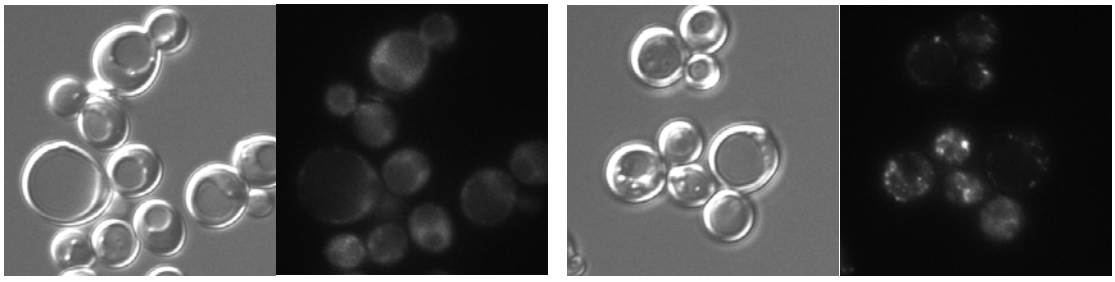
Although Vac14p is known to be one of the activators of Fab1p and possibly also Fig4p, Dr. Dove initially failed to demonstrate a direct functional interaction between Vac14p, Fab1p and Fig4p using yeast two-hybrid method (data not shown).

I next examined Vac14p localisation in yeast. To analyse its localisation *in vivo*, the chromosomal copy of the *VAC14* gene at its native locus was tagged in frame with GFP at the 3'-end. The fusion protein was expressed as a full-length protein under the control of the native *VAC14* promoter and is therefore likely to be expressed at near-normal physiological levels.

Vac14p-GFP localised predominantly to the vacuole membrane in wild-type BY4742 (Fig. 3.2). This result is consistent with Vac14p being a peripheral membrane-associated protein that regulates the levels of PtdIns(3,5)P_2 by activating Fab1p at the tonoplast. To determine what regulates Vac14p localisation, I expressed this fusion protein in yeast deletion strains lacking *fab1*, *fig4*, *vac7*, *atg18/svp1*, *ldb19*/*svp3* and/or *vps34*, all of which encode proteins that play a role in the metabolism or the function of PtdIns(3,5)P_2. Vac14p failed to decorate the enlarged vacuoles of *fab1Δ*, *fig4Δ* and *vps34Δ* cells (Fig. 3.2), so these three proteins are required for localisation of Vac14p. Vac14p localisation was not affected by deletion of the effectors Atg18p/Svp1p or Ldb19p/Svp3p.



WT (VAC14::VAC14-GFP) ***fig4Δ*** **(VAC14::VAC14-GFP)**



fab1Δ **(VAC14::VAC14-GFP)** ***vps34Δ*** **(VAC14::VAC14-GFP)**

Figure 3.2: Vac14-GFP expressed at physiological levels fails to localise on the vacuole membrane in fig4Δ, fab1Δ and vps34Δ cells.

The VAC14 gene at its native locus was tagged in frame with GFP at the 3'-end and expressed at physiological levels in wild type BY4742, and in *fig4Δ, fab1Δ and vps34Δ cells*

The mislocalisation phenotype of Vac14p is consistent with a recent identification of a Fab1p-Fig4p-Vac14p signalling complex which is tethered to vacuole membrane via the binding of the FYVE domain of Fab1p to PtdIns3*P* (Botelho *et al*., 2008). Since the Vac14p does not possess a transmembrane domain, its attachment to the vacuole membrane probably requires interactions with other proteins or lipids; quite possibly from Fab1p and Fig4p. Therefore Vac14p is mislocalised in both *fab1Δ* and *fig4Δ* cells. Interestingly, I also noticed that Vac14p's localisation pattern was different when comparing *fab1Δ* / *fig4Δ* and *vps34Δ* cells. Vac14p is completely diffuse and cytoplasmic in the former two strains but is concentrated into distinct vesicular dots in latter strain. It is unknown what organelle this dot corresponds to but my data would seem to indicate that in the absence of PtdIns3P/Vps34p, the whole Fab1p-Vac14p-Fig4p signalling complex is re-distributed to a subcellular compartment that these proteins are not normally resident in.

Unexpectedly, although Vac14p localisation is not obviously different comparing wild type and *atg18Δ* cells, over-expressed Atg18p can partially rescue the mislocalisation of Vac14p in *fig4Δ* (Fig. 3.3). This observation is interesting considering that Atg18p was proposed to have a negative feedback effect on PtdIns(3,5)P_2 generation whilst it was found to be a specific effector of PtdIns(3,5)P_2 required for membrane retrival from vacuole (Dove *et al.*, 2004). This result is possible evidence for the above proposal that Atg18p's feedback effect may be achieved by affecting the Vac14p-Fig4p complex's proper localisation.

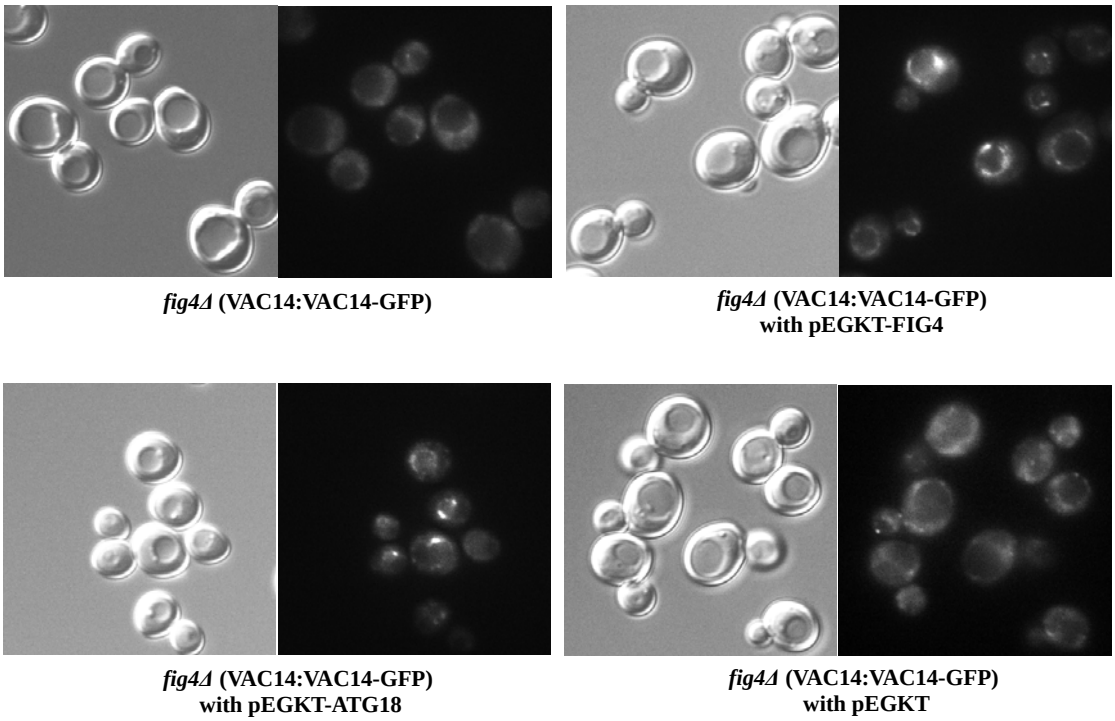


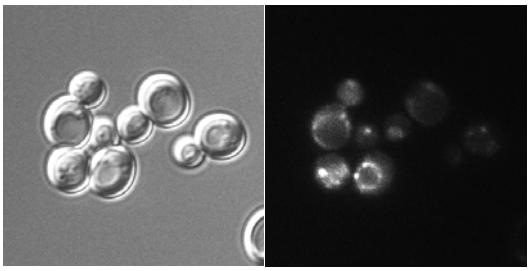
Figure 3.3: Over-expressed Atg18p partially rescues the mislocalisation of Vac14p in fig4Δ cells

The constructs pEGKT-ATG18 was introduced into *fig4Δ* (VAC14:VAC14-GFP) and induced to over-express by addition of 2% Galactose. Introduction of pEGKT-FIG4 and pEGKT were positive and negative controls, respectively

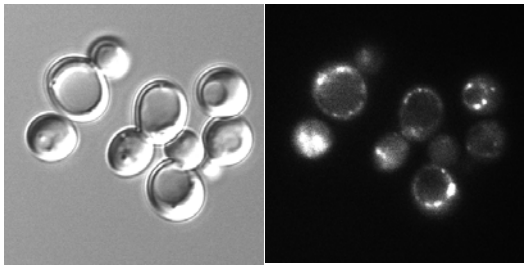
3.2.2 Both the CCT-like and PIPKIII-unique domains of Fab1p are required for Vac14p to localise to the vacuole membrane

To address which domain(s) of Fab1p might mediate the interaction with Vac14p, I introduced constructs harboring *FAB1* with mutations in various functional domains into *fab1Δ* (VAC14:VAC14-GFP) cells to see which of the Fab1p variants could support Vac14p localisation. The conserved domains being considered are defined in Michell *et al*., 2006. Fluorescence microscopy showed that Fab1p mutated in its FYVE domain (in conserved Cys residues, so as to lose its ability to bind PtdIns3*P*) or in the PIPKinase domain (to yield a catalytically inactive protein) retained an

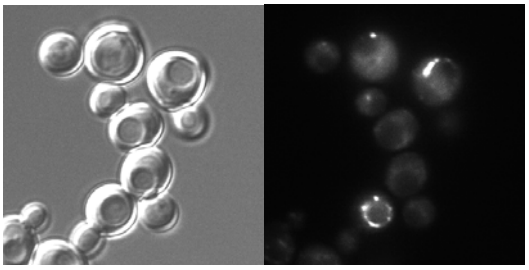
ability to partially correct the mis-localisation of Vac14p in *fab1*-null cells. By contrast, no Vac14p-GFP was associated with the vacuole in cells that expressed Fab1p in which a conserved central Gly residue in the CCT-like domain was mutated, and most of the Vac14p-GFP was not vacuole-associated in cells harbouring Fab1p carrying mutations in conserved Cys residues in the PIPKIII-unique region of the protein (Fig. 3.4).



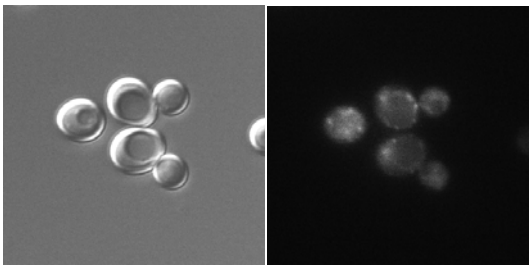
(Fab1p)



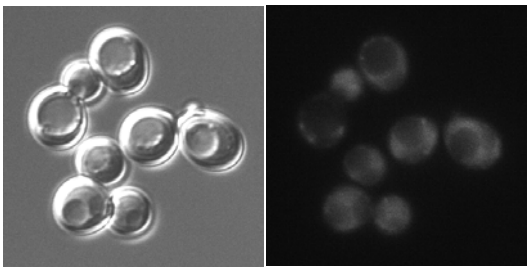
(Fab1p mutated on PIPkinase domain)



(Fab1p mutated on FYVE domain)



(Fab1p mutated on PIPKIII-unique domain)



(Fab1p mutated on CCT-like domain)

Figure 3.4: Vac14p-GFP distribution in fab1Δ introduced constructs harboring FAB1 with mutations in various functional domains

Constructs containing differential *FAB1* variants were introduced into *fab1Δ* (VAC14:VAC14-GFP) to identify what domain(s) of Fab1p may regulate Vac14 loading onto vacuole membranes. A construct containing wild type FAB1 was positive control

The finding that Vac14p also failed to localise on the vacuole in *fig4Δ* and *vps34Δ* cells seems to lend weight to the possibility that Fab1p alone is not enough to recruit Vac14p to vacuole. It is therefore quite possible that additional proteins are required to mediate Vac14p localisation other than Fab1p. The above proteins are likely candidates.

In the respect of Fig4p, it was shown to be a specific PtdIns(3,5)P_2 5-phosphatase *in vitro* and proposed to be able to convert PtdIns(3,5)P_2 to PtdIns3*P in vivo* (Rudge *et al.,* 2004). Fig4p localises on the vacuole membrane in wild type but not in *fab1Δ* cells, which is reminiscent of the localisation defects of Vac14p in the same mutant. Like Vac14p, Fig4p has no trans-membrane domain, so the same questions about Vac14p localisation apply to Fig4p. How can Fig4p be recruited to vacuole membrane? Is it Fab1p alone that achieves this or does it require additional proteins? To address this question, I constructed strains expressing both Fig4p-GFP and Vac14p-mCHERRY from their own promoters by doing standard yeast knock-ins. Microscopy clearly showed the co-localisation of Vac14p and Fig4p in some micro-domains of the vacuole membrane in wild-type cells (Fig. 3.5). This observation further supported the idea that Vac14p and Fig4p may form a complex and might be mutually dependent on each other for vacuole membrane loading. I tried to investigate the Fig4p localisation pattern in various deletion mutants as I have successfully done for Vac14p, but the Fig4p-GFP construct gave very weak fluorescence signals in all strains tested except for wild type, possibly because of the instability of Fig4p-GFP. Thus to date I have not yet managed to get any good images of Fig4p localisation except for in wild-type cells e.g. those displayed in Fig. 3.5. If time allowed I would construct a Fig4p-3XGFP construct as this should produce a brighter signal.

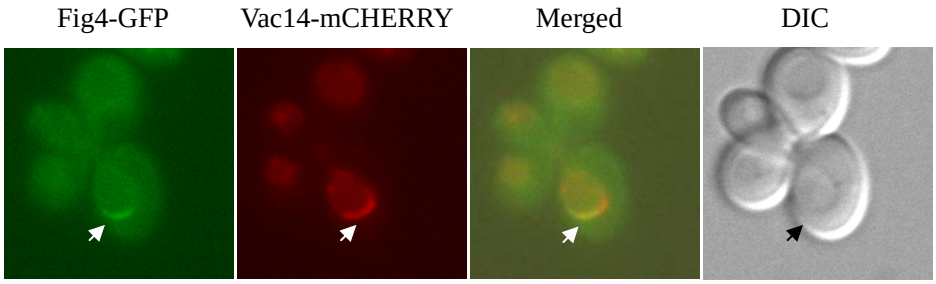


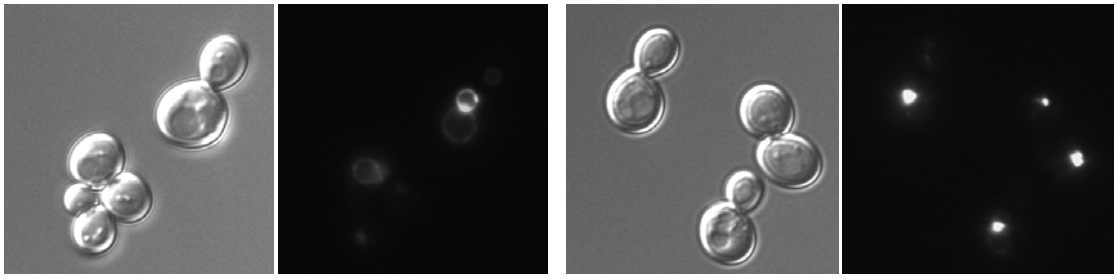
Figure 3.5: Co-localisation of Vac14p and Fig4p in micro-domains of the vacuole membrane A strain of BY4742 expressing VAC14:VAC14-mCHERRY and FIG4:FIG4-GFP, was constructed and visualized by light microscopy and fluorescence. Merged images were then produced in Photoshop.

3.2.3 Vac7p traffics to the vacuole membrane via the AP-3 pathway

I examined the localisation of Vac7p, an activator of Fab1p that seems to be confined to some yeasts. The over-expressed fusion protein yEGFP-Vac7p resides on the vacuole membrane in wild type cells, together with Fab1p, Vac14p and Fig4p. Unlike the other members of the Fab1p pathway, Vac7p is an integral membrane protein, and I therefore determined by what route Vac7p travels to the vacuole membrane from the Golgi.

By examining the localisation of Vac7p in several deletion mutants defective for essential participants in trafficking pathways to the vacuole, it was found that Vac7p resides on the vacuole membrane in *vps45Δ* cells but goes into the vacuole lumen in *apm3Δ* cells (Fig. 3.6). Vps45p is essential for protein traffic through the late endosome-dependent CPY pathway, and Apm3p is needed for the AP-3-dependent direct Golgi-to-vacuole pathway (Bryant *et al*., 1998; Stepp *et al*., 1997; Cowles *et al*., 1997). The fact that Vac7p enters the vacuole in *apm3Δ* cells is most readily

explained if Vac7p is shunted into the CPY pathway by bulk flow when a lack of Apm3p inactivates the AP-3 pathway. Once Vac7p has entered the CPY pathway, it probably lacks the negative sorting information required to keep it out of the intraluminal vesicles of the MVB and so travels to the vacuole lumen. This result, as well as the lack of effect of *VPS*34 deletion on Vac7p localisation, strongly suggests that Vac7p is normally delivered to vacuole membrane via the AP-3 pathway. It has been observed that the Vac14p-Fig4p complex regulates PtdIns(3,5)P_2 synthesis and turnover independently of Vac7p (Duex *et al*., 2006b). Our discovery of an AP-3 dependent Vac7p trafficking pathway suggests that Vac7p may control PtdIns(3,5)P_2 synthesis on the vacuole membrane whereas the Vac14p-Fig4p complex may do this job predominantly on endosomal compartments.



***vps45Δ* with pUG36-VAC7** ***apm3Δ* with pUG36-VAC7**

Figure 3.6: GFP-Vac7p fails to localise on the vacuole membrane in apm3Δ but not vps45Δ

pUG36-VAC7 was introduced into *apm3Δ* and *vps45Δ cells*. GFP-Vac7p expression was repressed by addition of 0.8mM of Methionine

3.2.4 PtdIns5*P* is present in live yeast

It has not been clear whether yeast normally contains PtdIns5*P*, because most HPLC analyses of PPIn do not distinguish between PtdIns4*P* and PtdIns5*P*. I have however, now devised an anion-exchange HPLC method that resolves the deacylation products of all three of the natural PtdIns*P* isomers. Figure 3.7 shows an HPLC trace of

deacylated lipids extracted from wild-type cells. This HPLC gradient can resolve Gro*P*Ins4*P* and Gro*P*Ins5*P* by virtue of a pH shift from 3.8 to 4.4 that allows these two isomers to be separated.

Fig. 3.7 clearly shows the presence of a peak that co-elutes with authentic [^{14}C]GroPIns5*P*. This authentic standard was made by HPLC purifying Gro*P*Ins(3,5)P_2 from [^{14}C]inositol radiolabelled yeast that had been subjected to a 0.9M NaCl stress for 10-15 minutes, followed by immediate methanol quenching. The resulting [^{14}C]GroPIns(3,5)P_2 was desalted using Dowex resin and eluted in 1.0 M TEAB (tetraethylammonium bicarbonate) a volatile buffer that was removed *in vacuo* using a Speed-vap low pressure drying device. Dried desalted [^{14}C]GroPIns(3,5)P_2 was then incubated with washed erythrocyte membranes (3-4 mg protein) at pH 7.5 in 1 ml of 12.5 mM HEPES/KOH/1 mM EGTA/5 mM EDTA for 4 h, see Dove *et al*., 1997). This treatment reproducibly removes the 3-phosphate from the inositol ring whilst leaving the 5-phosphate intact (the 5-phosphatase activity requires Mg^{2+} for activity).

The small quantity of [^{3}H]Gro*P*Ins5*P* indicates the likely presence of its parent lipid (PtdIns5*P*) in yeast cells. So far I have been unable to obtain enough of this material for formal chemical identification, but the behaviour of Ins(1,2)P_2 and Ins(1,6)P_2, the comparator InsP_2 isomers makes it unlikely that the deacylation products of the other possible but unknown PtdIns*P* isomers (PtdIns2*P* and PtdIns6*P*) would elute in this position. Its full characterisation must await the discovery of a treatment that increases the level of this inositide in cells.

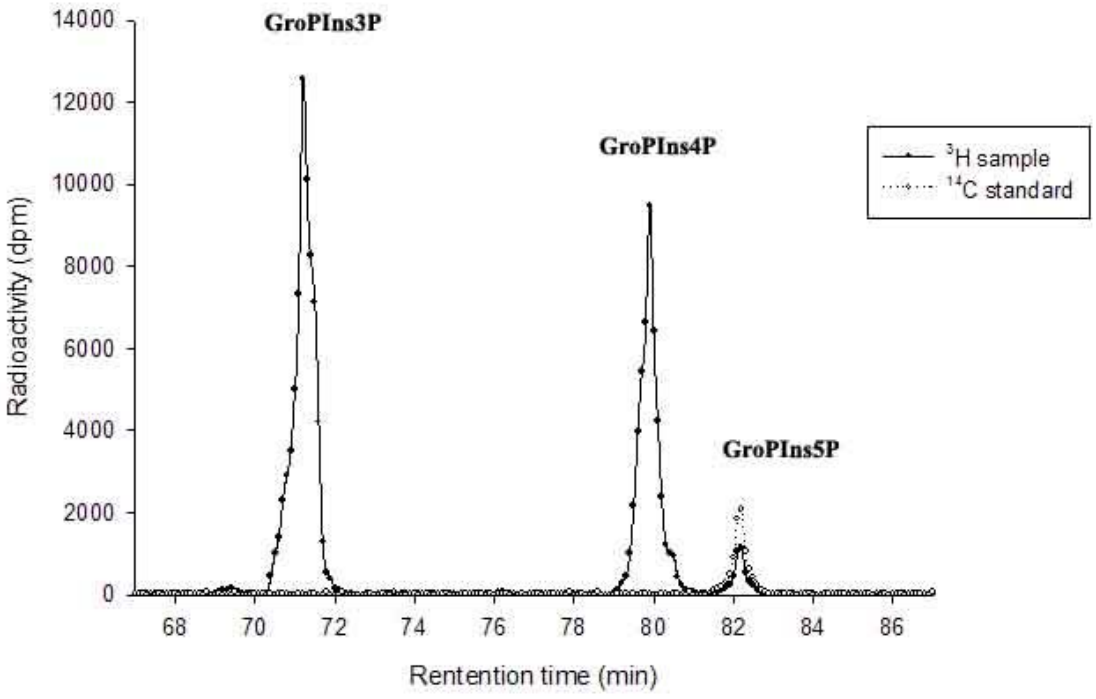


Figure 3.7 HPLC analysis of [^{3}H] inositol labelled lipids extracted from wild-type cells and resolved using a novel HPLC gradient. Note the extra peak that co-elutes with authentic [^{14}C] GroPIns5P

3.2.5 Purification of yeast Ymr1p and Fig4p from *E. coli*

If PtdIns5*P* does exist in budding yeast, Ymr1p might be a factor to generate it by 3-dephosphorylating PtdIns(3,5)P_2 *in vivo* as discussed in Chapter 1. To examine this possibility, I had to gain a better understanding of the substrate specificity of Ymr1p. To do this, I resolved to examine its catalytic activity by employing a malachite green-based colorimetric phosphatase assay. However, in order to set up this experiment, I had to first obtain a reasonable amount of purified Ymr1p by overexpressing the recombinant Ymr1p in *E.coli*. Therefore, I expressed GST-tagged Ymr1p in bacteria and purified the full length GST-fusion protein (Fig. 3.8). I then set up a colorimetric phosphatase assay using malachite green and the purified Ymr1p

but time precluded me from doing anythiug other than the most preliminary of characterisations of this enzyme.

Whilst I was characterizing Ymr1p, I was also interested in investigating another enzyme, Fig4p, the proposed PtdIns(3,5)P_2 5-phosphatase (Rudge *et al*., 2004) by utilising this same colorimetric assay. But unlike the Ymr1p purification, work on Fig4p presented a challenge as it appeared to be unstable in bacteria. Following the published method (Rudge *et al*., 2004) using a His6-tagged bacterial expression system, I just obtained a truncated protein. Similar results were obtained when the protein was expressed in *P. pastoris*. Expression in *S. cerevisiae*, using a construct where Fig4p was tagged as an N-terminal GST-fusion, showed that the enzyme could be made full length but the yield was low. It was then decided to return to the bacterial system and try to express GST-Fig4p in the same way as Ymr1p. This yielded some protein, although the yield was still less than the other proteins (Fig. 3.8). The first assay of purified GST-Fig4p by colorimetric assay showed that it can degrade PtdIns(3,5)P_2 but not PtdIns3*P* or PtdIns(4,5)P_2, a detailed characterization was beyond the scope of this study.

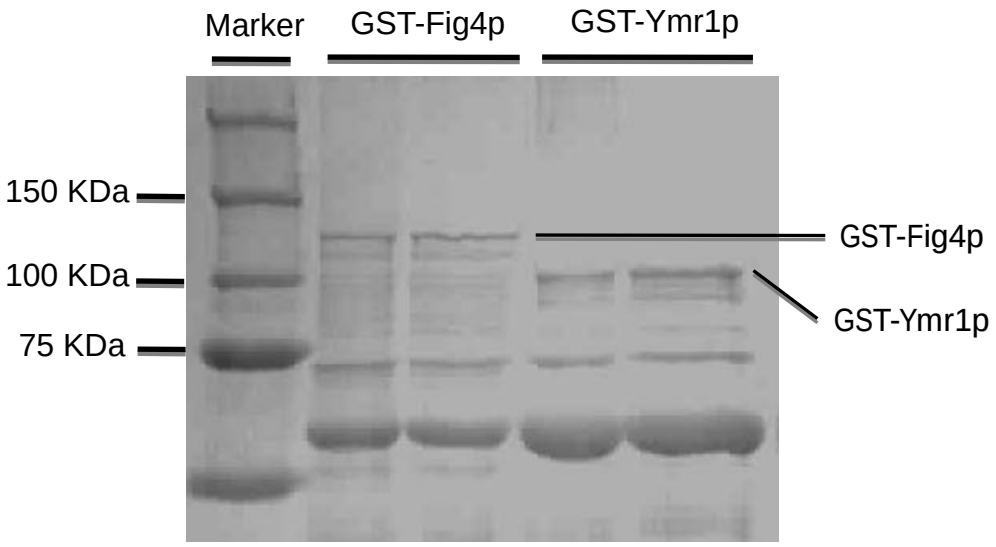


Figure 3.8: GST-Ymr1p (105.3 KDa) and GST-Fig4p (126.9 KDa) fusion proteins purified from E. coli and resolved by SDS-PAGE

The annotated bands were assumed to be the bone-fide Gst-Fig4p and Gst-Ymr1p proteins based on the expected molecular weight firstly, and being purified to do mass spectrometry for the futher verification and confirmation

3.3 Discussion

This Chapter has described studies of the regulation of PtdIns(3,5)P_2 metabolism in yeast, notably the roles of Vac14p and Vac7p in its synthesis and its possible metabolism to PtdIns5*P in vivo* via the myotubularin like protein, Ymr1p.

3.3.1 Involvement of Vac14p and Vac7p in the synthesis of PtdIns(3,5)P_2 by Fab1p

Our Vac14p-GFP localisation data suggest that the CCT-like and PIPKIII-unique domains of Fab1p are necessary for Vac14p to associate with the vacuole membrane. The most likely interpretation is that they directly bind to one another, but our method would not have distinguished whether intermediary proteins are involved.

To address the details of the Fab1p:Vac14p interaction, I made constructs that would

attach a CAAX motif (where C is Cysteine, A is an aliphatic residue, X is any amino acid, see Hancock 2003; Strahl *et al*., 2005) that directs prenylation and results in any target proteins so-tagged becoming resident at the plasma membrane. I then constructed fluorescently tagged constructs containing each of the four isolated functional domains of Fab1p with yEGFP at the 5-termini and a CAAX motif at the 3-terminus. The aim was to introduce these constructs into *fab1Δ* (VAC14:VAC14-2X mCHERRY) or *fab1Δ* (FIG4:FIG4-2X mCHERRY) strains in which either Vac14p or Fig4p would be tagged with mCherry, and so determine whether any of the Fab1p domains could recruit either Vac14p or Fig4p to the plasma membrane. Unfortunately, I only obtained cells expressing the plasma membrane-directed FYVE domain construct, probably because the chosen constructs were not stable in the absence of the rest of the Fab1p molecule.

Nevertheless, the recent description of the isolation of a protein complex comprising Fab1p, Vac14p and Fig4p used this strategy to recruit Vac14p to the vacuole membrane using a CCT domain tagged to a trans-membrane domain (Botelho *et al*., 2008). Our results are in general agreement with the conclusions of that study, though our work was obviously at a much earlier stage. In particular, I found that Atg18p can bind Vac14p and this was confirmed in the above work.

3.3.2 Myotubularin type 3-phosphatases of Ymr1p

Ymr1p is the sole member of the myotubularin-related phosphatase family present in yeast (Parrish *et al.*, 2004). Myotubularins were originally identified as a family of presumed protein tyrosine phosphatases (Taylor *et al.*, 2000a; Clague and Lorenzo, 2005). They were of note because mutations in genes encoding at least two of the myotubularins resulted in certain types of the human genetic disease X-linked

myotubular myopathy (a defect in the MTM1 gene) or Charcot-Marie Tooth disease (linked to mutations in MTMR2) (Sarnat, 1990; Berger *et al.*, 2002). Subsequently, it emerged that they could hydrolyse PtdIns3*P* and later it was also found that they could hydrolyse PtdIns(3,5)P_2 (Wishart and Dixon, 2002; Clague and Lorenzo, 2005). Hence, it is likely that defects in the metabolism of one of these lipids underlie the two genetic diseases described above (see below for more evidence).

Mutations in the gene encoding mammalian Fig4p have recently been shown to underlie a previously undefined genetic neuropathy that bears striking similarity with the Charcot-Marie Tooth disease caused by disruption of the myotubularin MTMR2 described above (Chow *et al.*, 2007). Since the only common substrate of both these phosphatases is PtdIns(3,5)P_2, it seems likely that defective metabolism of this lipid results in at least 3 neurological and musclo-skeletal diseases in humans.

Ymr1p likely functions in cytoplasm to vacuole transport (CVT) pathway and in the maintenance of the architecture of the endosomal system, possibly by regulating the localisation and levels of PtdIns3*P* (Parrish *et al.*, 2004). However, the *ymr1* null mutant strain has no obvious phenotypes and its functions only emerge when other PPIn phosphatases are simultaneously inactivated (Taylor *et al.*, 2000a). This suggests a nonessential function or possible redundancy amongst yeast lipid phosphatases given that budding yeast can express several Sac1p-like proteins, as well as a PTEN-like phosphatase (Tep1p), which are also thought to play a role in regulating PtdIns3*P* levels (Hughes *et al.*, 2000a). However, recent work has suggested that the mammalian enzymes can all hydrolyse PtdIns(3,5)P_2 (Clague and Lorenzo, 2005). Prior to our work, it was not known if this was also a property of the yeast Ymr1p

protein, indeed the enzyme had never been expressed and examined *in vitro*. It is also not known if any of the other proteins in the CVT or Vps34p pathways are adaptors for this phosphatase. The significance of myotubularin mediated degradation of PtdIns(3,5)P_2 is that it gives rise to another PPIn signal: PtdIns5*P*, that may have its own functions in cell regulation. Since PtdIns5*P* has never before been described in yeast, I was particularly interested in the possible presence of this lipid in *S. cerevisiae*. This is because the route of synthesis of PtdIns5*P* is not well established in any system but in yeast it would be a relatively trivial matter to establish, as long as I have an assay for this lipid.

In my current study, I first demonstrated that bacterially expressed GST-Ymr1p is active, and the preliminary colorimentric phosphatase assay suggested it can also specifically degrade PtdIns3*P* and PtdIns(3,5)P_2 but not PtdIns4*P* or PtdIns(4,5)P_2, in line with its previously described 3-phosphatase activity *in vivo* (Taylor *et al.*, 2000a; Pendaries *et al.*, 2003). In addition, the detection of PtdIns5*P* from intact cells by our HPLC analysis further supports the proposal that Ymr1p may be a *bona fide* PtdIns(3,5)P_2 3-phosphatase in budding yeast.

If time allowed I would exploit the observation that pH shifts allow separation of PtdIns4*P* and PtdIns5*P* by HPLC to optimize an HPLC method to separate PtdIns5*P* from PtdIns3*P* and PtdIns4*P*. I would then have liked to have looked at the role of Fab1p and Ymr1p in production of PtdIns5*P*, by radiolabelling *fab1* and *ymr1* knockout strains. I would also have liked to try to find stresses (e.g. hyper-osmotic, oxidative, UV, alkalinsation etc) that elevate the levels of the putative PtdIns5*P*.

Having identified a possible novel inositol lipid in yeast, an important experiment would be to confirm that this is indeed PtdIns5*P* using the periodate oxidation method. Putative GroPIns5*P* will be HPLC purified, desalted and oxidised by periodate. This opens the inositol ring wherever there are two adjacent free hydroxyls, yielding a polyol-phosphate. If I dephosphorylate the polyol-phosphate and identify the resulting polyol (PtdIns5*P* should result in the formation of Xylitol), then this will tell us what the original compound was. This would typically be done with [^{3}H]lipid as well as an authentic [^{14}C]PtdIns5*P* standard to account for losses and to ensure that the reaction proceeds in the control. It will then be necessary to identify the resulting [^{3}H]polyol by HPLC co-chromatography with authentic standards.

I would also have liked to have dual label yeast undergoing hyper-osmotic stress with [^{3}H]inositol to isotopic equilibrium and pulse-chase them with [^{32}P]orthophosphate. This would allow me to measure turnover rates of PtdIns(3,5)P_2 so that I can determine if Fab1p is activated or Fig4p is inhibited by hyper-osmotic stress (or both). Looking at the same cells using our new HPLC gradient may also allow us to determine if Ymr1p plays any role in PtdIns(3,5)P_2 degradation after stress.

Chapter 4: A Novel Osmotic Stress Pathway Mediated by Inositol Polyphosphates and Vps41p in *S. cerevisiae*

4.1 Introduction

Stress signalling occurs in response to changes in the extracellular environment and allows cells to survive by making appropriate adjustments to their metabolism and physiology. In budding yeast, one response to hyper-osmotic stress is activation of the Fab1p lipid kinase to produce PtdIns(3,5)P_2, a lipid required for membrane fission and other events. Hypo-osmotic stress, however, activates Plc1p, a phospholipase C that cleaves the PtdIns(4,5)P_2 to generate water-soluble inositol polyphosphates, including bisdiphosphoinositol tetrakisphosphate (which can be abbreviated either as $(PP)_2$-IP_4 or IP_8), a 'high energy' inositol pyrophosphate. Plc1p is required for vacuole fusion *in vitro* (Jun *et al,* 2004), suggesting that IP_8 may be required for hypo-osmotic stress-induced vacuole fusion. However, I also noticed that Plc1p and IPs are important in the response to hyper-osmotic stress since *plc1*Δ mutants cannot grow in hyperosmotic media. The same is true of *arg82*Δ and *kcs1*Δ mutants that are unable to generate inositol pyrophosphates. This has led others to suggest that Plc1p is activated by any event that causes plasma membrane deformation and this includes all osmotic imbalances (Cooper *et al*., 1999).

It is therefore possible that PPIns and their derived IPs are involved in the responses of cells to all osmotic stresses. The exact role of inositides in stress adaptation is still largely obscure but may involve the regulation of vacuolar membrane dynamics via fusion and fission. Indeed, many mutants with defective IPs metabolism display

aberrant vacuole morphology, although the molecular mechanism linking IPs to vacuolar homeostasis was undefined at the time I began this work.

The response of the yeast vacuole to external stresses makes it an excellent model system to study such phenomena because it is relatively large and its size and shape can be recorded by light microscopy. As discussed in Chapter 1, hypo- and hyper-osmotic stresses can provoke fusion and fission of vacuolar subcompartments, so allowing novel components of fusion/fission pathways to be identified by genetic screens.

A simple way to examine how cells sense and adapt to hyperosmotic stress is to add NaCl to the medium. Wild-type vacuoles become fragmented within 10 min and this fragmentation can be maintained for one hour or more (Duex *et al.*, 2006a; Dove and Johnson, 2007). In an original screen using this technique, it was found that when a yeast casein kinase Yck3p is deleted, cells still exhibit the hyper-osmotically driven and PtdIns(3,5)P_2-dependent vacuole fragmentation, but that this fragmentation is not maintained and the vacuoles quickly re-fuse (LaGrassa and Ungermann, 2005). Yck3p therefore seems to be required to maintain vacuoles in the fragmented state after PtdIns(3,5)P_2 levels have returned to basal. This study suggested that Vps41p, an important part of the HOPS tethering complex that is required for homotypic vacuole fusion, might be one target of Yck3p-mediated phosphorylation. It was suggested that phosphorylation of Vps41p might block the tethering together of vacuole subcompartments and so ‘freeze’ vacuoles in the fragmented state upon hyper-osmotic stress, but there was no direct evidence on this question when my study was initiated.
My starting point was the idea that Vps41p phosphorylation might be controlled by

the same upstream signalling pathway that activates Fab1p: both processes are activated by hyperosmotic stress, and it seemed possible that the control of the two processes might be integrated.

An immunoblot method was developed to detect Vps41p phosphorylation *in vivo*, and was used to rule out the possibility that hyper-osmotically activated Vps41p phosphorylation lies directly downstream of either the Hog1p or Fab1p/PtdIns(3,5)P_2 pathway. Instead, our data suggest that Vps41p may lie on a novel osmotic stress pathway that is under the influence of Plc1p-catalyzed generation of inositol pyrophosphate(s). The phosphorylation sites on Vps41p were characterized by FT-ICR mass spectrometry and their phosphorylation was shown to be required for Yck3p-mediated inhibition of vacuole fusion in salt-stressed cells. As a result of these studies, I propose a model in which Fab1p and Vps41p might lie on two parallel rapid response pathways to high extracellular osmotic potential whilst the Hog1p pathway mediates long-term adaptation.

4.2 Results

4.2.1 Vps41p is phosphorylated by Yck3p upon hyper-osmotic stress

In a screen of deletion strains whose vacuole fusion/fission fails to response properly to external hyper-osmotic stress, Ungermann and his colleagues (2005) observed that Vps41p, a subunit of HOPS complex which governs the homotypic vacuole fusion procedure, is phosphorylated by the casein kinase Yck3p upon hyper-osmotic stress. They suggested that this phosphorylated Vps41p might inhibit vacuole membrane fusion and therefore cause the hyper-osmotically induced vacuole fragmentation that had been triggered by Fab1p/PtdIns(3,5)P_2 to persist.

This suggested to us that phosphorylation of Vps41p by Yck3p is a part of a yeast hyper-osmotic signalling pathway. These results provoked us to ask whether I could use phosphorylation of Vps41p by Yck3p as a selective screen of yeast null mutants (ideally ~100), with which to identify novel components of the Fab1p salt stress pathway. In the original study, phosphorylation of Vps41p was identified by isolating vacuole fractions, incubating these with or without ATP under standard fusion conditions, resolving proteins by SDS-PAGE, and detecting Vps41p by immunoblotting: phosphorylated Vps41p migrates more slowly than the non-phosphorylated form (LaGrassa and Ungermann, 2005). The complexity of this method is not appropriate for large scale screens.

I therefore developed a simpler *in vivo* method by which to identify hyper-osmotically induced Vps41p phosphorylation. I constructed a C-terminally 3×HA-tagged Vps41p construct that could be integrated into the yeast chromosome and expressed at endogenous levels, extracted total yeast proteins, subjected them to SDS-PAGE separation and analysed these by western blot analysis using anti-HA antibody. This only employed routinely available techniques, and the key simplification was that I used a 0.1M NaOH-based cell lysis method to extract total cell protein: this involves no cell breakage protocol and removed the need for phosphatase inhibitors and ice-based handling of samples (see chapter 2 for a full description).

By using this rapid and convenient method, I could readily detect phosphorylated Vps41p in cells subjected to hyper-osmotic stress (0.9M NaCl for 15 min) *in vivo*, and confirmed that this hypertonically activated phosphorylation of Vps41p is dependent on the casein kinase Yck3p (Fig. 4.1). These data both confirmed that this method

works robustly and demonstrated for the first time that this pathway is active *in vivo*.

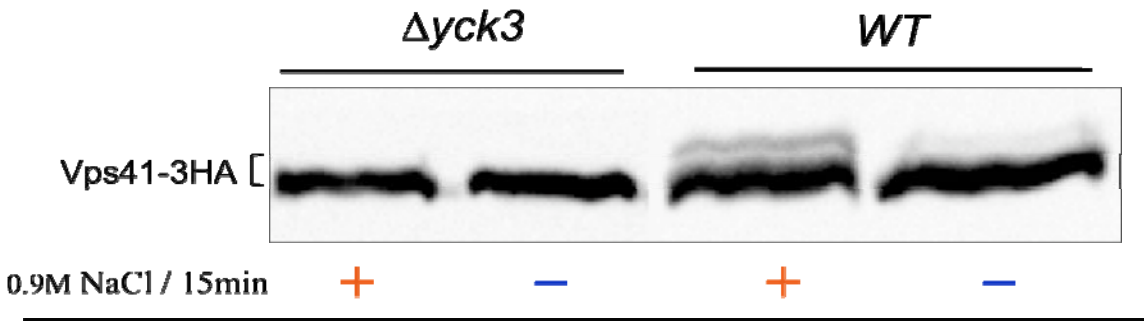


Figure 4.1: Vps41p is phosphorylated upon hyper-osmotic stress via Yck3p

Wild type and *yck3*Δ cells expressing Vps41-3HA at endogenous levels were grown to exponential phase and then subjected to hyper-osmotic stress (0.9M NaCl, 15 min) or mock treated. Cells were then extracted by treatment with NaOH and boiled in SDS sample buffer and then immunoblotted with Anti-HA antibody. The slightly retarded band was believed to be phosphorylated Vps41p based on previous studies (LaGrassa and Ungermann, 2005), and was also identified to be phosphorylated Vps41p in our later mass spectrometry results

Next, I examined the time-course for this response and the effects of varying osmolarity. Stress-induced Vps41p phosphorylation was extremely rapid, starting within less than one minute, and was sustained for at least one hour (Fig. 4.2). The quantification demonstartes that the phosphorylation of Vps41p attained its maximum level during the first five minutes of stress applicattion, and this level was then sustained for around 30 minuts, and afterwards began to decrease. When compared with the change in the steady-state level of PtdIns(3,5)P_2 upon hyper-osmotic stress (see Section 1.9), it was intriguing to note that the kinetics of both responses were similar, supporting our proposal that both PtdIns(3,5)P_2 and Vps41p were sequentially and synergistically controlling the hyperosmotic stress adaptation of budding yeast cells (see Section 4.3.4 and Fig. 4.19).

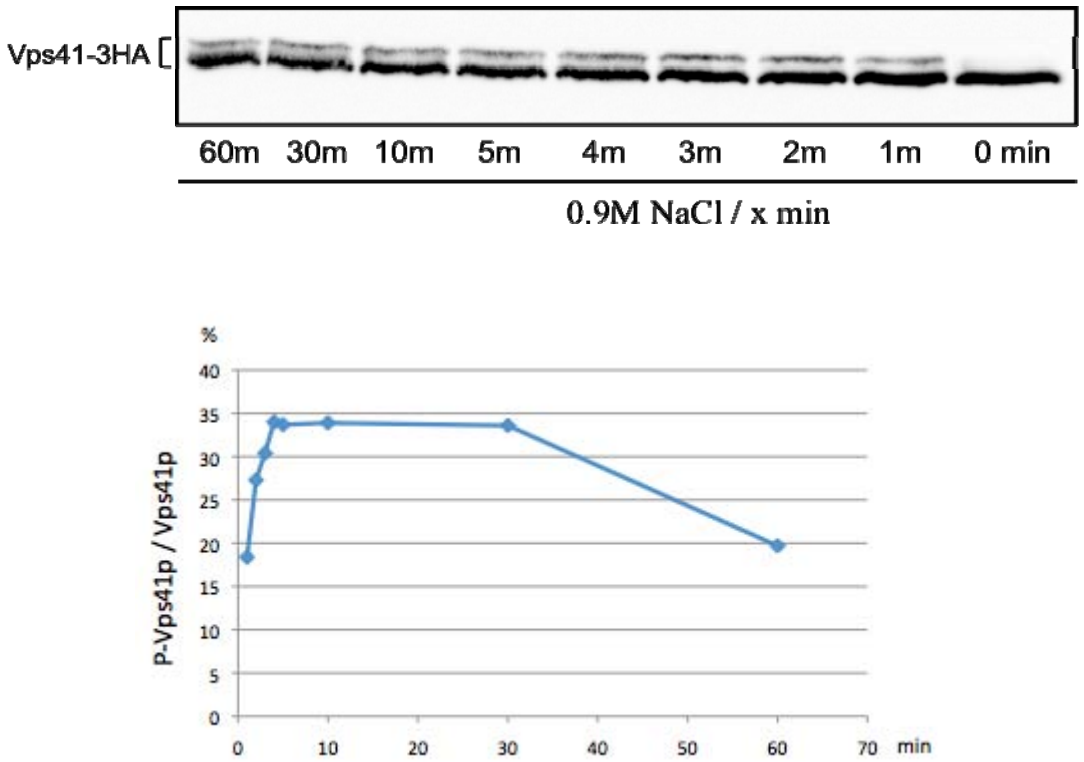


Figure 4.2: Time course of hyper-osmotically induced Vps41p phosphorylation

Top: Immunoblot identification of hyperosmotic induced Vps41p phosphorylation over time. BY4742(VPS41:VPS41-3HA) cells were grown to exponential phase in YPD, and then 5M NaCl stock solution added to a final concentration of 0.9M NaCl. Cells were grown on in this hypertonic media for 15 min and samples of equal volumes removed at each indicated time interval. Cells were immediately killed by addition of two volumes of methanol (this was done in order to control the time interval: controls indicate that this killling method does not affect the final result) and then subjected to SDS-PAGE/immunoblot analysis

Bottom: The change of ratio between phosphorylated Vps41p (P-Vps41p) and non-phosphorylated Vps41p (Vps41p) over time. The band intensity of all bands in the top blot were quantified by ImageJ (1.42) and the ratio of P-Vps41p and Vps41p calculated from the resulting two absolute intensity values (see Section 2.17)

In addition, the hyperosmotic induced Vps41p phosphorylation was sensitive to the hypertonic application, being able to respond to 0.8M NaCl shock (Fig. 4.3), even lower osmolarities (0.2M NaCl) provoked phosphorylation if the time point for sampling was changed (data not shown). This seemed appropriate for a short-term

response to stress: it was quick, fairly sensitive and prolonged, allowing the cells to survive a sudden external stress and buy time for the activation of a long-term response such as the Hog1p pathway. Like the rapid synthesis of PtdIns(3,5)P_2, phosphorylation of Vps41p appeared to behave appropriately for a rapid physiological response to external salt stress.

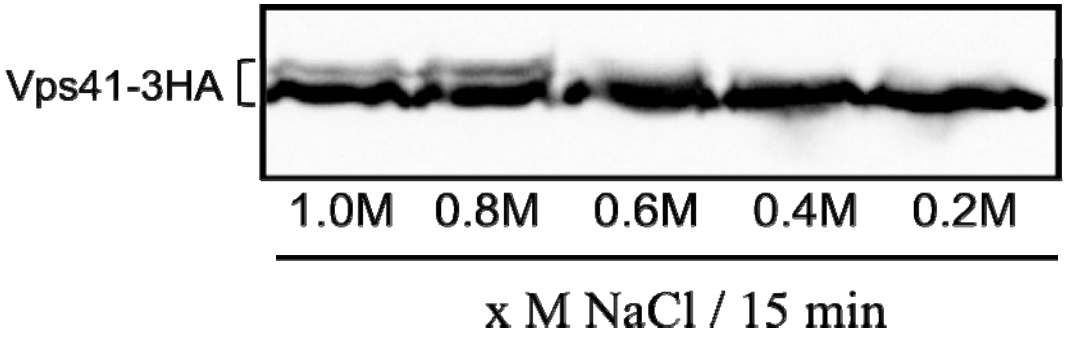


Figure 4.3: Osmolarity course of hyper-osmotic induced Vps41p phosphorylation

BY4742(VPS41:VPS41-3HA) cells were grown to exponential phase in YPD and divided into 5 equal volumes, and then 5M NaCl stock solution added to the indicated final concentrations of NaCl. Cells were then grown on in this hypertonic media for a further 15min and then subjected to immunoblot analysis

4.2.2 Hypertonically induced Vps41p phosphorylation is independent of PtdIns(3,5)P_2 synthesis by Fab1p and of activation of the Hog1p pathway

After verifying that our screening method worked, I next examined the *in vivo* Vps41p phosphorylation in some key deletion strains, to determine which pathways might activate this phosphorylation or facilitate its cellular functions downstream. I concentrated our first efforts on mutants that compromise known hyper-osmotic stress response pathways, particularly the Fab1p and Hog1p signalling systems.

The Fab1p PtdIns3*P* 5-kinase lipid kinase is activated upon salt stress, but the osmotic

sensor that lies upstream of Fab1p is unknown. When I examined the Vps41p phosphorylation response in several deletion strains which block the activation of Fab1p, none of them had any obvious effect on Vps41p phosphorylation (Fig. 4.4). These ineffective mutations included inactivation of Fab1p itself, of the Fab1p scaffold/activator Vac14p, of the dual functional Fab1p activator and PtdIns(3,5)P_2 phosphatase Fig4p, and of two putative PtdIns(3,5)P_2 effectors Svp1p/Atg18p and Svp3p/Ldb19p.

It was noted that although Vps41p phosphorylation still occurred in the above mutants, the quantification analysis demonstrated that the mutants (*fab1*Δ, Δ*vac14* and *fig4*Δ) deficient in PtdIns(3,5)P_2 synthesis had significantly less phosphorylated Vps41p after hyper-osmotic stress compared with those displaying a supranormal level of PtdIns(3,5)P_2 (*svp1*Δ and *svp3*Δ) (Fig. 4.4). This phenotype may suggest that an absence of PtdIns(3,5)P_2 could to some extent influence the Vps41p phosphorylation although it does not completely abolish it. This subtle modulation of Vps41p is reasonable when considering the common roles of Vps41p and Fab1p in inducing and maintaining vacuole fragmentation upon hyperosmotic stress.

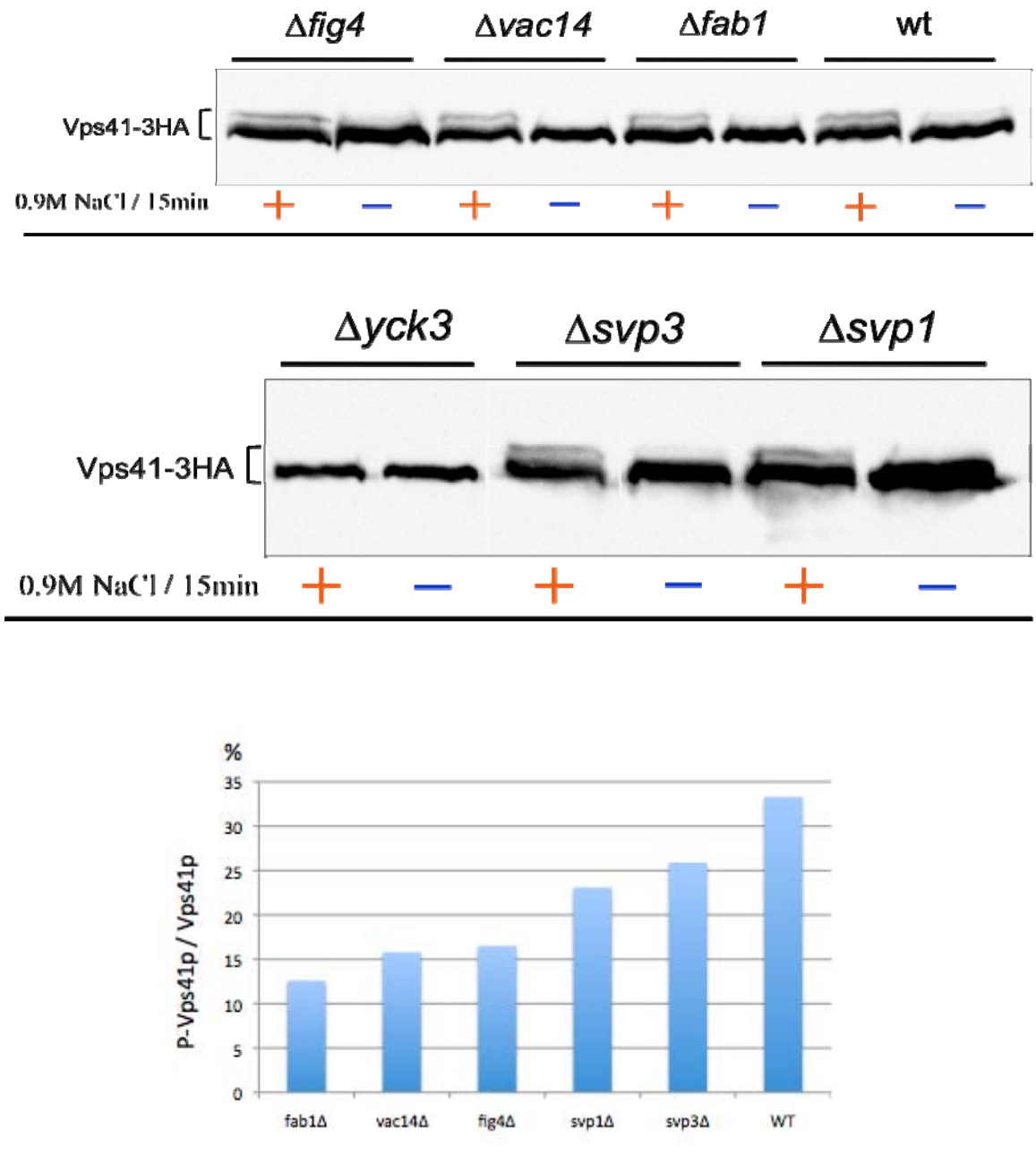


Figure 4.4: Hyperosmotic induced Vps41p phosphorylation is not downstream of the Fab1 pathway

Top: Immunoblot idenfication of hyperosmotic induced Vps41p phosphorylation in null mutants which compromised the Fab1p pathway. Cells with the indicated mutations and bearing a VPS41-3HA knock-in were shocked for 15 min with 0.9M NaCl and then killed and proteins extracted and subjected to SDS-PAGE and immunoblotting

Bottom: Comparison of the amounts of phosphorylated Vps41p (P-Vps41p) in Fab1p pathway disrupted null mutants. The band intensities of the top blot were quantified by ImageJ (1.42) and the ratio of P-Vps41p and Vps41p was calculated from the resulting two absolute intensity values (see Section 2.17).

Another hyper-osmotic response system is the Hog1p MAPK pathway. Yeast Hog1p is the functional homologue of mammalian p38 family of MAPKs. MAPK pathways are three tier protein kinase pathways that frequently end in regulation of the activities of nuclear transcription factors (O'Rourke *et al*., 2002). In Yeast, the Hog1p MAPK can be activated by two upstream branches that are initiated by plasma membrane sensor proteins: Sho1p and Sln1p (Fig. 4.5). Activation of Hog1p causes rapid translocation of phosphorylated Hog1p from the cytoplasm to the nucleus where it activates the expression of genes responsible for increasing the synthesis of glycerol, which then accumulates in the stressed yeast. The Hog1p pathway is the major mechanism for long-term adaption of yeast to high osmolarity.

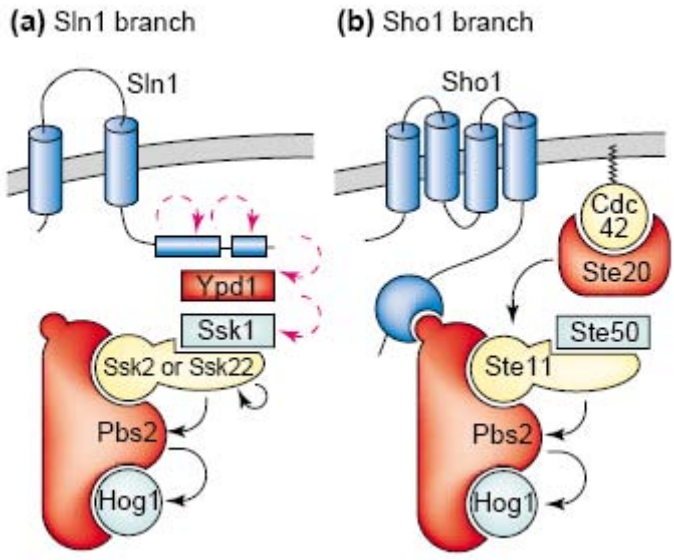
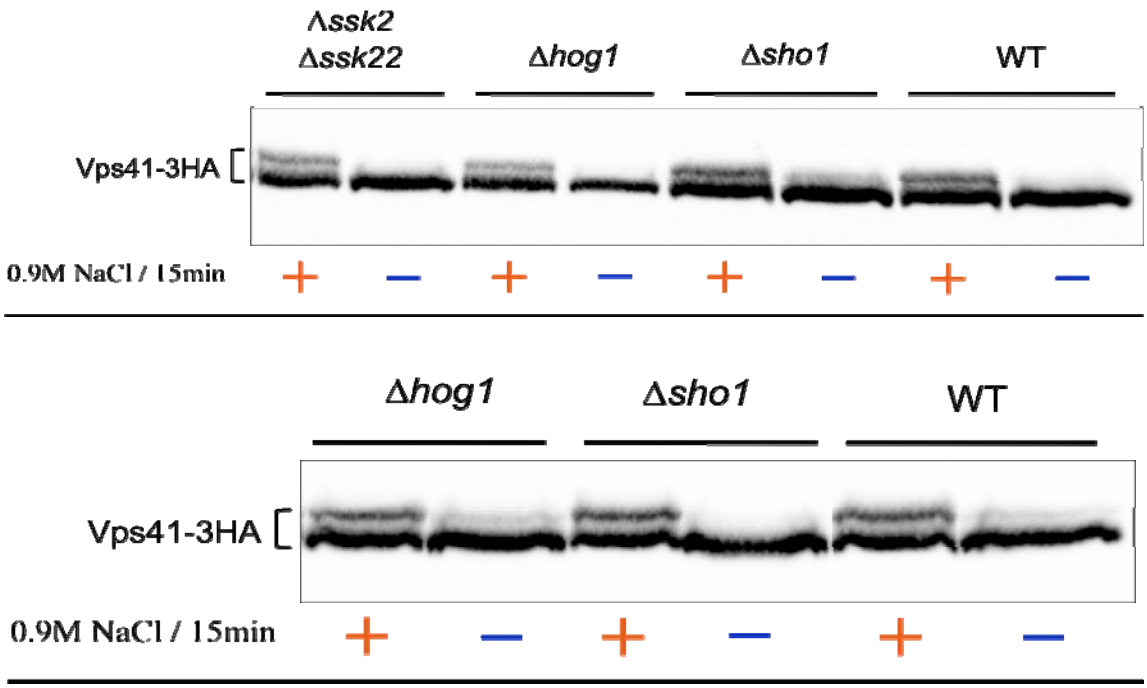


Figure 4.5: Yeast hyper-osmotic pathway II: Hog1p MAPK pathway (from O'Rourke et al., 2002)

Two upstream branches of the HOG pathway can each activate Pbs2p and Hog1p. (a) The Sln1p branch is composed of a phosphor-relay system that regulates the activity of the two partially redundant MAPKKKs, Ssk2p and Ssk22p. Phosphotransfer in the Sln1–Ypd1–Ssk1 system occurs in constant osmotic conditions to keep Ssk1p inactive. (b) The Sho1 branch uses a novel four membrane-spanning domain protein to recruit Pbs2p to the membrane through an SH3-polyproline interaction, after which the MAPKKK Ste11p is phosphorylated by Ste20p. Hog, high osmolarity glycerol; MAPKKK, mitogen-activated protein kinase (MAPK) kinase kinase.

When I investigated Vps41p phosphorylation in several null mutants that lacked either Hog1p or the upstream effector Sho1p, phosphorylation was unchanged, either by eye or when the bands were quantified using ImageJ, indicating that Vps41p phosphorylation is not downstream of activation of the Hog1p pathway, or of any other pathways that respond to Sho1p activation (Fig. 4.6).



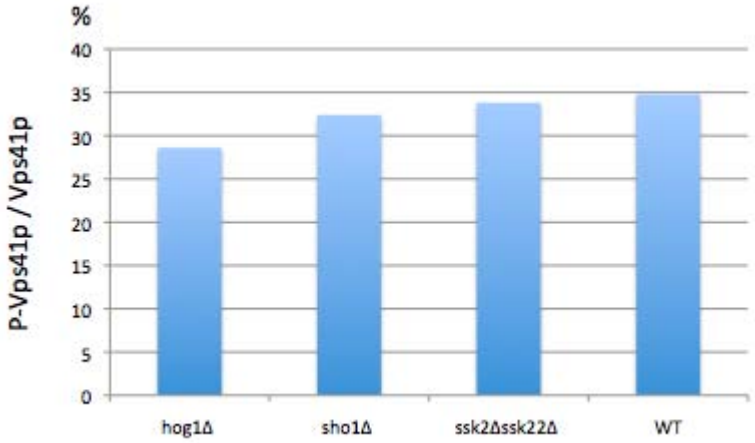


Figure 4.6: Hyper-osmotic induced Vps41p phosphorylation is not downstream of the Hog1p pathway

Top: Immunoblot analysis of Vps41p phosphorylaltion in mutants deficient for Hog1p pathway. The above blot shows Vps41p phosphorylation in the indicated deletion strains (W303 genetic background for top panel, BY4742 for bottom panel). Cells were given a hyper-osmotic stress for 15 min and then killed and proteins extracted and resolved on an SDS-PAGE gel and immuno-blotted with anti-HA.

Bottom: Comparison of the amounts of phosphorylated Vps41p (P-Vps41p) in Hog1p pathway disrupted null mutants. The band intensites from the top blot were quantified using ImageJ (1.42) and the ratio of P-Vps41p and Vps41p was calculated from the resulting two absolute intensity values so obtained (see Section 2.17)

In order to rule out the unlikely possibility that the Fab1p and the Hog1p pathways might be redundant regulators of Yck3p, I examined Vps41p phosphorylation in *hog1*Δ*fab1*Δ double mutants in which both pathways were simultaneously inactivated. Vps41p phosphorylation still occurred in this mutant (Fig. 4.7).

It therefore seems that hypertonically activated Vps41p phosphorylation is independent of the Fab1p and Hog1p pathways, so it may reflect the activation of a previously uncharacterized osmotic response pathway. The next question was whether I could identify other components of this novel pathway.

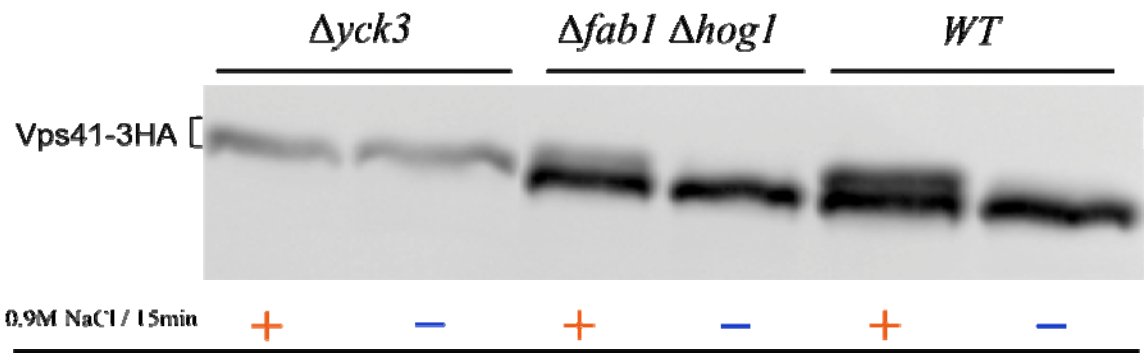


Figure 4.7: Vps41p phosphorylation occurs even when both Fab1p and Hog1p pathways are blocked

4.2.3 Synthesis of inositol 'pyrophosphates' is required for stimulation of Vps41p phosphorylation

Given the evidence that PPIns influence osmotic responses, particularly the PtdIns(3,5)P_2 pathway and the phospholipase C pathway, it seemed appropriate to test whether the latter might be involved in regulation of the phosphorylation of Vps41p. It is known that hypo-osmotic stress can activate Plc1p and as a result the accumulation of IP_6 in budding yeast (Perera *et al.*, 2004), and the synthesis of IP_8 is

selectively induced by hyper-osmotic stress in mammalian cells (Pesesse *et al.*, 2004; Choi *et al.*, 2007). Synthesis of inositol polyphosphates is catalyzed by sequential phosphorylations, by Arg82p, Ipk1p, Kcs1p and Vip1p, of the IP_3 that is released by Plc1p-catalyed PtdIns(4,5)P_2 hydrolysis (Fig. 4.8).

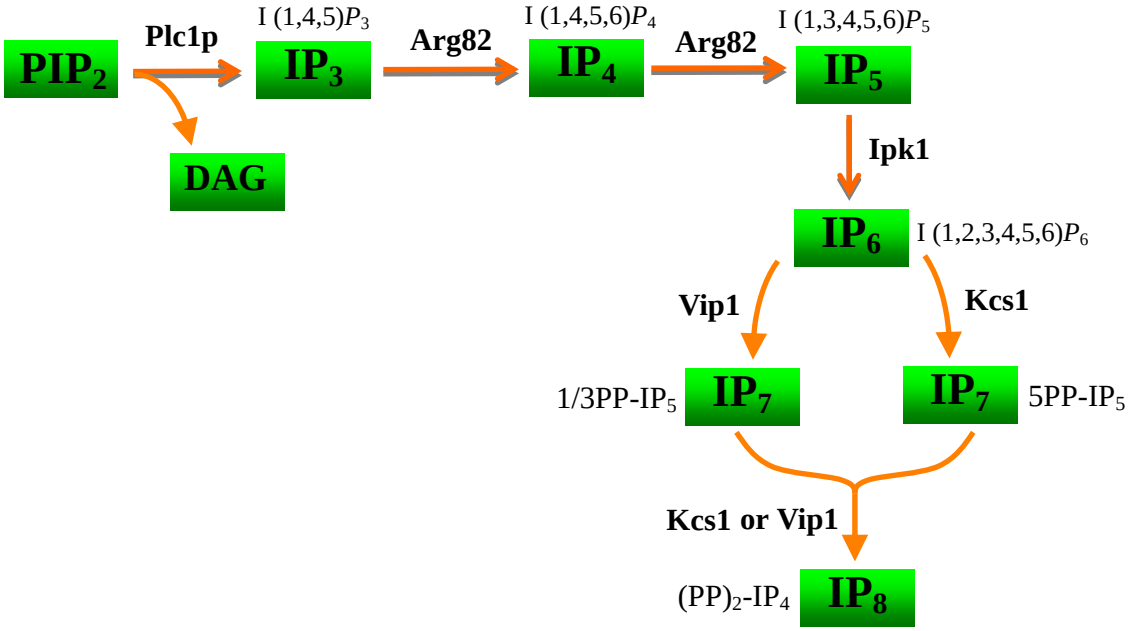


Figure 4.8: Synthesis of Inositol polyphosphates in budding yeast

When I examined Vps41p phosphorylation in hyper-osmotically stimulated *plc1*Δ, *arg82*Δ, *ipk1*Δ, *kcs1*Δ and *vip1*Δ cells, no Vps41p phosphorylation was detected in any of these mutants (Fig. 4.9). The simplest interpretation of this result is that an IP_8, one of the terminal products of this pathway may be necessary for hypertonically induced Vps41p phosphorylation, and so I initiated experiments to explore this possibility further.

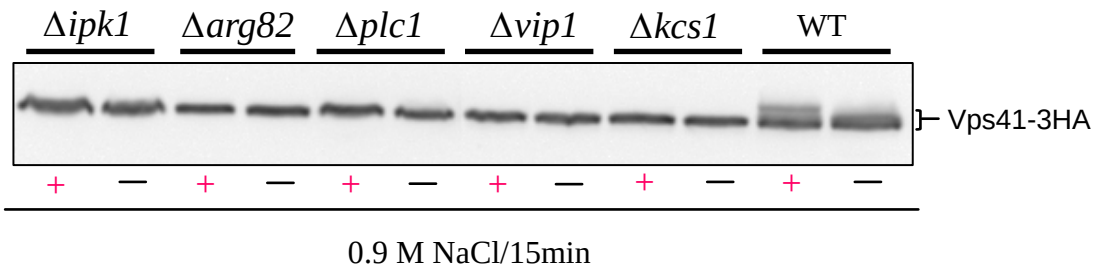


Figure 4.9: Inositol polyphosphate synthesis affects hyper-osmotically induced Vps41p phosphorylation

The indicated strains were grown to exponential phase and then given a hyper-osmotic shock (0.9M NaCl) for 15 min. Proteins were then extracted and resolved on SDS-PAGE and probed using an anti-HA antibody

4.2.4 Vps41p localisation is correlated to its phosphorylation

To comprehend the function of Vps41p in osmotic stress conditions, I first constructed endogenously expressed Vps41-GFP to observe its localisation pattern in cells. In wild type cells, Vps41-GFP is comparatively evenly distributed on the vacuole membrane that is in accord with its function during the vacuole membrane fusion and fission. In contrast, in *yck3*Δ cells, Vps41p is still retained on the vacuole, but there are now a number of large bright dots accumulating on or proximal to certain micro-domains of vacuole membrane which are absent in wild type cells. It is proposed that these micro-domains may be active sites of homotypic membrane fusion or some vesicular membrane fusion sites with vacuole. This differential distribution of Vps41p between wild type and *yck3*Δ enticed us to examine the Vps41p localisation in those inositol phosphate kinase null mutants where the Vps41p fails to be phosphorylated upon hyper-osmotic shock, such as *plc1*Δ, *arg82*Δ, *ipk1*Δ, *kcs1*Δ and *vip1*Δ. As expected, in all of these null strains, the Vps41p shared a similar localisation manner as in *yck3*Δ (Fig. 4.10).

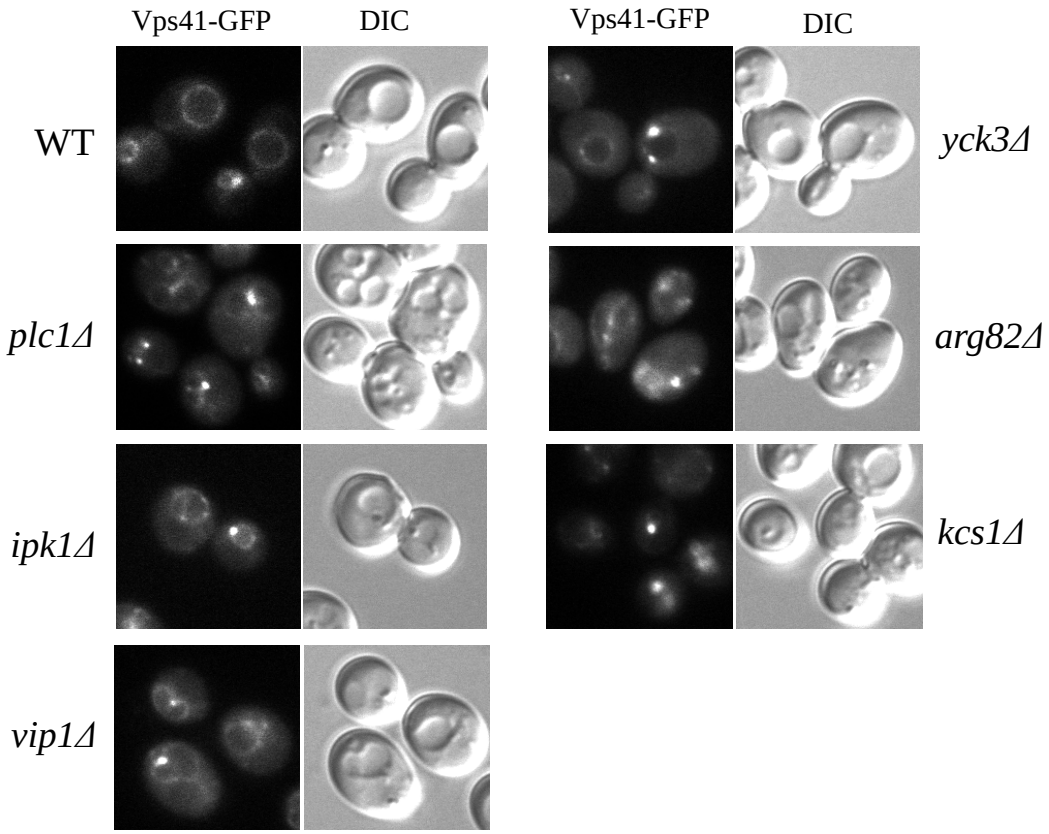


Figure 4.10: Vps41p localisation in wild type and IP null mutants

Vps41p-GFP was expressed in wild type, *yck3Δ* and several IP null mutant cells via knock-in and grown to exponential phase. The fluorescence signals were then detected by fluorescence microscopy

Since both Vps41p and Yck3p are vacuole-membrane localised proteins, they must take certain pathway to traffic to vacuole from Golgi. In budding yeast, there are two main routes leading to the yeast vacuole from Golgi. The first is called the CPY pathway. During this trafficking pathway, cargoes transit the late endosome or MVB and are only delivered to vacuole after MVB sorting. Alternatively, some cargoes can be conveyed directly from Golgi to vacuole by the AP-3 pathway. What pathway the cargoes can take depends on what sorting signals they have. In yeast, Yck3p is palmitoylated, then sorted to the vacuole membrane via the AP-3 pathway (Sun, *et al.*, 2004), whereas Vps41p is required by AP-3 pathway to form transport intermediates (Darsow *et al.,* 2001). Both proteins play functional roles in the AP-3 pathway.

In addition, inositol pyrophosphates are proposed to act as phosphate donors for a novel pathway of protein phosphorylation (actually they act as phosphate donors to phosphoserines previously phosphorylated by CK2 type protein kinases, and hence form serine pyrophosphates on target proteins) (Bhandari *et al.*, 2007). One of the substrates of inositol pyrophosphates was identified as a subunit of AP-3, namely Apl6p, although the physiological effect of this pyrophosphorylation is unknown (Bhandari *et al.*, 2007). Therefore, I needed to examine whether the AP-3 pathway functions properly in the IP synthesis disrupted null strains to exclude the possibility that the failure of Vps41p phosphorylation in these mutants is an indirect consequence of complete disruption of the AP-3 pathway.

In yeast cells, Sna4p can be used to examine the functional status of the AP-3 pathway. In wild type cells, the tetraspanin type membrane protein Sna4p is delivered to the vacuole outer membrane via the AP-3 pathway (Reggiori and Pelham, 2001). However, in AP-3 blocked *apl5*Δ cells, the Sna4p is mis-sorted into vacuole because it contains a cryptic MVB sorting signal that directs it into the vacuole if the protein enters this compartment. Similar to wild type, the trafficking of Sna4p displays wild-type localisation in *plc1*Δ and *kcs1*Δ cells (Fig. 4.11, similar results were obtained with *arg82*Δ, *ipk1*Δ and *vip1*Δ mutants (data are not shown)). So I can rule out the possibility that the phosphorylation and localisation defects of Vps41p in any of the IP synthesis blockade null strains results simply from gross disruption of the AP-3 pathway. So far the available data thus suggests that Vps41p localisation is correlated to its phosphorylation state, and that inositol pyrophosphates influence Vps41p phosphorylation through Yck3p in some fashion.

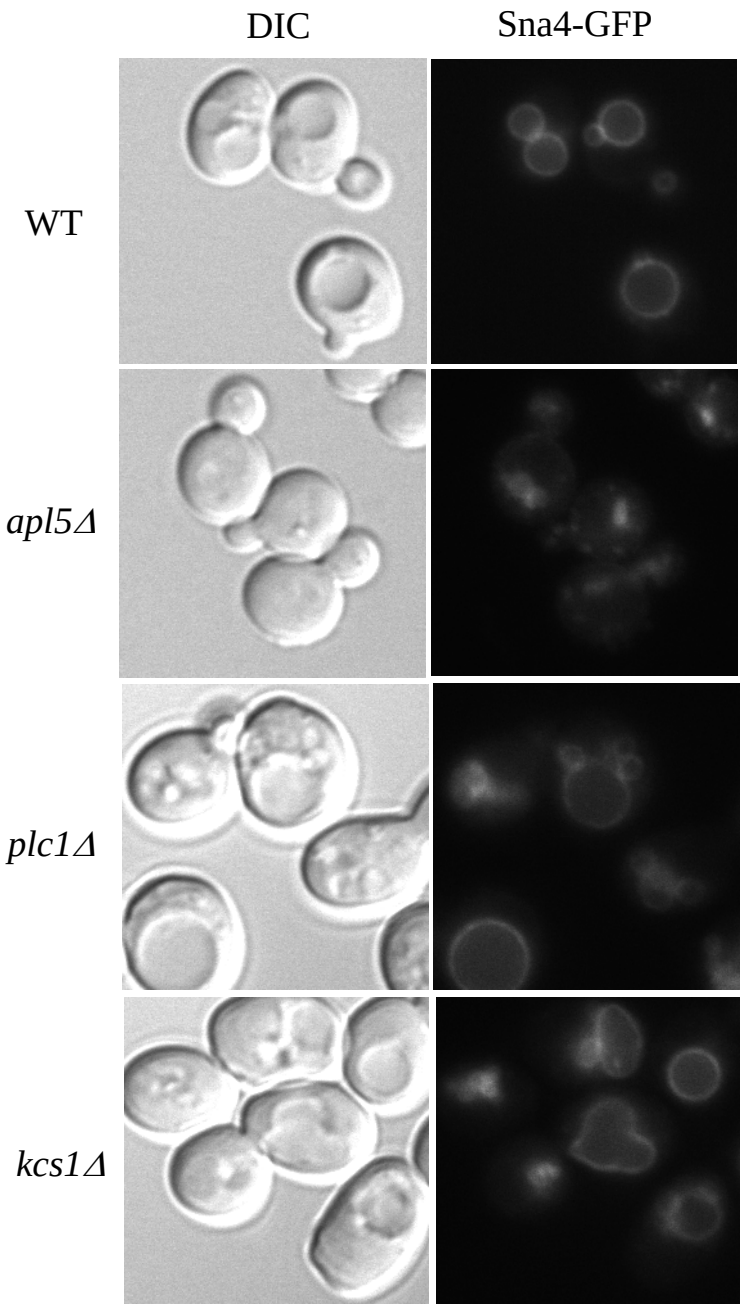


Figure 4.11: The AP-3 pathway functions properly in plc1Δ and kcs1Δ

Endogenous levels of Sna4p-GFP were expressed in wild type, *apl5*Δ, *plc1*Δ *and kcs1*Δ by knock-in to confirm that AP-3 pathway is working properly in IP null mutants. Cells were grown to exponential phase and visualised by fluorescence microscopy

In addition to being affected by IPs, deletion of *VPS41* itself seems in turn to affect synthesis of IPs and PPIns. As shown in Table 4.1, PtdIns4*P*, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and IP_6 levels (and presumably inositol pyrophosphates like IP_7 and IP_8, though I did not measure these latter two molecules directly) are drastically reduced in a *vps41*Δ strain. Indeed the lack of IP_6 is almost as profound as that found in *plc1*Δ mutants, as is the effect on PtdIns(4,5)P_2 levels, since deletion of *PLC1* reduces the levels of this lipid in some strain backgrounds for unknown reasons. This may suggest that Vps41p may feedback regulate synthesis of IP_6, much as Svp1p/Atg18p affects synthesis of PtdIns(3,5)P_2. This suggests some bidirectional regulatory links between Vps41p and Plc1p.

Strain --->	Wild-type basal	Wildtype salt	*vps41Δ basal*	*vps41Δ salt*
Inositol lipid				
PtdIns	3403000	3410102	3498579	3479290
PtdIns3P	92602	69266	95447	84270
PtdIns4P ↓	64789	57799	19838	25418
PtdIns(3,5)P2 ↓	7863	27141	697	8901
PtdIns(4,5)P2 ↓	39370	43315	11949	9745
InsP6 ↓	6800	10500	150	250

Table 4.1: inositol lipids and IP_6 levels in [^{3}H]Inositol radiolabelled cells

Cells were grown in media lacking inositol but containing radiolabel (^{3}H *myo*-inositol) for 5-6 generations and then lipids and InsPs extracted and quantified by anion exchange HPLC. Peak areas were calculated by using the integration function in the Flo-one software. Counts in different strains were then standardized to total counts using Excel. The above are representative results of one experiment that was repeated 3 times

4.2.5 Identification of the Vps41p phosphorylation sites

Before attempting to investigate the upstream control of Vps41p phosphorylation, I resolved to identify the exact Vps41p phosphorylation sites. Therefore, I immuno-purified Vps41p from total cellular lyates (Fig. 4.12). Note that phosphorylated

Vps41p can be detected by immunoblot but cannot be observed by coomassie staining, largely due to the low abundance or stiochiometry of phosphorylated Vps41p and to the poor sensitivity of coomassie statining. Inadequate separation from non-phosphorylated Vps41p was also probably partially to blame as well, as SDS-PAGE separations were sometimes variable. In order to identify the phosphorylation sites by FT-ICR mass spectrometry, therefore, I excised the Vps41p bands including the phosphorylated and non-phosphorylated Vps41p in the SDS gel (framed by the red rectangular in Fig. 4.12) based on the expected molecular weight of Vps41-3HA (approx. 120KDa) for the analysis by FT-ICR mass spectrometry. A list of the individual peptides were identified with reference to individual MS spectra given in Table 4.2 by searching against the non-redundant NCBI database together with the predicted phosporylation site localisation assessed by the SEQUEST algorithm. The distribution of all identified pepties in the full Vps41p sequences was depicted manually in Fig. 4.13.

The CID-FT-ICR mass spectrometry method is able to identify the presence of the phosphorylation modifications in certain peptides by observing its loss from the parent ion, but can not tell the exact modification sites because any labile modification is lost during the fragmentation (see Section 4.3.3 for more discussion). In order to obtain some ideas of where the phosphorylation may occur which will facilitate the subcequent biochemical and cellular experiments testing, the CID-MS data were analysed by the SEQUEST algorithm. As shown in the Table 4.2, the software inferred that at least one phosphorlations may occur on the four serines within the peptide sequence 20- TIDES*NS*IS*DENNVDNKREDVNVTS*PTK -47 but it can not tell which one(s) is/are the *bone fide* phosphorylation site(s).

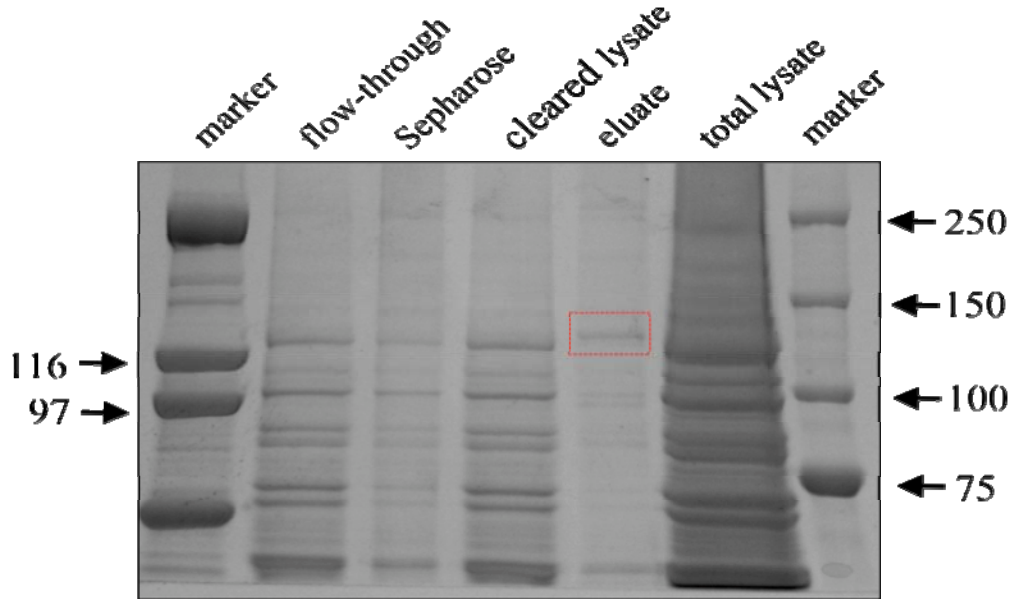


Figure 4.12: Immuno-purification of Vps41p

Approximately 300ml of BY4742 (VPS41:VPS41-3HA) cells were grown to early exponential phase in YPD media and subject to hyper-osmotic stress by further incuabating in YPD with 0.9M NaCl for 15 min at 30 °C. Then total cell lysates were extracted based on the method described in section 2.17 and Vps41p (both phosphorylated- and non-phosphorylated versions) was immuno-precipitated by anti-HA antibody - conjugated protein G sepharose, and detected by coomassie staining on an SDS-PAGE gel

Table 4.2: The peptides of Vps41p uncovered by CID-FT-ICR mass spectrometry

(Asterisks in peptides indicate sites of phosphorylation assigned by SQUEST, the start and finish numbers of each peptide in whole protein sequence were added manually)

Peptide	Mass (*m/z*)	Mass error (ppm)	Charge state	Fragmentation type	E-value	Coverage
20- TIDESNS*ISDENNVDNKR -37	2129.89828	1.66768	3	CID	3.62E-06	0.16
20- TIDES*NSISDENNVDNKR -37	2129.89828	1.66768	3	CID	4.04E-05	0.18
20- TIDESNSISDENNVDNKR -37	2049.93188	3.53002	3	CID	6.14E-08	0.41
20- TIDESNSISDENNVDNK -36	1893.83081	4.85939	2	CID	2.30E-07	0.38
654- IEAASEPTASSKEEGSNITYR -674	2240.06763	3.17590	3	CID	3.90E-09	0.44
20- TIDESNSIS*DENNVDNKREDVNVTSPTK -47	3200.42268	0.09819	4	CID	1.43E-05	0.17
20- TIDESNSISDENNVDNKREDVNVTS*PTK -47	3200.42268	0.09819	4	CID	1.43E-05	0.19
20- TIDESNSISDENNVDNKREDVNVTSPTK -47	3120.45630	2.15820	4	CID	3.69E-07	0.19
48- SVSCISQAENGVASR -62	1564.73840	1.12260	2	CID	3.80E-07	0.60
823- HNNYNVESAIEVCSSK -838	1850.83374	2.65662	3	CID	5.93E-06	0.52
211- MFVSGGMAGDVVLSQR -226	1685.79855	1.71194	2	CID	1.94E-06	0.53
935- HIVQENSLSLEVR – 947	1523.81763	1.47318	2	CID	7.09E-10	0.63
469- LSINDYHLGK -478	1159.61060	1.51480	2	CID	1.18E-05	0.18
156- SSILCINTDGK -166	1207.59875	0.34266	2	CID	2.38E-07	0.44
675- TELVHLYLKENK -686	1486.82642	-1.02383	3	CID	1.90E-04	0.59
901- AILTCSDETSEIYLK -915	1742.85168	3.52933	2	CID	7.24E-11	0.57
675- TELVHLYLK -683	1115.64587	-0.17618	2	CID	2.84E-05	0.47
483- TTPEYYLISSNDAIR -497	1742.85950	2.85622	3	CID	9.60E-05	0.34
948- DNILVIINDETKK -960	1514.84241	2.28773	2	CID	1.98E-08	0.33
285- SQLLNIPFPSR -295	1271.71069	1.28528	2	CID	3.62E-06	0.38
919- GMSDDLQIDNLQDIIK -934	1833.88987	3.76573	2	CID	1.46E-07	0.53
948- DNILVIINDETK -959	1386.74744	1.88287	2	CID	2.15E-06	0.49

MTTDNHQNDSVLDQQSGERTIDES*NS*IS*DENNVDNKREDVN
VTS*PTKSVSCISQAENGVASRTDESTITGSATDAETGDDDDD
DDDDDDEDEDDEDEPPLLKYTRISQLPKNFFQRDSISSCLFGD
TFFAFGTHSGILHLTTCAFEPIKTIKCHRSSILCINTDGKYFA
TGSIDGTVIIGSMDDPQNITQYDFKRPINSVALHSNFQASRMF
VSGGMAGDVVLSQRNWLGNRIDIVLNKKKKKKTRKDDLSSDMK
GPIMGIYTMGDLILWMDDDGITFCDVPTRSQLLNIPFPSRIFN
VQDVRPDLFRPHVHFLESDRVVIGWGSNIWLFKVSFTKDSNSI
KSGDSNSQSNNMSHFNPTTNIGSLLSSAASSFRGTPDKKVELE
CHFTVSMLITGLASFKDDQLLCLGFDIDIEEEATIDEDMKEGK
NFSKRPENLLAKGNAPELKIVDLFNGDEIYNDEVIMKNYEKLS
INDYHLGKHIDKTTPEYYLISSNDAIRVQELSLKDHFDWFMER
KQYYKAWKIGKYVIGSEERFSIGLKFLNSLVTKKDWGTLVDHL
NIIFEETLNSLDSNSYDVTQNVLKEWADIIEILITSGNIVEIA
PLIPKKPALRKSVYDDVLHYFLANDMINKFHEYITKWDLKLFS
VEDFEEELETRIEAASEPTASSKEEGSNITYRTELVHLYLKEN
KYTKAIPHLLKAKDLRALTIIKIQNLLPQYLDQIVDIILLPYK
GEISHISKLSIFEIQTIFNKPIDLLFENRHTISVARIYEIFEH
DCPKSFKKILFCYLIKFLDTDDSFMISPYENQLIELYSEYDRQ
SLLPFLQKHNNYNVESAIEVCSSKLGLYNELIYLWGKIGETKK
ALSLIIDELKNPQLAIDFVKNWGDSELWEFMINYSLDKPNFTK
AILTCSDETSEIYLKVIRGMSDDLQIDNLQDIIKHIVQENSLS
LEVRDNILVIINDETKKFANEFLKIRSQGKLFQVDESDIEIND
DLNGVL

Figure 4.13: The distribution of identified peptides in the full Vps41p sequences

The identified peptides were highlighted by yellow, the SEQUEST assessed phosphorylation sites are labelled by star *****, the total amino acid coverage is 21.4% of the full protein's sequence. Note, the blue-highighted four serines were not mapped in our study but identified by another group (Cabrera *et al.*, 2009, see Section 4.3.3 for more discussion)

In practice it is not possible to guarantee the accuracy of data generated by mass spectrometry results and database searching alone. Therefore, in order to test and verify the MS result, I employed site-directed mutagenesis to construct a *vps41*^{S24,26,28,44A} variant in the genomic DNA of wild type. As expected, Vps41^{S24,26,28,44A} failed to be phosphorylated upon hyper-osmotic stress (Fig. 4.14). The *vps41*^{S24,26,28,44A} allele also recapitulated the vacuole morphology phenotype of *yck3*Δ when expressed as a knock-in: i.e. it fragmented its vacuoles in response to salt

but then these vacuoles immediately re-fused indicating that phosphorylation of Vps41p may indeed be the means by which Yck3p blocks homotypic vacuole fusion during osmotic stress (Fig. 4.15 & 4.16). The localisation of this Vps41p variant, the *Vps41*^{S24,26,28,44A} allele, also displayed the characteristic Δ*yck3*-like dot morphology in some but not all cells (Fig. 4.17). These data reinforce the mass spectrometry results indicating that Serines 24, 26, 28 and 44 are likely *bona fide* phosphorylation sites.

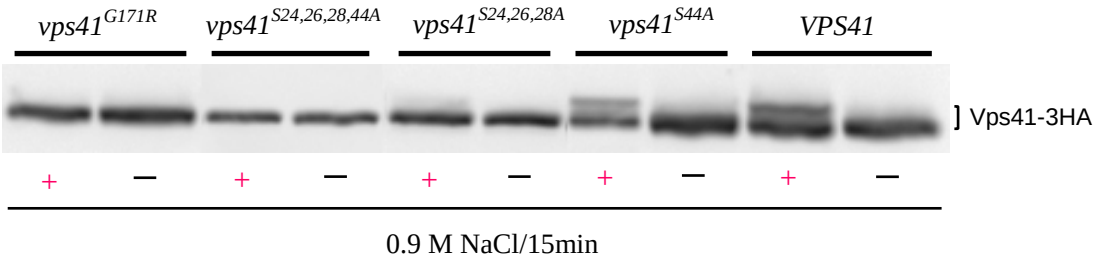


Figure 4.14: Response of Vps41p mutants to hyper-osmotic Stress

The site-specific mutagenesis method described in section 2.15 was used to construct three *vps41* mutants in the genomic DNA of BY4742: BY4742 (VPS41:VPS41^{S44A}-3HA), BY4742 (VPS41:VPS41^{S24,26,28A}-3HA), BY4742 (VPS41:VPS41^{S24,26,28,44A}-3HA) and BY4742 (VPS41:VPS41^{G171R}-3HA), abbreviated as: *vps41*^{S44A}, *vps41*^{S24,26,28A}, *vps41*^{S24,26,28,44A} and *vps41*^{G171R} respectively. The hyper-osmotic stress induced Vps41p phosphorylation status for each variant was analysed as described in section 2.16 with the wild type Vps41p acting as a positive control.

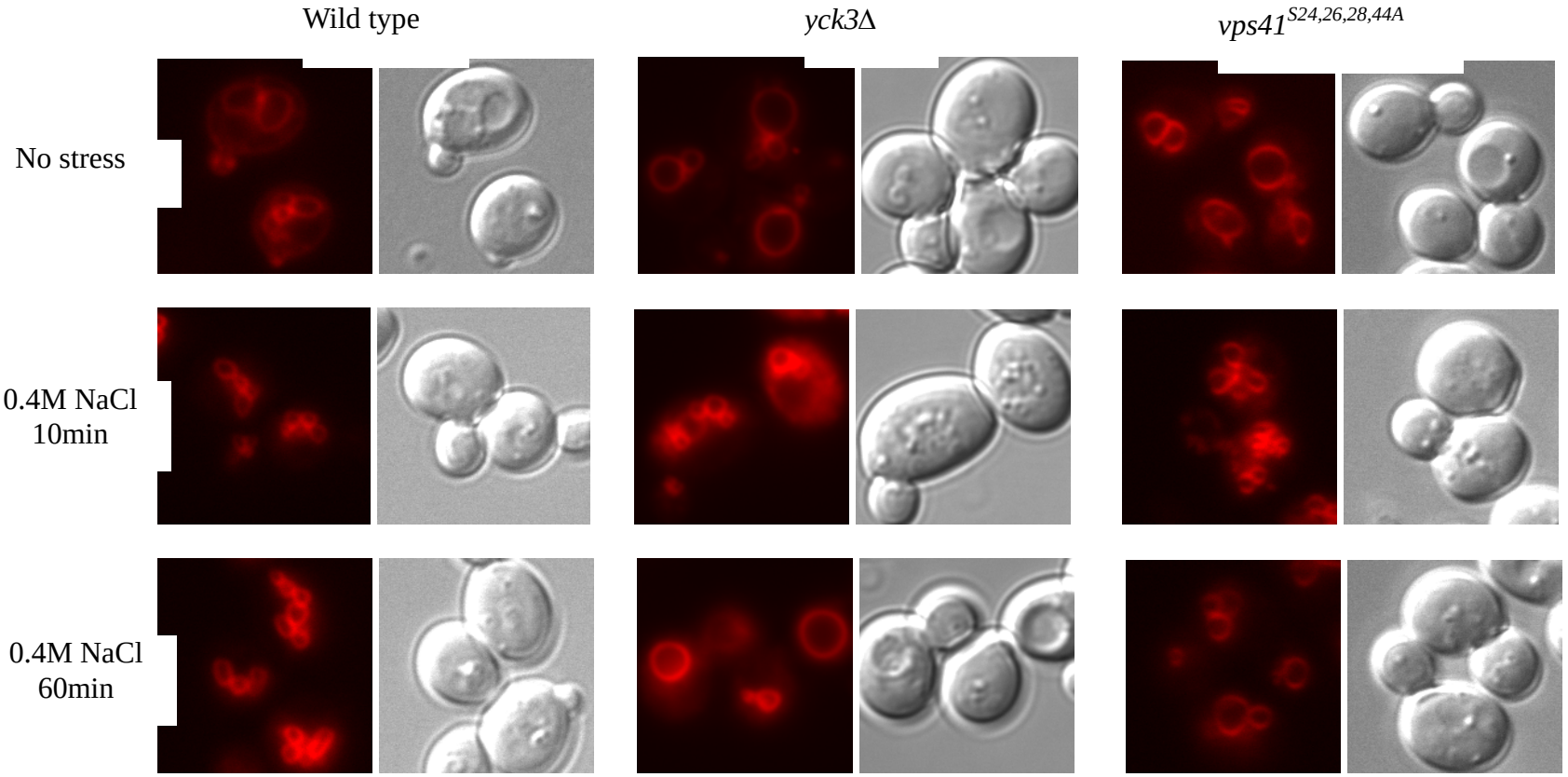


Figure 4.15: The vacuole morphologies of wild type, yck3Δ and vps41^{S24,26,28,44A} response distinctly upon salt stress. Cells of the indicated genotypes were grown to exponential phase and stained with the vital dye, FM4-64 to visualize vacuoles by fluorescence micoscopy (see Materials and Methods). Fields of cells were then photographed before, 10 and 60 mins after a hyper-osmotic stress and the number of vacuoles in each cell quantified (see below).

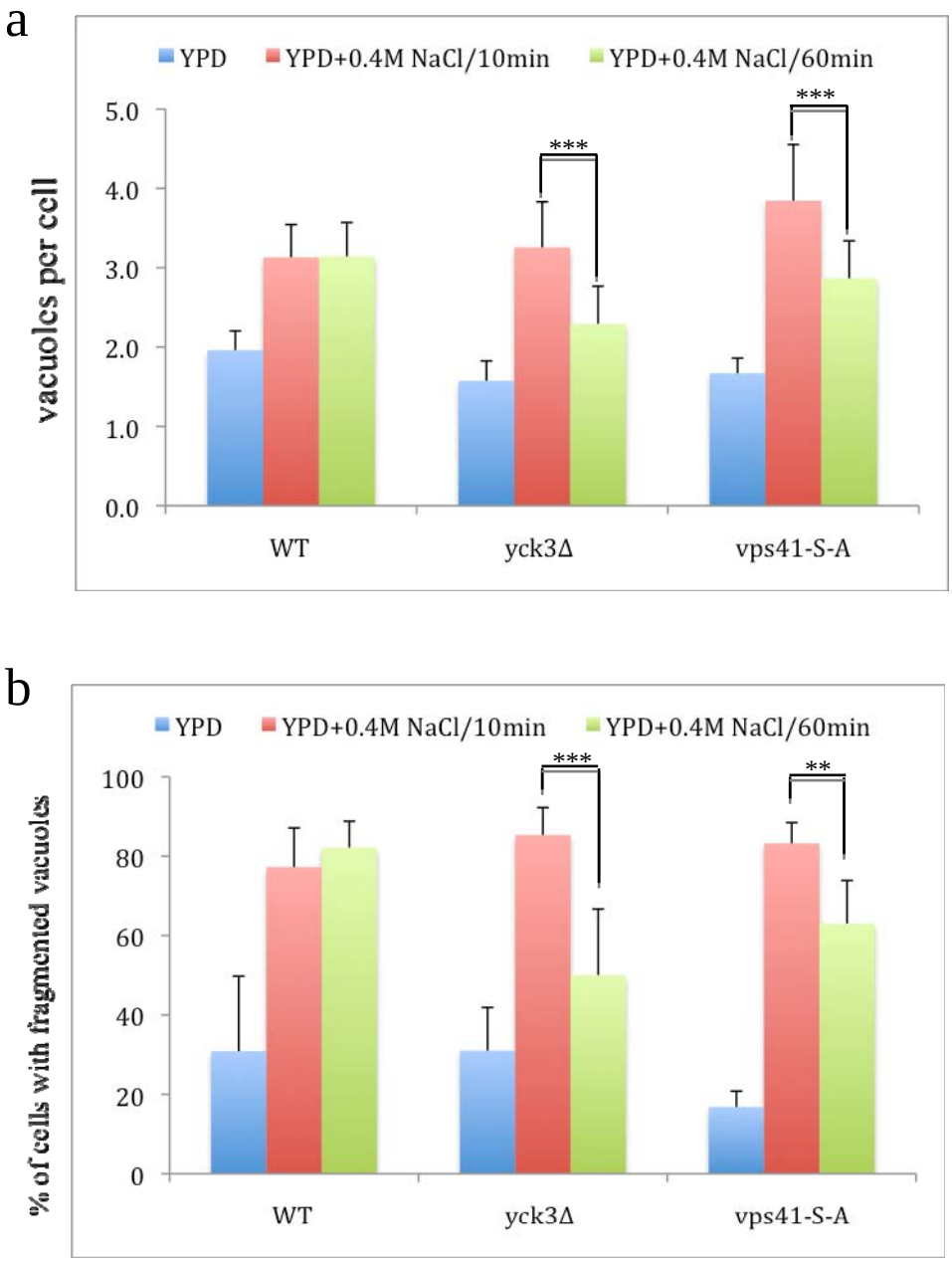


Figure 4.16: a. The vacuole number per cell in wild type, yck3Δ and vps41^{S24,26,28,44A} upon salt stress; b. The percentage of cells containing fragmented vacuoles in wild type, yck3Δ and vps41^{S24,26,28,44A} upon salt stress

Wild type(BY4742), *yck3*Δ and *vps41*^{S24,26,28,44A} were stained with FM4-64, exposed to hyper-osmotic stress (YPD+0.4M NaCl) for the indicated times (10 or 60min), then vacuole morphology was scored as either average vacuole number in each cell (a) or percentage of cells with fragmented vacuoles according to LaGrassa & Ungermann (2005) (b). Error bars indicate mean ± SD. At least 8 independent experiments were conducted and 300 cells were counted for each condition. Statistical significance is denoted by asterisks. **, $P \leq 0.01$; ***, $P \leq 0.001$

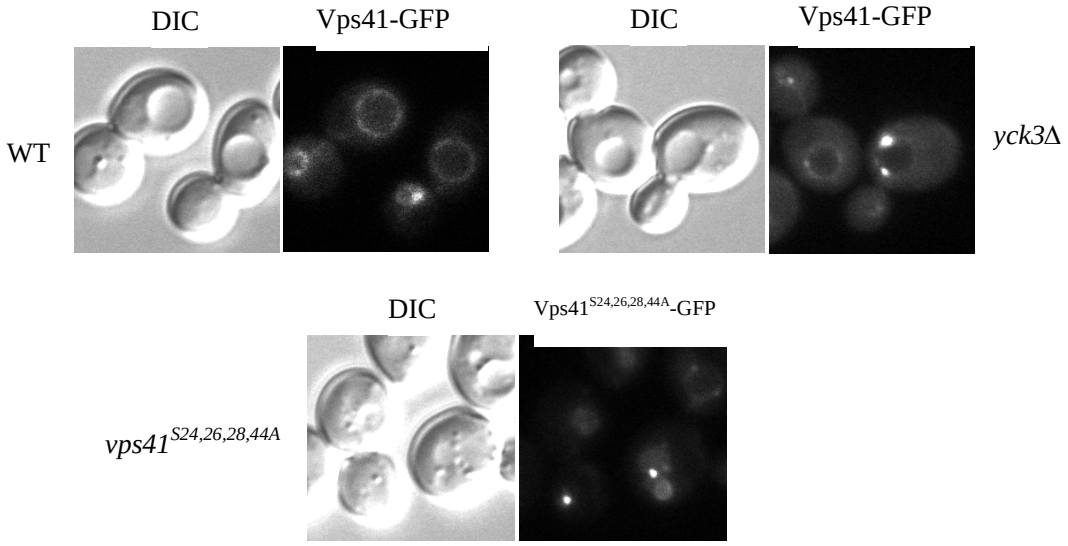


***Figure 4.17: Vps41^{S24,26,28,44A} localisation similar to Vps41p in yck3*Δ**

Cells of the indicated genotypes, bearing a *VPS41-GFP* knock-in (expressing Vps41-GFP at endogenous levels) were grown to exponential phase and the localisation of Vps41p established by fluorescence microscopy using a 100 x objective.

It was reported previously that Vps41p is required for the formation of transport vesicles at the Golgi complex in the AP-3 pathway (Rehling *et al*., 1999). It was later determined, by the same lab, that Vps41p exerts this function by homo-oligomerization and interaction with AP-3 through two distinct domains (Darsow *et al.*, 2001): an N-terminal Apl5p binding domain and C-terminal multimerization domain. In order to delineate the relationship between these two domains and the phosphorylation domain that I just identified (Fig. 4.18), I tried to re-create the two *vps41* variants described in Darsow *et al*, 2001, at the genomic level by knock-in: *vps41*^{G171R} and *vps41*^{I832T} cripple the Apl5p binding domain and multimerization domains respectively. I constructed the *vps41*^{G171R} mutant and the immunoblot result demonstrated that this Vps41p variant failed to be phosphorylated upon salt stress (Fig. 4.14), probably due to the mis-sorting of the kinase Yck3p in this mutant. I have not so far succeeded in constructing the *vps41*^{I832T} allel that compromises the Vps41p

multimerization domain, but I expect this Vps41p variant would also display the same lack of phosphorylation as *Vps41*^{G171R} and for much the same reason as discussed above.

Figure 4.18: Schematic domain structure of Vps41p

4.3 DISCUSSION

Previous work indicated that Vps41p is phosphorylated by Yck3p upon hyper-osmotic stress *in vitro*. I extended this by developing a new method to detect Vps41p phosphorylation *in vivo* and succeeded in mapping the stress-induced phosphorylation sites of Vps41p. I used this method to test various null mutants in the hope of identifying upstream regulators of this pathway and ruled out both the Hog1p and Fab1p/PtdIns(3,5)P_2 pathways, either singly or in combination. Our data also indicate that Plc1p-catalyzed generation of inositol pyrophosphates may be the long-sought upstream regulators, though I were unable to identify the sensor system upstream of Plc1p. Radiolabelling also suggests that a bidirectional interaction is occurring between Vps41p and Plc1p, (or with synthesis of PPIns upstream of it). Thus I propose that Plc1p, through inositol pyrophosphates, may regulate the phosphorylation of Vps41p in response to osmotic stress.

4.3.1 Detection of hyper-osmotic induced Vps41p phosphorylation by two distinct methods

In the original study, Vps41p phosphorylation was detected by a complex *in vitro* method: vacuole-containing membrane fractions were isolated and then incubated under standard fusion conditions in the presence or absence of ATP followed by SDS-PAGE and immuno-blotting of Vps41p. When ATP was present, Vps41p becomes retarded in its SDS-PAGE mobility corresponding to its increased phosphorylation status. However, when ATP is absent, only a single band corresponding to hypo-phosphorylated Vps41p is observed. There are a number of disadvantages to this method that make it difficult to increase the throughput, as would be required for even a directed screen.

1). Both protocols for vacuole isolation and the *in vitro* vacuole fusion reaction are relatively complicated and not amenable for screening of large numbers of mutants. In addition, there are many possible artifacts associated with *in vitro* assays of this type.

2). The manipulation of ATP to control the phosphorylation status of Vps41p is not physiological and may also introduce artifacts and could be prone to misinterpretation.

In order to circumvent the above problems, I developed a novel method to detect phosphorylated Vps41p: I constructed an epitope-tagged *VPS41* by knock-in (in the future, when anti-Vps41p sera are available, this step can be omitted), then gave the growing cell a hyper-osmotic stress followed by standard immuno-blotting protocols. I think this method is more physiological and rapid and will therefore lend itself to directed screens designed to isolate additional components of this pathway in future studies.

4.3.2 Regulation of hyper-osmotic induced Vps41p phosphorylation

To date, Yck3p is the only protein identified whose deletion totally abolishes Vps41p phosphorylation *in vitro* (LaGrassa and Ungermann, 2005) and *in vivo* (our current data). In the original work, Yck3p was suggested to be the direct kinase mediating Vps41p phosphorylation, chiefly based on the following observations: **a.** In the presence of excess Yck3p, Vps41p was constitutively phosphorylated *in vivo*; **b.** Yck3p and Vps41p are associated on isolated vacuoles; c. When wild type vacuoles containing tagged Vps41p were incubated together with *yck3Δ* vacuoles containing

untagged Vps41p, became phosphorylated *in trans* (LaGrassa and Ungermann, 2005). These data do not exclude the possibility that Yck3p is not the direct kinase but is in fact required for activation of the *bona fide* kinase *in vivo* or that Yck3p is required to constrictively phosphorylate Vps41p at a "priming site" that is required for subsequent, stress-induced phosphorylation by another protein kinase (or even an inositol pyrophosphate, see later). Indeed casein kinases are often found in exactly this role as priming agents. Therefore, evidence should be obtained from the purified Vps41p and Yck3p reaction *in vitro*, although such *in vitro* kinase assays are also fraught with the potential for artifacts. However, the mobility of Vps41p on a gel after such an *in vitro* phosphorylation should at least confirm that the mobility shift observed after stress *could* be mediated by Yck3p, as this is currently uncertain. Fortunately, it was recently reported that both Vps41p and Yck3p have been successfully purified as recombinant proteins from insect cells and bacteria, respectively (Brett *et al*, 2008; Hickey *et al*, 2009), thus, it is very likely that this issue will be resolved in the near future.

One particular reason for being cautious about the event causing the Vps41p band-shift in response to stress is the recent discovery that inositol pyrophosphates can donate directly one phosphate to proteins in a non-enzymatic reaction (Saiardi *et al*., 2004; Bhandari *et al.*, 2007). The available evidence suggests that proteins to be phosphorylated in this way must first be "primed" by being phosphorylated by CK2 isoforms. The serine residue phosphorylated by CK2 is then apparently further phosphorylated by the inositol pyrophosphate directly to create a phospho-anhydride (or pyrophosphate) bond. Hence it is possible that the observed band shift in Vps41p is not a direct result of Yck3p phosphorylation but could instead be a combination of

phosphorylation and inositol pyrophosphate phosphate donation. Whilst this proposition is attractive, there are a number of objections to this model. The first is that Yck3p is a CK1 like enzyme and, despite the similarity in name, CK1 and CK2 protein kinases are only very distantly related and share very little sequence homology or biochemical properties. CK1 isoforms form their own sub-family of protein kinase whilst CK2s are part of the CMGC family that includes Cyclin-dependent kinases, Map kinases, Glycogen synthase kinases and Cdk-like kinases (Gross and Anderson, 1998; Meggio and Pinna, 2003). The consensus phosphorylation sequences for the two families of enzymes are quite different, although both prefer to phosphorylate serines that are present in a background of acidic amino-acids. However, all phosphorylations mediated by inositol pyrophosphates in yeast appear to occur in the presence of a priming kinase activity that can use GTP as a phosphate donor (Bhandari *et al*., 2007), indicating that the priming activity is CK2-like as this family of protein kinase can utilize both GTP and ATP as a substrate, whereas CK1 isoforms cannot (Tuazon and Traugh, 1991). So unless yeast CK1s display fundamentally different properties to mammalian enzymes, and can use GTP, Yck3p is unlikely to be merely a priming kinase for inositol pyrophosphate mediated phosphorylation of Vps41p.

In addition to the Yck3p, I propose that inositol pyrophosphates (probably IP_7 and/or IP_8) may be regulators of hyper-osmotic stress evoked Vps41p phosphorylation. Such a role for these molecules has been hinted at from previous studies because mutants in various inositol phosphate kinases display altered vacuole morphology and because these mutants grow very slowly or not at all in high salt for unknown reasons (Perera *et al*, 2004; Pesesse, *et al.*, 2004; Choi *et al.*, 2007). In addition, one of the proposed

targets of inositol pyrophosphate mediated phosphorylation was identified as Apl6p (Bhandari *et al.*, 2007): a subunit of the AP-3 complex and therefore on the same pathway as Vps41p, although no functional role for this phosphorylation has yet been identified. Indeed I show here that ablation of inositol pyrophosphate synthesis does not cause a gross defect in the AP-3 pathway, since Sna4p is trafficked normally in *plc1*Δ, *arg82*Δ and *kcs1*Δ mutants (Fig. 4.11). This was an important result because I then observed that hyperosmotic-activated Vps41p phosphorylation did not occur in single mutants of enzymes mediating the IPs synthesis pathway. If I had not done the earlier Sna4p experiment, it would have been easy to speculate that the failure to phosphorylate Vps41p was merely due to disruption of the AP-3 pathway (which would also result in mislocalisation of Yck3p).

Our data do not allow us to determine if Plc1p and IPs are required for a constitutive event (e.g. priming) that is needed for the subsequent stress mediated phosphorylation of Vps41p or whether inositides directly control the phosphorylation in response to stress. There have been reports that IP levels are increased by the application of osmotic stresses (both hyper and hypo osmotic stresses) but the changes are relatively modest (3-5 fold) although these are whole cell measurements and it is unclear how much of basal and stimulated IP production is nuclear and whether nuclear pools can access the cytoplasm (Perera *et al*., 2004; Choi K, *et al*., 2005; Choi JH, *et al*., 2007). If, for example, basal IP production in *S. cerevisiae* is substantially nuclear, as would be inferred by the number of constitutive nuclear cell functions that seem to require IPs, then it is possible that stimulated levels of some inositol pyrophosphates might be increased 10-fold by stress and this might be enough to act as a second-messenger type switch if the affinities of the relevant inositol phosphate targets was in the

appropriate range. However, this remains speculation until these direct targets are identified and their affinities measured (see Chapter 5 for a more extensive discussion).

The mechanism of IP-mediated control of Vps41p phosphorylation is similarly unclear. There are reports that IPs, particularly IP_4, can regulate casein kinase activity in animal cells, through binding to the regulatory β-subunit of these enzymes, but this report again identified the CK2 family as the targets of IPs and not CK1s like Yck3p (Solyakov *et al*., 2004). It remains possible that Yck3p is directly regulated by inositol pyrophosphates or it may indeed be that once Yck3p has phosphorylated Vps41p that the inositol pyrophosphates are then required to directly phosphorylate the same serines on Vps41p non-enzymatically, forming a serine pyrophosphate that is required to block vacuole-vacuole fusion. Our MS data did not indicate that Vps41p was pyrophosphorylated, but it remains possible that serine-pyrophosphate containing peptides were somehow not identified or destroyed during the procedure for technical reasons, as they are known to be much more labile than simple phosphoserine residues. Further experiments are required to clarify this point.

Our discovery that IPs may regulate Vps41p phosphorylation, and therefore control vacuole fusion and fission, may provide a key to unravel the underlying mechanisms for the phenotypes of the IP null strains. Indeed, *plc1*Δ, *arg82*Δ and *kcs1*Δ mutants were all recently isolated in a screen for deletion mutants displaying highly aberrant vacuoles (Seeley *et al*., 2002), suggesting that IPs are indeed required for vacuole fusion/fission under some circumstances. This observation has also been noted in more directed studies of vacuole morphology in *arg82*Δ and *kcs1*Δ mutants, where

aberrant morphology was also reported.

The discovery that levels of PtdIns4*P*, PtdIns(4,5)P_2 and IP_6 (and probably IP_7 and IP_8) are extremely low in *vps41*Δ mutants was surprising and may indicate that some of the phenotypes of this strain are attributable to low Plc1p signalling. The reason for this effect is not clear but it is tempting to speculate that Vps41p contributes to the feedback regulation of steady-state levels of PPIn signals in the Plc1p pathway in much the same way as Svp1p/Atg18p does for the Fab1p pathway. Indeed, both proteins are seven bladed β–propellers and both exhibit a blade junction with a very similar sequence over just the area where PtdIns(3,5)P_2 is reported to bind in Svp1p/Atg18p (Fig. 4.19)

```
VPS41:  DGxxxATGSIDGTVIIGSMDDPQNITQYDFKRPIN
ATG18:  DGxxxATAS-DKGTIIRVFDIETGDKIYQFRRGTY
```

Figure 4.19: Amino-acids of the junction of blades 4 and 5 of Atg18p compared with the corresponding junction between two blades of Vps41p

The AP-3 binding site of Vps41p is underlined. The PPIns binding site of Atg18p is also underlined

Previous studies have indicated that Vps41p might bind several inositol lipids in a weak and fairly nonspecific fashion but our unpublished data have not corroborated this so-far since Vps41p expresses poorly in bacteria and appears to produce partially mis-folded, albeit soluble protein, that may not display the normal properties of Vps41p (Heath and Dove, unpublished). The use of CCT-like chaperones in *in vitro* translation reactions might be a profitable next step to overcome this technical expression problem, as many β-propellers require CCTs for correct folding and these proteins are absent from prokaryotes.

4.3.3 Identification of hyper-osmotically induced Vps41p phosphorylation sites

In order to investigate the Vps41p phosphorylation phenomenon further, it was imperative that the identification of the phosphorylation sites was undertaken. I therefore immuno-purified phosphorylated Vps41p from hyper-osmotically stimulated cells. Our mass spectrometry data identified the presence of phosphorylation within peptide of 20-TIDESNSISDENNVDNKREDVNVTSPTK-47. The SEQUEST algorithm analysis of the MS data suggested the four serine amino acids at 24, 26, 28 and 44 as likely phosphorylation sites. The *vps41*$^{S\text{-}A}$ mutants harboring different combinations of serine-alanine transitions exhibited different degrees of phosphorylation of Vps41p, with the mutant harboring all four serine to alanine mutations phenocoping most of defects of *yck3Δ* cells, whereas mutants with fewer serine mutations displayed less severe defects as expected, further confirming that these four serine sites are Vps41p's intrinsic phosphorylation sites upon osmotic stress.

The coupling of high mass accuary FT-ICR mass spectrometry and the tandem mass spectrometry and CID enables the identification of post-translational modifications like phosphorylation. However, CID achieves peptide fragmentation by heating the sample to a higher Boltzmann temperature, resulting in the loss of any labile modification during this step and this procedure can’t locate the exact modification sites. In addition, the use of SEQUEST analysis on CID data just gives a low confidence site localization, and it is also difficult to analyse multiply and/or doubly phoshorylated peptides. Finally, the low stoichiometry typically associated with phosphorylation event means that phosphopeptides are often overlooked in data-dependent CID analyses. However, the Electron capture dissociation (ECD) mass

spectormetry provide an alternative to CID on the identification of exact modification sites (Zubarev *et al*., 1998). ECD is a radical-driven fragmentation technique and retains the labile side-chain modifications intact upon peotide backbone cleavage. However, ECD enables unambiguous assignment of the sites of modification, and also exact localization of any double phosphorylations. The downside to this technique is the increased sample purity and abundance required. If I had had more time, I would have liked to have optimized my immuno-purification method in order to get a much purer protein sample, and also try to enrich the phosphopeptide by use of phosphor-specfic antibodies or chromatographic enrichment. I would then have tried to determine the exact phosphorylation sites of Vps41 by ECD-FI-ICR mass spectrometry.

After I identified these phosphorylation sites but before publishing our result, another group also reported the identification of the osmotic stress sentitive phosphorylation sites but mapped them to four different serine residules: S367, 368 371, 372. They adopted a different method from us by searching available databases for CK1 (Yck3p) sites within the Vps41p sequence. They mutated the four serines to alanines and the resulting Vps41p mutants were expressed in the *vps41Δ* background. Membrane fractions were then purified from these mutants and Vps41p phosphorylation was detected by an ATP-dependent mobility shift on SDS-PAGE gels following an *in vitro* assay (Cabrera *et al*, 2009). Their data also showed no phosphorylation of Vps41p after these mutations were made, so how to reconcile our data with those of Cabrera *et al*.?

The observed differences between our data obtained by mass spectrometry and the

data obtained from database searching are difficult to explain if Yck3p is the *bona fide* kinase that directly phosphorylates Vps41p in response to stress. This is, however, an unproven assumption, as is the assumption that Yck3p is a prototypic CK1 with the same consensus sequence as that found in other organisms: the exact consensus and substrate preferences of Yck3p are untested experimentally and conclusions based purely on *in silico* analysis are often highly questionable. Indeed, Yck3p is a fairly divergent member of the CK1 family and could easily display unusual substrate specificity. In addition, since Vps41p is a β-propeller, making mutations and then drawing conclusions based solely on the mutants in *in vitro* kinase assays is dangerous as subtle changes in amino-acid sequence can drastically affect the structure of the propeller and can make interpretation difficult, especially when the expected result is a negative one: i.e. no phosphorylation. Our mutants are in an area of the propeller, close to the N-terminus, where point mutations would not be expected to affect the gross structure of the whole propeller. Cabrera's mutations (2009), in contrast, are deep in the propeller, though it is difficult to evaluate the effect, these mutations might have without a proper 3-D model of this protein.

In contrast, our identification of the phosphorylations sites has been validated by both subsequent immuno-blot examination of phosphorylation of Vps41p *in vivo*, and by examination of vacuole morphology in response to stress. This further strengthens the possibility that Ser 24, 26, 28 44 are phosphorylated *in vivo,* though it is possible that they are constitutively phosphorylated as I did not subject Vps41p from unstressed cells to the same analysis. If this were to be true, then our Vps41p mutants still blocks phosphorylation, although the effect could be indirect (e.g that our sites are "priming" phosphorylation sites). Since our phosphorylation sites and those described by

Cabrera (2009) are very distant in the primary amino-acid sequence, it seems unlikely that one set of sites is the “priming” site for the other, unless these sequences are closely apposed in the 3-dimensional structure.

Intriguingly, our phosphorylation sites do not conform to the consensus for CK1 and this suggests that I have identified the sites phosphorylated by a novel kinase, and not those directly attacked by Yck3p, unless this enzyme has an atypical consensus phosphorylation sequence. On balance, this might infer that the sites of Cabrera *et al*. are those phosphorylated by Yck3p and that these act as priming sites for our phosphorylations, which are responsive to hyper-osmotic stress. This would imply that the Yck3p phosphorylations are constitutive and that a second unidentified kinase mediates the phosphorylations I have uncovered. If this were the case, then it will be important to determine which of these kinases is downstream of Plc1p and the inositol phosphates. Finally, ECD mass spectrometry should be used to map the phosphorylation sites unequivocally.

4.3.4 Three hyper-osmotic pathways in the budding yeast

In budding yeast, two pathways have previously been known to respond to extracellular hyper-osmotic stress. One is called Hog1p MAPK pathway, which is centered on three tiers of protein kinases whose sequential phosphorylations eventually influence transcription factors to synthesize increased levels of glycerol that maintain the osmotic balance of the cells under hyper-osmotic conditions. The other is called Fab1p pathway, which is responsible for the generation of the phospholipid PtdIns(3,5)P_2 and appears to regulate vacuole fission and possibly vacuole acidity in response to stress. In this case, it is suggested that efflux of ions,

osmolytes and water from vacuole to cytoplasm results in a short term benefit to the cell in the face of intense dehydration. It is also possible that the regulated reduction in vacuole membrane area makes the rupture of this organelle much less likely during osmotic imbalances. It has been demonstrated that Fab1p and Hog1p pathways work independently under hyper-osmotic stress and it now appears that Vps41p is similarly independent. It is still possible that Fab1p and Yck3p share a common upstream activator but that the pathway diverges before it reaches these two proteins, but I consider this unlikely because of the fact that Plc1p appears to control Vps41p phosphorylation.

Although the PtdIns(3,5)P_2 accumulation and Vps41p phosphorylation have a lot in common upon osmotic stress, I failed to demonstrate any form of co-regulation. Our final model is depicted in (Fig. 4.20): upon hyper-osmotic stress, three pathways, Fab1p, Vps41p and Hog1p are activated almost at the same time but they take effect in a defined sequence. Fab1p and Vps41p come into operation much earlier than Hog1p by controlling vacuole fission and inhibiting re-fusion respectively. These two pathways are therefore responsible for a rapid but short-term adaptation to stress. Hog1p is then required for long-term adaptation and for the resumption of growth and cell division.

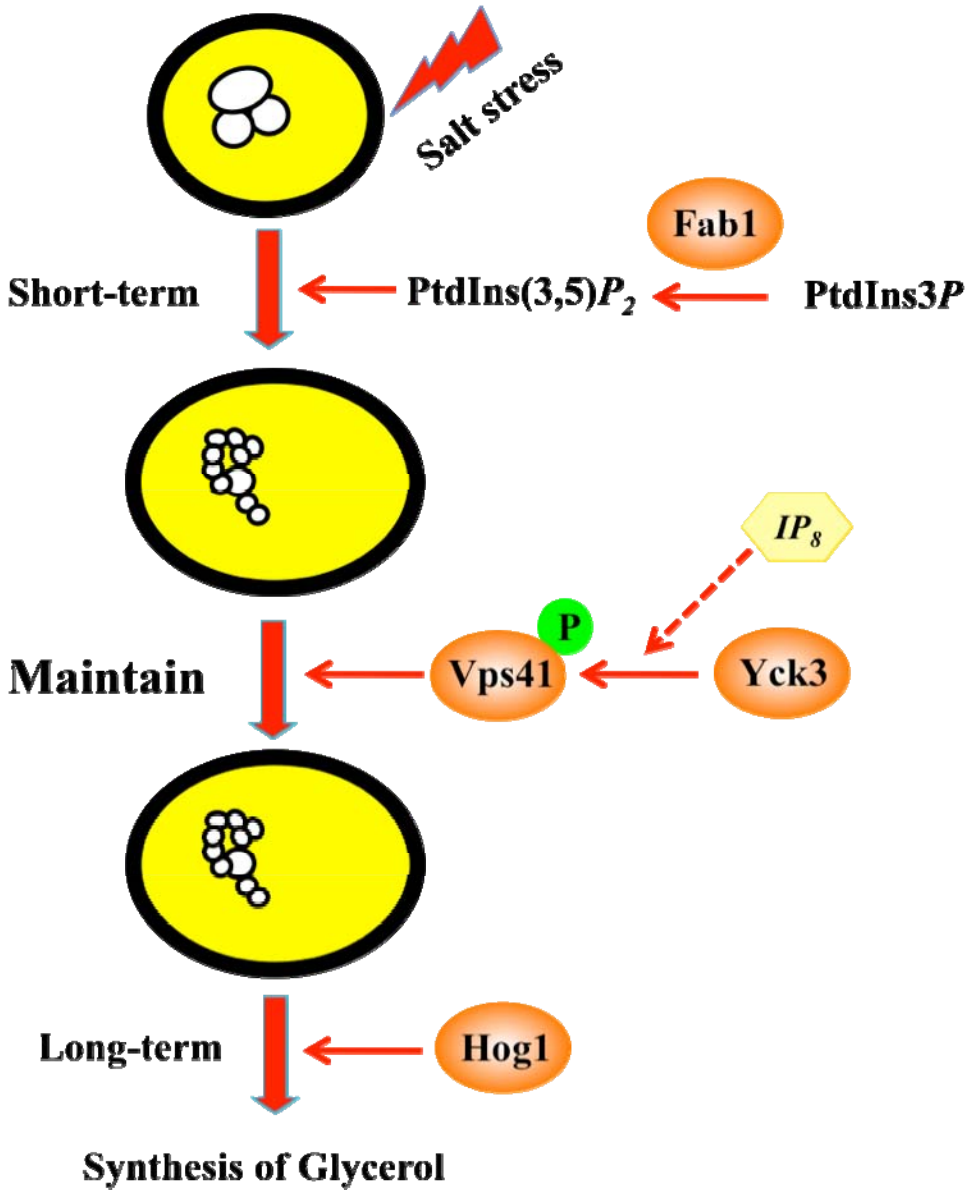


Figure 4.20: Model of hyper-osmotic stress pathways in budding yeast

Hyper-osmotic stress first activates the Fab1p pathway and the resulting PtdIns(3,5)P_2 fragments the vacuole to prevent immediate rupture. Plc1p is also activated and the resulting IP_8 promotes phosphorylation of Vps41p, which blocks fusion and keeps the vacuoles in a fragmented state after levels of PtdIns(3,5)P_2 return to normal after 10-60 mins of stress (the exact time is determined by the intensity of the osmotic stress). The Hog1p pathway then has time to activate gene transcription and elevate cytoplasmic levels of glycerol, resulting in a restoration of water balance with the external medium

Chapter 5: General discussion and future work

5.1 Myotubularins: what is their real function?

Following the successful purification of Ymr1p from bacteria, I also tried to examine its substrate preference using a malachite green-based colorimetric phosphatase assay. The premilinary observation showed that this enzyme can utilize PtdIns3*P* and PtdIns(3,5)P_2 but not PtdIns4*P* or PtdIns(4,5)P_2 as substrates. Since the assay is not yet optimized it is not possible to say what the preferred substrate is, although under present conditions it appears to prefer PtdIns3*P*. Therefore, optimizing this colorimetric assay in order to convince the above observation would be a major concern in my future work.

I am interested in the study of the substrate specificity of Ymr1p because of the confusion surrounding the role of the myotubularin family of enzymes in animal cells. I had hoped to identify a myotubularin that would not attack PtdIns(3,5)P_2 because this would have been a valuable tool for expressing in animal cells, to probe which substrate(s) of myotubularins is/are important in various pathological processes. It is known that the mammalian enzymes (e.g. MTM1 and MTMR2) will degrade both PtdIns3*P* and PtdIns(3,5)P_2 (Clague and Lorenzo, 2005). In the case of the latter lipid, myotubularins may form PtdIns5*P*: indeed this is the only widely accepted route to this novel inositol lipid.

As indicated in Chapter 1, myotubularins are inactivated in a number of human genetic diseases, including the muscle disease, X-linked myotubular myopathy and a number of Charcot-Marie-Tooth (CMT) like neuropathies, particularly CMT4 (Bolino

et al., 2000; Berger *et al*., 2002). In the case of these latter diseases, gross outfoldings of the myelin sheath coating the affected nerve cells causes a severe reduction in the speed of the action potential. This appears to result from the aberrant morphology of Schwann cells (the cells responsible for forming the myelin sheath), which have excessive plasma membrane area (Bolino *et al*., 2004). One recent study suggests that MTMR2, a myotubularin inactivated in CMT4B, antagonizes the action of the exocyst complex that delivers a membrane cargo to the plasma membrane for secretion (Bolis *et al*., 2009).

This implies that myotubularins might be regulating endocytosis somehow. Intriguingly mutations in mammalian Fig4p, the enzyme that degrades PtdIns(3,5)P_2, can also give rise to a form of CMT, known as CMT4J (Chow *et al*., 2007). This result suggests that it is alterations in inositol lipids caused by myotubularin/Fig4p inactivation that causes CMT4B because Fig4p is not known to associate with myotubularins. Somewhat non-intuitively, FIG4 mutations reduce the levels of PtdIns(3,5)P_2 in all organisms tested because this enzyme is an essential part of the complex that correctly localises the Fab1p lipid kinase and keeps it in an active conformation, partnered with Vac14p (Duex *et al*., 2006a). Hence if I compare what happens to inositol lipids in myotubularin null and fig4$^{-/-}$ null strains some patterns begin to appear as following table.

Gene inactivated / Lipid	**FIG4**	**myotubularin**
PtdIns3*P*	increased	increased
PtdIns(3,5)P_2	30-40%	increased
PtdIns5*P*	~20%	~20% or less

This suggests that either too much PtdIns3*P* or too little PtdIns5*P* is responsible for the CMT4 type phenotype and results in excess exocytosis or a reduction in

compensatory endocytosis. Since PtdIns3*P* has a well defined set of functions in various cellular processes that all tend to send membrane away from the cell surface and towards to the lysosome/vacuole (e.g. autophagy, the Cvt pathway, protein sorting to the lysosome/vacuole and MVB sorting) (see Section 1.6.1), I would suggest that too much PtdIns3*P* would be unlikely to result in the delivery of more membrane to the cell surface, indeed, the opposite might be expected.

In contrast, very little is known about PtdIns5*P* and it putative functions, making alterations in the metabolism of this lipid the prime candidate for the cause of CMT4 type neuropathies. Yet if PtdIns5*P* synthesis occurs via myotubularins from PtdIns(3,5)P_2, how does this get to the cell surface: after all Fab1p/PIKfyve are implicated in events at or around the late endosome in animal cells: deep within the cell (Shisheva, 2008). However, our ideas of where PtdIns(3,5)P_2 is actually made in a cell are vague because none of the effectors so far identified have provided us with a probe that is suitable for live cell imaging. In effect, I am only guessing where this lipid is made from the effects that I observe on phenotypes. *A priori*, PtdIns(3,5)P_2 (and consequently PtdIns5*P*) could be made anywhere that PtdIns3*P* is present in the cell. The distribution of PtdIns3*P* was recently defined by using a tandem FYVE domain construct linked to immuno-gold to probe ultrathin sections of fixed cells under EM (Gillooly, *et al.*, 2000). The results suggest that PtdIns3*P* is present at the TGN, late endosome/MVB and lysosomal/vacuolar surface, as well as the nucleus of animal but not yeast cells.

So PtdIns(3,5)P_2 could indeed be made close to the cell surface, and there is already evidence that it can influence cell surface trafficking. For example, the increased cell

surface residency of a number of ion and nutrient channels in response to various agents requires PIKfyve (the mammalian Fab1p) (Klaus *et al*., 2009a and b)**,** and there is evidence in yeast for a requirement for Fab1p in the trafficking of Chs3p, a chitin synthase that cycles between the cell wall/plasma membrane and the Snc1p positive recycling endosome (Phelan *et al*., 2006).

This suggests that increased PtdIns(3,5)P_2 is required for exocytosis somehow and it is tempting to speculate that PtdIns5*P* then balances the membrane delivered to the plasma membrane by stimulating endocytosis, either directly or possibly via type II PtdIns5*P* 4-kinases. I favour the view that PtdIns5*P* itself may be the regulator of endocytosis because I have shown here that Ymr1p can indeed make this lipid and that it is present in yeast cells, despite the absence of a type II PtdIns*P* kinase, though this is not well supported at present.

In regard to possible endocytic functions for PtdIns5*P*: it is notable that several bacterial pathogens produce extracellular enzymes (e.g IpgD) that they inject into cells to produce PtdIns5*P* (Niebuhr *et al*., 2002). Could this be to facilitate their uptake into cells via endocytosis: clearly further research is needed but it is an attractive hypothesis?

Indeed it fits in with a larger hypothesis that has been suggested in our lab a number of times: that one important function of PPIns is not just to regulate the direction and on/off nature of membrane trafficking, but that these lipids balance membrane flow from one organelle to the next so that there is no net gain or loss of membrane from one organelle to another (Dove *et al*., 2009).

To understand this idea, consider how type II PtdIns5*P* 4-kinases are supposed to work: as the last step in the complicated metabolic scheme indicated below

PtdIns ------> PtdIns3*P* -------> PtdIns(3,5)P_2 ------> PtdIns5*P* ------> PtdIns(4,5)P_2.

This metabolism seems an absurd way to synthesize a signal until I consider that there may be a cell biological reason for these inter-conversions: that each transition to a new PPIn may accompany a parallel transition to a new compartment/organelle. Now what if I go further and ask if PPIns are counting molecules that ensure that organelles do not grow or shrink as a result of endomembrane trafficking? So one important function of the PtdIns(3,5)P_2 to PtdIns5*P* transition could be to regulate plasma membrane area. This would mean that somehow the plasma membrane turns all arriving PtdIns(3,5)P_2 into PtdIns5*P* and this then results in the same amount of membrane leaving the cell surface as arrived at the plasma membrane, thus maintaining membrane area and tension. All the PtdIns5*P* does is signal the change in direction and when there is no myotubularin then this flow membrane away from the plasma membrane is blocked and the plasma membrane expands, as happens in mtm2$^{-/-}$ Schwann cells (Bolis *et al.*, 2009). Clearly some mechanisms must exist to balance membrane entry and exit from organelles and I would like to speculate that PPIns are a possible candidate. In this regard it is noticeable that in yeast, nearly all the membrane trafficking pathways leading into the vacuole utilise PtdIns3*P* and the only known pathway causing membrane to leave the vacuole is dependent on PtdIns(3,5)P_2 (Dove *et al.*, 2004). Since this lipid couplet are also interconnected metabolically, the same logic could apply with a certain fraction of PtdIns3*P* turned into PtdIns(3,5)P_2 to balance out the inward flow. In support of this hypothesis, increasing the levels of active Fab1p causes the vacuole to fragment and shrink, losing membrane area whilst decreasing the amount of active Fab1p causes the vacuole to swell and gain

membrane area (Bonangelino *et al.,* 2002).

5.2 Vac7p localisation

The idea that there might be different pools of PtdIns(3,5)P_2 in cells suggested to us that I might be able to find components of the Fab1p machinery that were only involved in the regulation of one pool but not others. It was therefore gratifying when I found that Vac7p takes the AP-3 dependent pathway to the vacuole, suggesting that it cannot participate in the MVB sorting functions of Fab1p/PtdIns(3,5)P_2 unless it traffics to back from the vacuole to the Pep12p positive endosome with the cargo. I think this is unlikely because Vac7p obviously lacks the negative sorting information required to keep it out of the forming intraluminal vesicles of the MVB and so sending it to the Pep12p positive endosome (which matures into the MVB) would result in the destruction of this protein.

I therefore believe that Vac7p only regulates Fab1p on the vacuole and since Vac7p is required for the increase of PtdIns(3,5)P_2 seen after hyper-osmotic stress (Gary *et al.*, 2002; Duex *et al*., 2006b), this suggests that most of the newly synthesized lipid is made on the vacuole membrane.

Vac7p trafficking through the AP-3 pathway may also suggest something else: that it and Atg18p are connected with AP-3 dependent sorting from Golgi to vacuole. As shown in Chapter 4, during the comparison between Vps41p and Atg18p domain structure, Atg18p has a domain close to the lipid binding site that looks very like the AP-3 binding site of Vps41p. Although Fab1p and PtdIns(3,5)P_2 are not involved in the regulation of AP-3 pathway, perhaps Vac7p and Atg18p are, because they are

binding partners for one another and because Atg18p may bind AP-3 (Efe *et al*., 2007). It is tempting to speculate that Atg18p might mediate membrane retrieval from the vacuole back to the Golgi in a PtdIns(3,5)P_2 independent but Vac7p dependent manner. Or perhaps Vac7p and Atg18p are required for forward trafficking of proteins and membrane towards the vacuole. Experiments are underway in the lab to determine the substance of these speculations.

5.3 The yeast endosomal system

Much of the work in this thesis has focused on membrane trafficking and has used the budding yeast, *S. cerevisiae* as a model organism, for reasons that are primarily technical and have been elaborated earlier (see Section 1.8). In using yeast I had the clear intention of discovering broad mechanistic insights into membrane trafficking that I might then be able to apply to trafficking in other eukaryotes in general. Our expectations were not unreasonable, given the fact that much of our understanding of eukaryotic endomembrane biology came from studies conducted in yeast (Bowers and Stevens, 2005). Indeed, the Fab1p pathway, originally described by ourselves and others from *S. cerevisiae* (Odorizzi *et al*., 1998; Cooke *et al*., 1998), has proved to be remarkably conserved in most eukaryotes (see review in Dove *et al*., 2009)

Yet I quickly became aware of a problem with such an evolutionary view: the yeast endosomal system is seemingly similar to its mammalian counterpart and yet this superficial similarity may be deceptive (Dove *et al*., 2009 and references therein). In order to port observations from yeast to animals it is necessary to have a clear idea of which compartment in yeast corresponds to which compartment in animal cells (Ali *et al*., 2004).

In *S. cerevisiae*, there is a compartment, marked by the SNARE Snc1p that is immediately under the plasma membrane. This has led many to assume that this compartment is the yeast "early endosome" (Prescianotto-Baschong and Riezman, 2002) though more cautious researchers have also called it the "post Golgi endosome" (PGE) in order to avoid this assumption (Weisman, 2003). Similarly, the endosomal structures lying close to the vacuolar membrane that are marked by the SNARE Pep12p, have also been dubbed "late endosomes" by many researchers (Schimmoller and Riezman, 1993), though this organelle is also variously referred to as the "pre-vacuolar endosome (PVE) or pre-vacuolar compartment (PVC) in an attempt to sidestep the issue (Ali *et al*., 2004). Even the vacuole itself has not escaped, and is almost universally assumed to be the yeast lysosome, mostly because of a lack of any other obvious compartment to fulfill this requirement.

Functionally, these designations may be correct in that the vacuole undoubtedly performs the degradative and protein recycling functions that in animal cells are mediated by the much smaller and considerably more acidic lysosomes. Indeed this is the reason that I have used the designation vacuole/lysosome in this thesis. However, great care needs to be exercised before making any glib assumptions about organelle identity based purely on morphological criteria because a wealth of evidence suggests that fungi have discarded, truncated or modified a whole host of cellular functions during the course of their rapid evolution as micro-organisms (Galagan, *et al*., 2005)**.**

For example, in the field of PPIns, it seems certain that fungi once had PI3KC1s but have completely lost them through the course of evolution (Mitra *et al.*, 2004). We know this because most fungi still encode a PTEN-like enzyme (Ptn1p) that in *S.*

pombe at least, retains the ability to degrade PtdIns(3,4,5)P_3. This assertion is also given added weight because slime-moulds, which diverged from animals after fungi, all retain PI3KC1s and indeed make substantial quantities of PtdIns(3,4,5)P_3 that allow them to regulate the process of chemotaxis (King and Insall, 2009). Despite these facts, early researchers studying inositol lipid metabolism concluded that PtdIns(3,4,5)P_3 synthesis and the pathways it regulated never evolved in fungi based simply on the absence of a gene for the requisite lipid kinase (Auger *et al*., 1989).

Similarly, *S. cerevisiae* probably also once had as many introns interrupting the coding sequences of its genes as other eukaryotes do, but that they lost them as a result of adaptation to their unicellular habitat (Seoighe and Wolfe, 1999).

So taking anything at face value presents dangers in yeast and the identity of compartments is no exception. If I cannot trust the morphology of these organelles to guide as to their identity, then it seems that genomics might offer another approach. Recent analyses of yeast Rab GTPases paint a surprising picture of the yeast endocytic system that is not what might have been anticipated (Pelham, 2002).

For example, the Snc1p positive “early endosome” has a Rab that is most similar to Rab11: the Rab GTPase that in animal cells controls entry to the recycling endosome. This organelle is quite distinct from a mammalian early endosome and has a disparate subset of cargos passing through it that is quite different from that found in the early endosome (Pelham, 2002).

Similarly, the Pep12p positive organelle close the vacuole, that was assumed to be a

"late endosome" has a Rab GTPase, Vps21p, which is most similar to Rab5: the mammalian early endosomal Rab (Schimmoller and Riezman, 1993). This suggests that it is indeed the yeast early endosome, although some EM studies imply that it can mature into a Pep12p negative organelle that might be the compartment known in animal cells as the MVB (Ali *et al*., 2004).

If I extend the analysis to the vacuole I find that the vacuolar Rab, Ypt7p is most similar to Rab7: a mammalian Rab controlling entry to and residing on, the animal late endosome. This strongly suggests that the yeast endocytic system may in fact be truncated and that yeast have no true lysosomes. This assertion is also borne out by the fact that the vacuole has a similar pH to that found in animal late endosomes (pH 6.0) and this contrasts strongly with the pH found in the much more acidic animal lysosome (pH 5.5-5.0).

The vacuole as the late endosome may explain why ablation of PtdIns(3,5)P_2 synthesis in animal cells, does not appear to affect lysosomal functions in most organisms: the late endosome is more often distorted, as would be expected if the vacuole was indeed the late endosome or was principally derived from it (Jefferies *et al*., 2008).

5.4 Why does the yeast vacuole respond to osmotic stress?

In this work I have highlighted the fact that the vacuole is a highly dynamic organelle in terms of its size and surface area to volume ratio. This dynamism is particularly evident when I consider the response of the vacuole to osmotic perturbations. It responds to hypo-osmotic stress by swelling and fusing to form one large

compartment in a HOPS dependent fashion and to hyperosmotic stress by fragmenting into smaller organelles and reducing in volume in a Fab1p and CORVET dependent manner (see Section 1.9 and Fig. 1.18).

I have used this dynamic property to begin to dissect the mechanisms that regulate membrane fusion and fission: two fundamental processes that probably underlay all membrane trafficking and organelle inheritance in eukaryotes. I am not the only one to exploit this and a slew of studies have taken advantage of the yeast vacuole in this way (Peters *et al.*, 2004; Baars *et al.*, 2007). Yet the fact remains that very little is known about why the vacuole responds to osmotic insults as it does. These responses are presumably adaptive, although the evidence for this is rather sketchy: indeed the fragmentation of the vacuole carried out by the Fab1p/PtdIns(3,5)P_2 pathway is dispensable under laboratory conditions because *fab1Δ* cells, that completely lack PtdIns(3,5)P_2, can grow perfectly well in media containing a high concentration of osmolytes (Yamamoto *et al.*, 1995).

However, *fab1*Δ yeast do in fact give us a clue as to why yeast vacuoles respond to high tonicity as they do. This clue to the function of the Fab1p pathway is revealed when *fab1*Δ mutants are taken from high osmolarity to low osmolarity: many *fab1*Δ cells die when this experiment is performed (Yamamoto *et al.*, 1995). This suggests that the true function of this pathway may be to protect cells against the consequences of a return to normal osmotic conditions after a hyper-osmotic shock. Perhaps this occurs by packaging up the vacuole into tiny sub-compartments so that the vacuole is less likely to burst when normal tonicity is restored: smaller and more curved membrane areas are intrinsically more stable than larger ones because the surface area

to volume ratio dictates that a smaller compartment is subject to less pressure because it is enclosing a proportionately lower volume of fluid relative to a larger compartment (Hohmann, 2002a and b).

Another likely function of the vacuole fusion and fission is to regulate vacuolar volume. Although simple fusion and fission of the same membrane area does not result in a change in total membrane area, it changes vacuolar volume to a very large degree. Indeed if I model the vacuole as a sphere with an arbitrary surface area of 100 units^2 and break it up into 8 smaller spheres of equal size, that together have the same total surface area as the original vacuole/sphere, then the total volume encompassed by all vacuoles decreases from ~94 units^3 when it is one large organelle to only ~32 units^3 when it is divided into 8: a more than 3 fold reduction in volume. Since the vacuole fragments much more than this, there is a much larger volume decrease. Thus although the vacuole loses water at the exact same rate as the cytosol during hyper-osmotic stress, there is still a large amount of water left after all passive water flow has ceased. So that when the vacuole subsequently undergoes a regulated surface area to volume ratio increase via the Fab1p pathway, the excess water is probably extruded into the cytoplasm to reduce tonicity briefly and this probably co-incides with the point where the aquaporins are closed and cell permeability is reduced. This may be viewed as an acute and immediate response that merely buys the cell a few minutes whilst the Hog1p pathway is activated, eventually raising the cytoplasmic osmotic potential by the production of glycerol (Hohmann, 2002a and b).

Similarly, it is likely that during excess water influx, e.g. during hypo-osmotic stress, the cell acts to decrease the surface area to volume ratio of the vacuole, effectively

increasing vacuolar volume enormously and thus containing much of the water and concentrating the cytosol. It is possible the vacuole may actively transport or hydrolyse vacuolar contents in order to draw water inside it during these episodes of sudden dilution and this is again likely to be an acute response (Hohmann, 2002a and b).

5.5 Are most higher inositol polyphosphates acting as true signals or are they just allosteric regulators?

In Chapter 4 I described a possible function for inositol pyrophosphates in the regulation of Vps41p phosphorylation and therefore in control of homotypic vacuole fusion. As alluded to in Chapter 4, I have not yet uncovered the precise molecular event that is regulated by these inositol pyrophosphates and this leads to an interesting question: are most "higher" inositol phosphates really acting as signals or second messengers? "Higher" IPs, for the purposes of this discussion, are those containing more than 5 phosphates on the inositol ring.

This question arises because, despite many statements to the contrary, there is currently no substantiated example of a "higher" IP acting as a *bona fide* second messenger and this appears to be the case because the concentration of many of these molecules is always high, even in apparently unstimulated cells.

The role of higher IPs appear analogous to that of the lipid PtdIns(4,5)P_2: they regulate many processes (e.g. nuclear RNA editing, mRNA export etc) and are required for the non-enzymatic pyro-phosphorylation of a number of proteins (See section 1.7 and Bhandari *et al*., 2007), but it seems likely that these events happen

constitutively in many cases. PtdIns(4,5)P_2 is needed to keep the actin cytoskeleton intact and is also an essential co-factor for a number of ion channels but again these appear to be constitutive functions. The advantages for this mode of regulation are understandable because most PtdIns(4,5)P_2 is in the plasma membrane and it appears to be acting as a compartment maker. One could imagine that the KCNQ potassium channel family, for example, that requires PtdIns(4,5)P_2 for full activity, are not active when being trafficked through the ER and Golgi and only acquire full activity upon arriving at the plasma membrane: i.e. when they encounter and bind PtdIns(4,5)P_2 (Loussouarn *et al.*, 2003) This mode of regulation seems desirable and logical and indeed, local changes in PtdIns(4,5)P_2 (e.g. during receptor stimulation) do appear to modulate channel activity, albeit in a subtle manner.

So can I learn anything about higher IP function from the PtdIns(4,5)P_2 paradigm? These are soluble molecules and so restricting their subcellular localisation seems unlikely but this would be required if they are to act in the same way as PtdIns(4,5)P_2: as compartment markers. Is it possible, for example, that the “basal” higher inositol phosphates are confined to the nucleus? At face value, this seems absurd because there are pores in the nuclear envelope that would make confining these soluble molecules unlikely. Yet there are two possible mechanisms that could both contribute to the maintenance of a nuclear pool of higher IPs, which could then act as constitutive “compartment markers.”

The first fact to consider is that higher IPs are not as mobile as might be expected through pores. Indeed, there is an old observation that IP_6 can be placed in dialysis tubing with a 50,000 Da cutoff and yet will not diffuse out of the tubing appreciably

over 20 h, despite being much smaller than 50 kDa in size (Van der Kaay and Van haastert, 1995). The authors of this work originally suggested that a large hydration shell of ordered water might surround the highly charged inositides and restrict their diffusion through the pores of the tubing. They showed that diffusion out of the tubing was inversely related to charge and that if very high salt concentrations were applied then diffusion rate was increased: presumably because of charge suppression. If the same phenomenon is operating at nuclear pores, which also have a molecular weight cutoff of about 30 kDa, then higher IPs generated in the nucleus might be retained and unable to diffuse into the cytoplasm and vice-versa.

If the above is combined with a second mechanism: high concentration of higher IP phosphatases in the cytoplasm, this could effectively separate the cell into at least two regions where IP signalling could occur independently of the other compartment.

This situation is really a prerequisite for true “higher” IP signalling because the resting concentration of these molecules in cells is often already high (particularly IP_5 and IP_6 but not IP_7 and IP_8). Yet if all of this resting higher IP is in either the nucleus or the cytoplasm, then true signalling could occur in the other compartment because it could be almost devoid of the higher IP by the mechanisms indicated above. This would then provide a situation in which an inactive effector protein could be allosterically regulated by a rise in the concentration of a higher IP upon some sort of stimulation. Our lab is currently generating Plc1p constructs that are restricted to either the nucleus or cytoplasm to test some of the above hypotheses and to see if pools of spatially restricted higher inositol phosphates are really possible *in vivo*.

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