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Oral examination:

The Role of Protein Palmitoylation for Protein Function at the yeast vacuole

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For my beloved mother

Declaration

I herewith declare that I wrote this thesis independently and used no other sources and aids than those indicated.

_____ August 21, 2007

(Kanagaraj Subramanian)

List of publications

Hou, H*., Subramanian K*., LaGrassa, T. J*., Markgraf, D., Dietrich, L. E., Urban, J., Deckar, N., and Ungermann, C. (2005). The DHHC protein Pfa3 affects vacuole-associated palmitoylation of the fusion factore Vac8. *PNAS* 48, 17366-17371.

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Summary

In eukaryotic cells, organelles communicate with each other by means of regulated vesicular transport. Vesicles loaded with cargo bud from the donor compartment and deliver their load upon fusion with the target membrane. Most of the proteins involved in the vesicular trafficking are peripheral membrane proteins, recruited from the cytosol. These proteins depend on membrane receptors, lipids or lipid anchors for binding to the membranes. In my research, I focused on the lipid modifications that alter the physical and functional properties of the protein. One type of lipid modification is palmitoylation, which facilitates the membrane association of the protein. Palmitoylation is the reversible addition of palmitate to the cysteine residue of the protein through a thioester linkage. Besides functioning as membrane anchor, palmitate influences the protein's functions. For my study, I used the budding yeast vacuoles as a model system, which is equivalent to lysosomes in higher organisms. The yeast vacuolar protein Vac8 is palmitoylated and required for vacuole fusion, morphology and inheritance. Specifically, I studied two important issues of Vac8 palmitoylation: (i) how does palmitoylation affect the function of Vac8? (ii) how is palmitoylation of Vac8 regulated?

1. Role of palmitoylation for Vac8 function. Vac8 contains an N-terminal Src homology (SH) 4 domain region, which harbors the sites for myristoylation and palmitoylation. For most of the proteins, a combination of myristate and one palmitate is sufficient for stable membrane anchoring. Interestingly, Vac8 has three cysteines in the SH4 domain and so we asked whether these cysteines have any individual function. To study this, we mutated the cysteines individually or in combination to alanines and examined the different functions of Vac8. Surprisingly, Vac8 with a single cysteine showed a dramatic difference in the localization and functions. The position of cysteine is critical for the palmitoylation of Vac8. Furthermore, we investigated the importance of palmitoylation for Vac8 function. We targeted Vac8 to the vacuole via the Src SH4 domain (myristate/polybasic domain). This protein localized to vacuoles, but did not complement Vac8 function. Only if a single cysteine was added to the Src SH4

domain complete rescue of function was observed. Based on these data, we suggest that palmitoylation determines the function of Vac8.

***2. Effects of DHHC protein Pfa3 on Vac8 palmitoylation*.** The SNARE Ykt6 mediates the palmitoylation of Vac8 in a non-enzymatic reaction. However, the enzymatic acylation of Vac8 is poorly understood. The vacuolar protein Pfa3 belongs to the DHHC family and is known to possess protein acyltransferase activity. We examined protein palmitoylation at the vacuole. Pfa3 contributes specifically to the palmitoylation of Vac8. Interestingly, it does not affect other palmitoylated proteins at the vacuole. Pfa3 is required for vacuole fusion, but not for vacuole inheritance. Besides protein acyltransferase activity, Pfa3 acts as the vacuole-sorting factor for both Vac8 (myristate/palmitate) and Src (myristate/polybasic domain) SH4 domains. Taken together, our data suggest that the DHHC protein Pfa3 affects the palmitoylation and functions of Vac8.

Zusammenfassung

Der Stoffaustausch zwischen den Organellen eukaryotischer Zellen basiert auf dem geordneten Transport von Vesikeln. Diese entstehen durch Abschnürung der Membran des Donor-Kompartiments, nachdem die zu transportierende Fracht spezifisch angereichert worden ist. Die Vesikel fusionieren nach Erreichen des Ziel-Kompartiments mit dessen Membran und geben ihren Inhalt an das Innere des Kompartiments ab. Ein Großteil der Proteine, die eine Rolle beim vesikulären Trafficking spielen sind periphere Membranproteine, die aus dem Cytosol rekrutiert werden. Diese Proteine benötigen Membranrezeptoren, spezifische Lipide oder Lipidanker, um an die Membran zu binden. In meiner Doktorarbeit habe ich Lipidmodifikationen, die die physikalischen und funktionalen Eigenschaften von Proteinen beeinflussen, näher untersucht. Palmitoylierung ist eine Lipidmodifikation, die die Membranassoziierung von Proteinen erleichtert. Es handelt sich dabei um eine reversible Anbindung eines Palmitatrestes durch eine Thioesterbindung an die Thiolgruppe eines Cysteins im Zielprotein. Neben seiner Funktion als Membrananker beeinflusst der Palmitatrest die Funktion des angebundenen Proteins. Als Modellsystem wählte ich die Vakuole der Bäckerhefe *Saccharomyces cerevisiae*, die in ihrer Funktion den Lysosomen höherer Eukaryoten entspricht. Das vakuoläre Hefeprotein Vac8 ist palmitoyliert und wird in der Zelle für die Vakuolenfusion, die Kontrolle der Vakuolenmorphologie und die Weitergabe von Vakuolen an die Tochterzelle bei der Zellteilung, im folgenden Vererbung genannt, benötigt. In meinen Studien habe ich zwei wichtige Aspekte der Vac8-Palmitoylierung im Besonderen untersucht: (i) wie beeinflusst die Palmitoylierung die Funktion von Vac8 und (ii) auf welche Weise wird die Palmitoylierung von Vac8 reguliert?

1. Der Einfluß der Palmitoylierung auf die Funktion von Vac8. Vac8 beinhaltet eine N-terminale Src homology (SH) 4 Domäne, in der sich die Aminosäurereste befinden, die myristoyliert und palmitoyliert werden. In den meisten Fällen werden Proteine jeweils einmal myristoyliert und palmitoyliert, um stabil in der Membran verankert zu werden. Vac8 besitzt demgegenüber drei potentielle Palmitoylierungsstellen (Cysteine 4, 5 und 7) und es stellte sich die

Frage, ob die drei Cysteine individuelle Funktionen erfüllen. Um diese Frage zu beantworten wurden Mutanten generiert, in denen entweder einzelne oder mehrere Cysteine durch Alanin ersetzt wurden. Dabei stellte sich heraus, das Mutanten mit nur einem Cystein, respektive einer Palmitoylierungsstelle, einen stark veränderten Phänotyp hinsichtlich der Proteinlokalisation und –funktion aufweisen, wobei die Position des Cysteins entscheidend für eine effiziente Palmitoylierung ist. Zur Untersuchung der Bedeutung der Palmitoylierung für die Funktion von Vac8 wurde das Protein mit der Src SH4 Domäne versehen, die myristoyliert wird aber statt der Palmitoylierungsstelle einen Abschnitt mit mehreren basischen Aminosäuren enthält. Diese Chimäre lokalisierte zwar wie erwartet auf der Vakuolenmembran, konnte aber eine Vac8 Deletion nicht funktionell komplementieren. Nur durch die Addition eines einzelnen Cysteins in der Src SH4 Domäne konnte die Wildtyp Funktion wiederhergestellt werden. Aufgrund der gewonnen Daten lässt sich daher sagen, dass die Palmitoylierung die Funktion von Vac8 entscheidend beeinflusst.

2. Der Effekt des DHHC Proteins Pfa3 auf die Palmitoylierung von Vac8. In vorhergehenden Studien wurde gezeigt, dass das SNARE-Protein Ykt6 die Palmitoylierung von Vac8 in einer nicht-enzymatischen Reaktion vermitteln kann. Demgegenüber ist die enzymatische Acylierungsreaktion von Vac8 bislang noch weitgehend unverstanden.

Das vakuoläre Protein Pfa3 gehört zur Familie der DHHC Transmembranproteine, deren Mitglieder Proteinacyltransferase-Aktivität aufweisen. Daher wurde es als ein Zielprotein gewählt, dessen Einfluss auf die Palmitoylierung von Vac8 näher untersucht wurde. Tatsächlich konnte ich in meinen Studien nachweisen, dass Pfa3 spezifisch zur Palmitoylierung von Vac8 beiträgt. Eine Palmitoylierung anderer vakuolärer Proteine durch Pfa3 konnte demgegenüber nicht nachgewiesen werden. Pfa3 wird nicht für die Vererbung der Vakuole benötigt, muss aber für eine effiziente Vakuolenfusion vorhanden sein. Neben seiner Funktion als Proteinacyltransferase fungiert es als Vakuolen-Sortierungsfaktor für Vac8 und Proteine mit einer Src SH4 Domäne.

Zusammenfassend lässt sich sagen, dass das DHHC Protein Pfa3 die Palmitoylierung und die Funktion des Vac8 Proteins beeinflusst.

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I. Introduction

Unlike a prokaryotic cell, which generally consists of a single intracellular compartment surrounded by a plasma membrane, a eukaryotic cell is subdivided into functionally distinct, membrane-enclosed compartments. Each compartment or organelle, contains its own characteristic set of enzymes and complex molecules, and interconnect with other compartments to carry out the various cellular functions.

I.1 The endomembrane system

The endomembrane system includes the endoplasmic reticulum (ER) and the contiguous nuclear envelope (NE), the Golgi apparatus, lysosomes, secretory granule and early/late endosomes. These organelles are highly organized and give a unique architectural feature to the cell. All these organelles form a dynamic network that allows the constant exchange of materials between them and also with the extracellular space. This exchange of materials between the organelles is largely accomplished by means of small transport vesicles and has become known as vesicular transport. Vesicles selectively carry the material from one organelle and targeted to a specific compartment and subsequently deliver their load upon fusion to their limiting membrane (Rothman, 1996). Correct delivery of proteins to their appropriate locations is therefore essential for the identity of an organelle.

According to the vesicular transport model, vesicles continually bud off from one membrane “donor” and fuse with another compartment “acceptor” carrying membrane components and soluble molecules referred as *cargo* (Bonifacino and Glick, 2004). Vesicle mediated transport of molecules in between the intracellular compartments is classified into two types: a) *the biosynthetic-secretory pathway* and b) *endocytic pathways* (Figure 1). In the *biosynthetic-secretory pathway*, the newly synthesized protein enters into the secretory pathway by translocation into the ER, where the protein gets folded with aid of chaperones and transported via vesicles to the Golgi complex. Further post-translational modifications occur in

the Golgi, and cargo is targeted to the plasma membrane, where it gets secreted to the extracellular space. A minor set of proteins particularly digestive enzymes reaches the vacuole via endosomes. In the *endocytic pathways*, the extracellular materials are ingested in vesicles mediated by the plasma membrane receptor proteins. These vesicles are targeted to early endosomes, from which materials may recycled back to the cell surface or onward to late endosomes, into multivesicular bodies, and finally, to the lysosome, where they are degraded (Mellman, 1996).

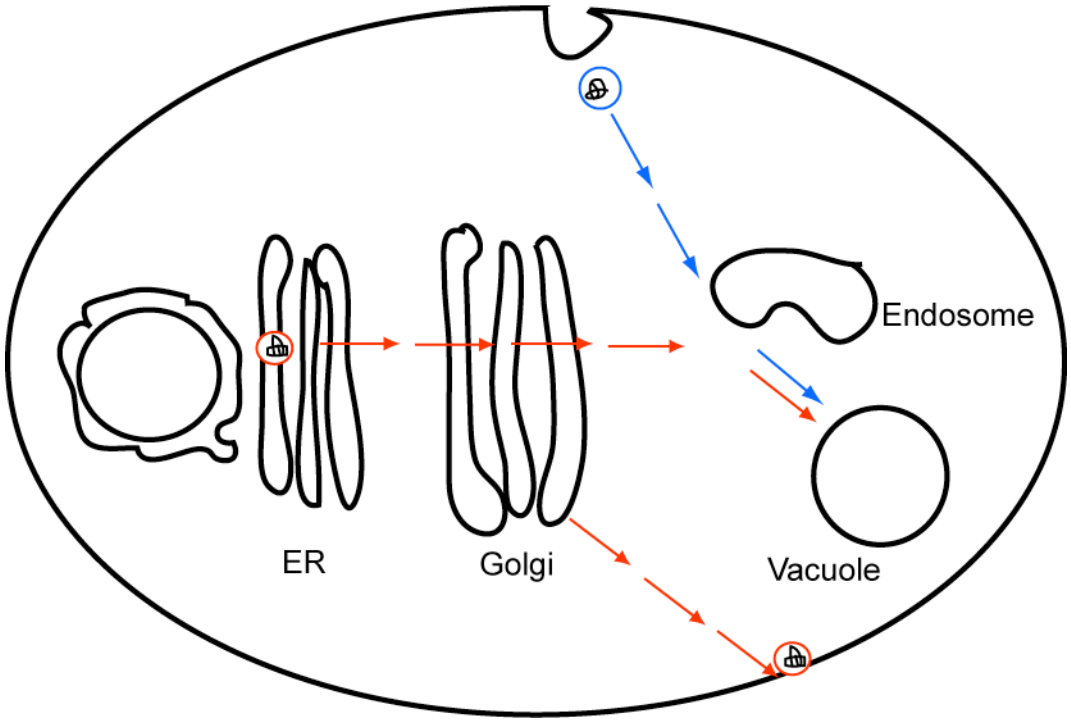

Figure 1. Intracellular vesicular transport in the endomembrane system

The organelles of the endomembrane system are inter-connected by vesicular transport.
(a) *Biosynthetic-secretory pathway* (red arrow): The newly synthesized protein translocates into the endoplasmic reticulum (ER), where it gets folded and modified. In the Golgi, further modification of protein occurs and proteins are secreted to the plasma membrane by exocytosis. Soluble digestive enzymes get targeted to the endosomal/vacuolar system.
(b) *Endocytosis* (blue arrow): The extracellular material from the plasma membrane is taken up by the receptor and gets targeted to the vacuole through the endosomes.

I.2 Vesicular transport between organelles

Vesicular transport of the protein to the target compartment is mediated by various protein complexes and lipids. Each vesicular transport can be divided into four main essential steps. They are as follows: (a) the formation of vesicles at the donor compartment by budding, (b) transport of the cargo loaded vesicles by motor molecules, (c) tethering of vesicles to the target membrane and, (d) fusion of vesicles with the target membrane (Figure 2).

I.2.1 Budding

The budding of transport vesicles and the selective incorporation of cargo into the forming vesicles are both mediated by protein coats (Bonifacino and Lippincott-Schwartz, 2003; Kirchhausen, 2000). These coats are dynamic structures that cycle on and off membranes. Protein coats are recruited from the cytosol to the donor compartment. The coats deform the flat membranes into round buds, resulting in the release of coated vesicles. Coat proteins also participate in the recognition of sorting signals present in the cytosolic domains of transmembrane cargo proteins. The different coats mediate vesicle budding and cargo selection at different stages of the exocytic and endocytic pathways. There are three types of coat proteins that exist in the intracellular vesicular transport between different compartments. The first coat protein to be identified was clathrin, a scaffold protein restricted to the post-Golgi locations including the plasma membrane, the *trans*-Golgi network (TGN), and endosomes (Pearse, 1975). Further studies have identified the existence of two non-clathrin coats that mediate the vesicular transport in the early secretory pathway. One of these coats, COPII, mediates transport of vesicles from the endoplasmic reticulum (ER) to the Golgi (Barlowe et al., 1994), while another coat COPI, is involved in inter-Golgi transport and retrograde transport from the Golgi to the ER (Letourneur et al., 1994). In addition, several other non-clathrin coats have been identified (Godi et al., 2004; Wang et al., 2006).

I.2.2 Transport

After budding, vesicles are transported to their final destination on cytoskeletal tracks consisting of microtubules and actin. The molecular motors kinesin, dynein, and myosin all have been implicated in this process (Matanis et al., 2002; Short et al., 2002). Rabs and Rab effector interacts with the molecular motors and target the vesicles to the specific compartment. For example, Rab6 GTPase binds to dynactin; a multisubunit complex required for the activity of dynein that regulates the selective recruitment of vesicles to Golgi membranes (Short et al., 2002).

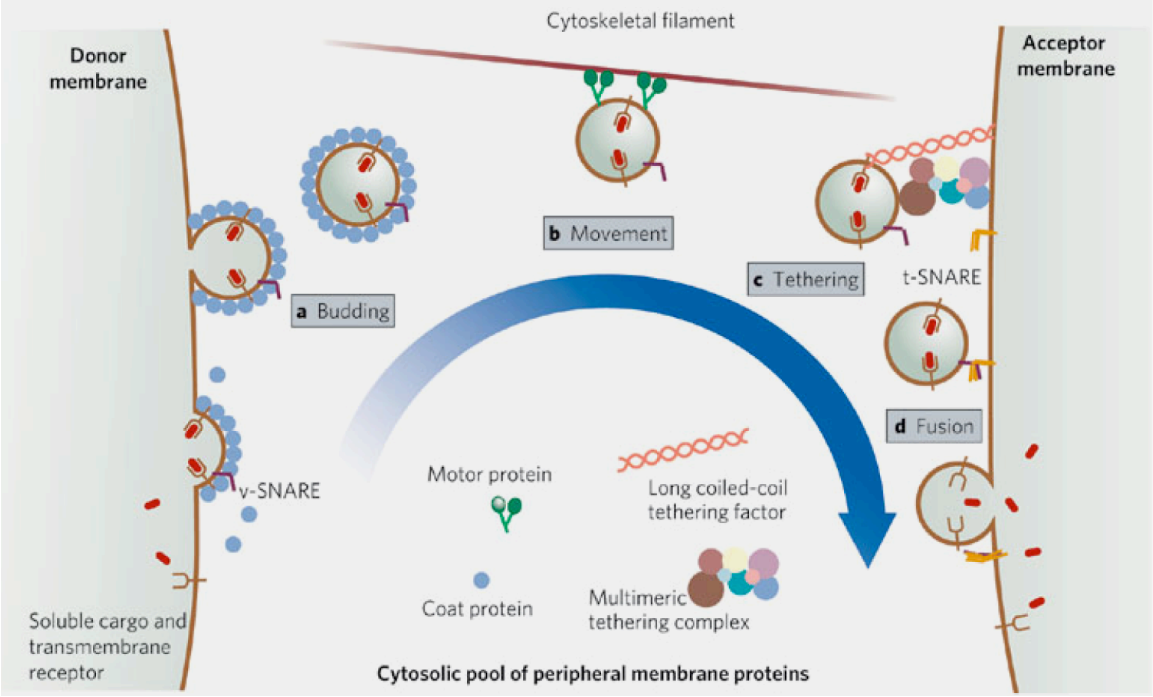

Figure 2. Schematic representations of the steps of vesicular transport

a. Coat proteins are recruited to the cytosolic face of the donor membrane and induce the formation of a vesicle. The coat recruits SNAREs and transmembrane receptors bound to their cargo. **b**. After uncoating, motor protein can be recruited to enable the vesicle to travel along microtubules or actin filaments. **c**. At its destination, the vesicle becomes tethered to the acceptor membrane by the tethering factors. **d.** The SNAREs on the vesicle and acceptor memembrane

form a complex which drives membrane fusion and hence delivery of the contents of the vesicle. Taken from (Behnia R & Munro S, 2005).

I.2.3 Tethering

Tethering is the initial interaction between a vesicle and its target membrane mediated by a set of proteins referred to as tethering factors. Tethers act together with Rab GTPases and play a critical role in determing the specificity of vesicle targeting. Tethering factors are classified into two broad categories: (a) a long, coiled-coil proteins and (b) large, multisubunit complexes.

Long coiled-coil proteins

The long coiled-coil tethers form homodimeric complex with lengths up to several times the diameter of a vesicle, and are able to tether the vesicle to the organelle. For example, yeast Usol and its mammalian homologue p115 are required for the tethering of ER-derived vesicles to the Golgi membranes (Allan et al., 2000; Barlowe, 1997).

Multisubunit tethering complexes

To date eight conserved multisubunit tethering complexes have been identified in yeast known to be involved in vesicular tethering in distinct trafficking steps. It includes COG (endosome-early golgi), TRAPPI (ER-Golgi), Dsl1 (Golgi-ER), TRAPPII (intra-Golgi/endosome-late Golgi), and the exocyst (Golgi-plasma membrane; endosome-plasma membrane) involved in *in vivo* secretion (Andag et al., 2001; Reilly et al., 2001; Whyte and Munro, 2002). In addition, three complexes CORVET (late Golgi-endosome), HOPS (endosome-vacuole) and GARP/VFT (endosome-late Golgi) are required for vacuolar sorting of proteins (Conibear et al., 2003; Peplowska et al., 2007; Peterson and Emr, 2001). Tethering factors acts at distinct stages of the trafficking that serve as a link between the vesicles and the target compartment. Tethers can be Rab effectors and Rab GEF, interacts with SNAREs proteins mediates the lipid mixing of vesicles to the membrane (Price et al., 2000).

Rab GTPases

Rabs belong to a family of small GTPases, which include the Ras proteins. Eleven Rabs in yeast and >60 Rabs in mammalian cells have been identified (Pfeffer, 2001). Rabs continuously cycle between the cytosol and membranes. They associate with the membrane by virtue of the double prenylation at their C-terminal end (Leung et al., 2007). In the cytosol, Rabs are in the inactive state, bound to GDI (Guanine nucleotide displacement inhibitor). They are recruited to the membrane mediated by GDF (GDI displacement factor) and results in the localization of Rabs to the membranes (Dirac-Svejstrup et al., 1997) (Figure 3).

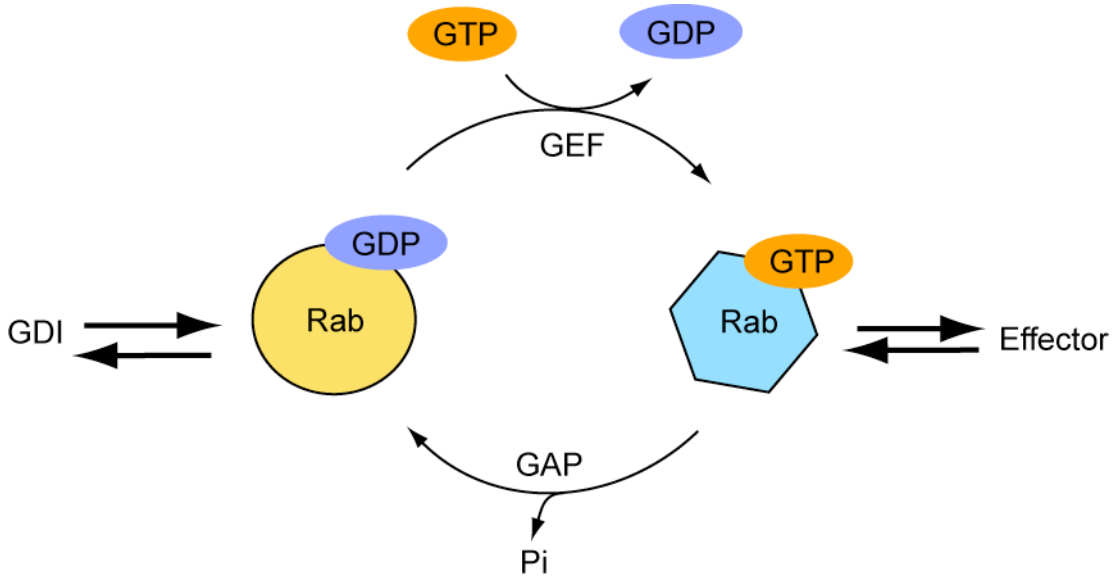

Figure 3. Rab GTPase cycle

The Rab GTPases switch between GDP- and GTP-bound forms mediated, which have different conformations. Conversion of GDP to GTP-bound form is mediated by GTPase exchange factor (GEF). Conversion of GTP to GDP-bound form occurs by ATP hydrolysis catalyzed by GTPase-activating protein (GAP). GTP-bound form interacts with the effector molecules, whereas the GDP-bound form interacts with GDP-dissociation inhibitor (GDI). See text for more details.

Rab proteins interconvert between the active GTP bound form and inactive GDP form. Membrane bound Rab-GDP is activated by GEF (Guanine nucleotide Exchange Factor) to GTP bound form (Grosshans et al., 2006). Activation of the Rab is terminated to the inactive GDP form by specific GAP (GTPase-activating protein) and GDI extracts the inactive Rabs from the membrane mediated by the membrane associated Hsp90 protein (Chen et al., 2005). Rab effectors recognize the Rab proteins in the active state, determines the specific recognition of vesicles at the target compartment.

I.2.4 Fusion

Fusion factors – SNAREs, SM proteins

SNAREs

SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are the family of proteins, involved in membrane fusion. To date, 25 members in *Saccharomyces cerevisiae*, 36 in humans, and 54 members in *Arabidopsis thaliana* have been identified (Bock et al., 2001). SNAREs are defined by a characteristic SNARE motif, an evolutionarily conserved stretch of 60-70 amino acids that are arranged in heptad repeats. At the C-terminal region, the SNARE motif is linked to the transmembrane domain through a short linker. A subset of SNAREs lacks transmembrane anchors but feature hydrophobic post-translational modifications that mediate membrane anchorage (e.g., neuronal SNAP-25). SNAREs are classified as v-SNAREs (vesicle membrane SNAREs) and t-SNAREs (target membrane SNAREs) based on the localization on ‘donor’ compartment and ‘acceptor’ compartment. When appropriate sets of SNAREs from the transport vesicle and the acceptor compartments are combined, the α-helical SNARE domains interwind together to form bundles of the core complexes. The centre bundle contains 16-stacked layers of interacting side chains. These layers are hydrophobic, except the central ‘0’ layer that contains three highly conserved glutamine (Q) residues and one highly conserved arginine (R) residue (Sutton et al., 1998). These conserved residues are almost

unchanged throughout the SNARE family, leading to a reclassification of SNARE proteins into Q- and R-SNAREs (Fasshauer et al., 1998; Weimbs et al., 1997).

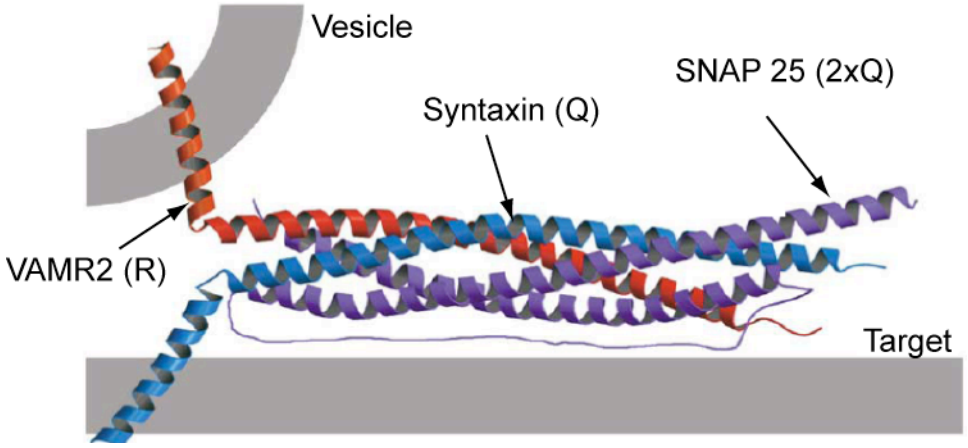

Figure 4. Assembly of SNARE complexes

SNAREs from both transport vesicle and the target membrane bind each other via their SNARE domains. These align in a parallel manner and start zippering from their N- to C-terminal direction. Three Q-SNAREs and one R-SNAREs form the SNARE complexes (Q/R hypothesis). The figure shows neuronal SNARE complexes, required for synaptic vesicle fusion. Taken from Sutton et al., 1998.

Initially SNAREs are present in both membranes. During fusion, the assembly of SNAREs proceeds in a zipper-like fashion from the N-terminal end of the SNARE motifs toward their C-terminal membrane anchors clamps the membrane together and initiates fusion (Figure 4). The so-called *trans*-complexes overcome the energy barrier for fusion. After fusion, the transmembrane regions of the SNAREs are present in the same membrane, resulting in *cis*-complexes (Figure 5). The SNAREs in the cis-complexes are disassembled and reactivated by the ATPase NSF with the soluble NSF-attachment (SNAP) protein as a cofactor (Sollner et al., 1993). A number of accessory components and regulatory factors modulate the action of SNAREs.

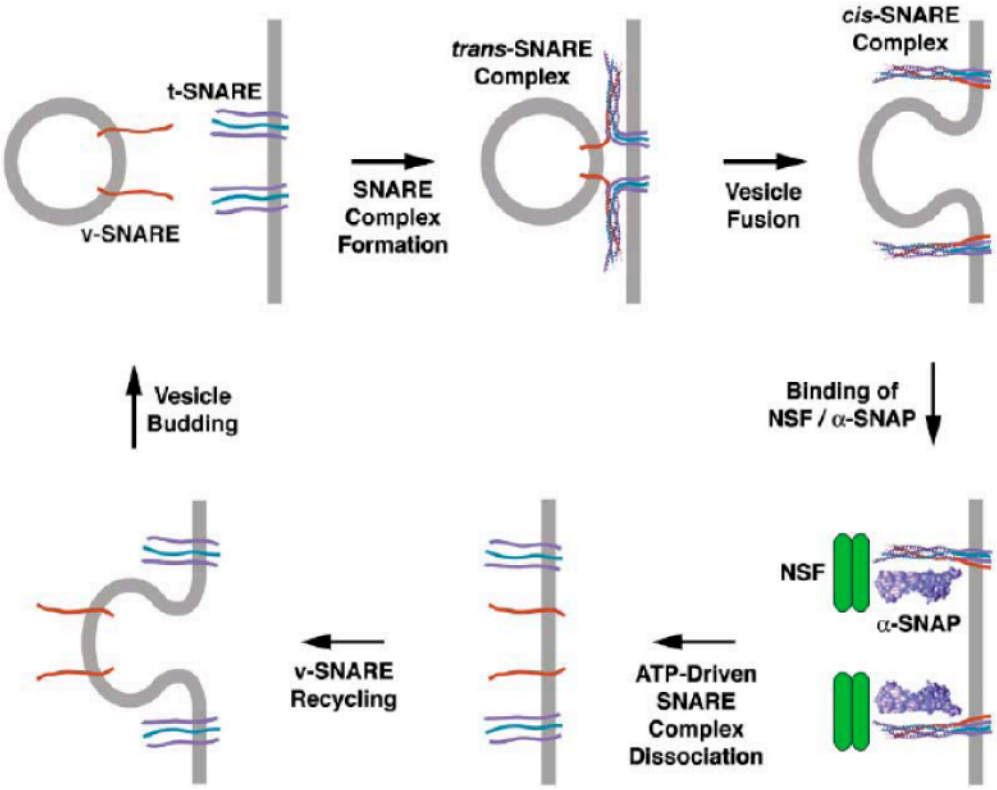

Figure 5. The SNARE cycle

A *trans*-SNARE complex assembles when a monomeric v-SNARE on the vesicle binds to an oligomeric t-SNARE on the target membrane, forming a stable four-helix bundle that promotes fusion. The result is a *cis*-SNARE complex in the fused membrane. NSF hydrolyzes ATP to dissociate the complex in the presence of α-SNAP. Unpaired v-SNAREs are released and go for another round of complex formation. Taken from (Bonifacino and Glick, 2004).

I.3 Post-translational modification of proteins

Many proteins are modified at different compartments of the cell for their effective functions. Modifications occur either co-translationally or post-translationally to the residue of the target proteins. Protein modifications determine the targeting, subcellular localization and function of the many proteins. One such modification is the lipidation, in which a lipid group is attached to the specific residue of the protein. Here, I will focus on the types and mechanisms of lipid modifications.

I.3.1 Protein Lipidation

Lipid modifications of protein were first described in the lipid extracts obtained from brain tissue and brain tumor tissue (Folch and Lees, 1951). The lipid extracts contained proteins, which are covalently modified by fatty acid moieties so called proteolipids. Many peripheral membrane proteins are anchored to the membrane via lipid groups that are distinct from those employed by the transmembrane proteins. Lipid modifications facilitate the attachment of soluble proteins to biological membranes, enable protein-protein interactions and in some cases, the shuttling of proteins between the plasma membrane and the cytosol or other membranes.

Protein lipidation can be found in diverse groups of organisms from bacteria to mammals. In *Escherichia coli* the pore-forming hemolysin (HlyA), represents the bacterial toxins and activated intracellularly by amide linkage of fatty acids to two internal lysine residues and anchored onto the surface of the host cell lipid layer (Stanley et al., 1998). In eukaryotic and viral system, several proteins are modified by lipid moieties and alter the physical and functional properties of the modified proteins (Qanbar and Bouvier, 2003; Rousso et al., 2000). Protein lipidation is subdivided into three major categories, they are: (a) Myristoylation, (b) Prenylation and (c) Palmitoylation.

Myristoylation

Myristoylation describes the covalent attachment of myristate, a 14-carbon saturated fatty acid, which is added to the N-terminal glycine residue through an amide linkage (Figure 6). It generally occurs during protein synthesis, after the initiating methionine is removed by methionine amino peptidase from the N-terminus (Boutin, 1997). In proapoptotic protein BID, myristoylation occurs post-translationally during apoptotic cell death, a process termed ‘morbid myristoylation’ (Mishkind, 2001).

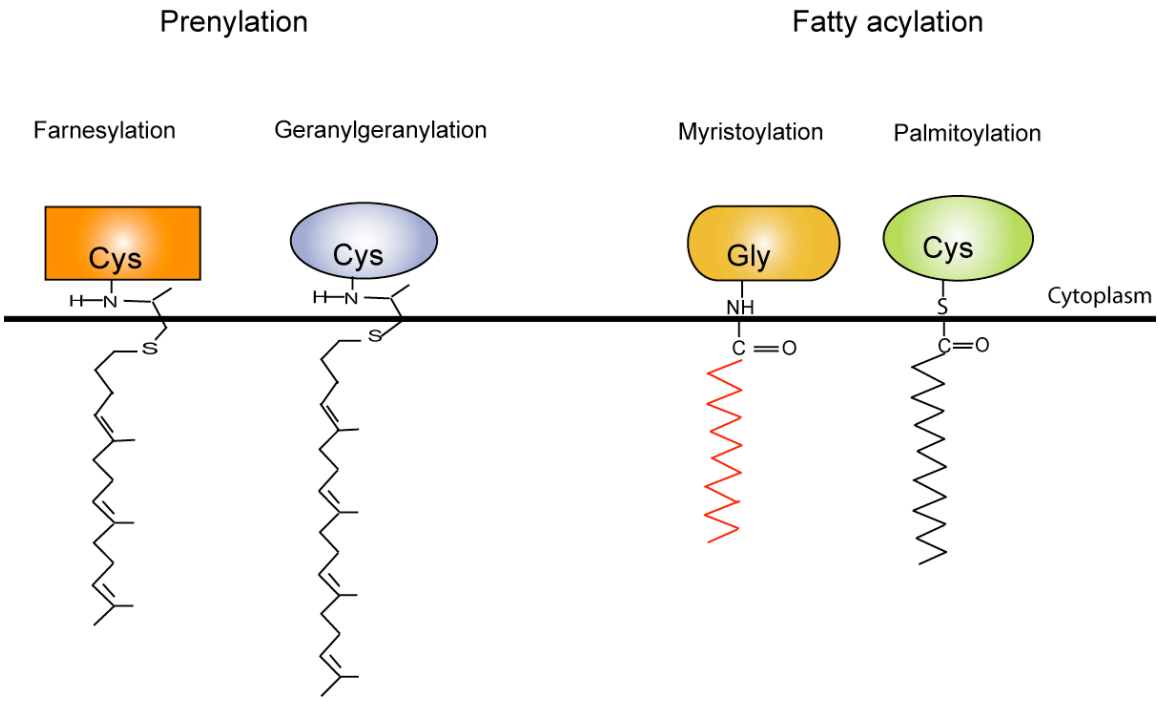

Figure 6. Types of protein lipid modifications

The structure of common lipid modifications. (A) In prenylation, the isoprenoid polymers are attached to the cysteine residue of the protein by thioether linkage. It can be a 15-carbon farnesylation or 20-carbon geranylgeranylation. (B) Fatty acylation of proteins. N-Myristoylation occurs by the addition of 14-carbon myristate to the glycine residue of the protein through amide linkage. Palmitoylation of protein is the attachment of 16-carbon palmitate to the thiol group of the cysteine residues. Palmitoylation is reversible and dynamic, whereas myristoylation and prenylation form a stable and irreversible linkage to the proteins.

N- myristoyltransferase (NMT), the enzyme catalyzes transfer of myristate from myristoyl-CoA to the amino-terminal glycine, recognizes the consensus sequence Met-Gly-x-x-x-Ser/Thr (Towler et al., 1988). In most cases, addition of myristate will not anchor a myristoylated protein firmly to the membrane (McLaughlin and Aderem, 1995). Although the modification is stable, and hydrophobic in nature, the myristoylated proteins have a high on and off rate from the membranes (K_d^{eff}=80 μM). Therefore, it requires an additional

membrane-moiety for effective localization, which can be supported by a stretch of polybasic amino acids or palmitate.

Prenylation

Protein prenylation is a post-translational modification that occurs in the cytosol and at the ER membrane. It involves the thioether linkage of 15-carbon (farnesyl) or 20-carbon (geranylgeranyl) isoprenyl group to one or more cysteine residues at or near the C terminus of the protein (Resh, 2006b). Many prenylated proteins contain a C-terminal 'CaaX box' (Cysteine-Aliphatic-Aliphatic-X). The 'X' amino acid determines whether the cysteine within the CaaX box is farnesylated by farnesyltransferase (FTase) or geranylgeranylated by geranylgeranyltransferase (GGTase-1). Both farnesyltransferase and geranylgeranyltransferase are heterodimeric enzymes that share a common α-subunit but have a distinct β-subunit that determine the substrate specificity. If 'X' is a methionine or a serine, the protein gets farnesylated (e.g., H-Ras, N-Ras). When 'X' is a leucine the protein becomes geranylgeranylated (e.g., Rho proteins) (Yalovsky et al., 1999). Following prenylation, the terminal -aaX is cleaved by Rce1 endoprotease (Michaelson et al., 2002) and C-terminal prenylated cysteine is carboxymethylated by isoprenylcysteine carboxyl methyltransferase (ICMT) in the endoplasmic reticulum (ER). A second Geranylgeranyltransferase (GGTase-II) is specific for Rab-GTPases with C-terminal CC, CCX or CXC motif (Lane and Beese, 2006; Leung et al., 2007). GGTase-II consists of α- and β-subunits similar to those of FTase and GGTase-I. However, the catalytic component requires a third component, the Rab Escort protein (REP) for full activity. To be prenylated, Rab proteins must first be complexed with REP.

Palmitoylation

Palmitoylation describes the covalent attachment of palmitate, a 16-carbon fatty acids to cysteine residues of a target protein through a thioester linkage (S-palmitoylation). It is unique among other lipid modifications because of its reversibility and dynamic nature. Fatty acids thioesterified to proteins include fatty

acids other than palmitate. These can be saturated, monounsaturated and polyunsaturated species of varying chain length (Linder and Deschenes, 2003). Thus, S-acylation or thioacylation are the general terms used to describe this process. In my thesis, the term palmitoylation will be used to refer to S-acylation. Unlike myristoylation and prenylation, there is no consensus sequence for palmitoylation.

I.4 Mechanisms of protein palmitoylation

Since the discovery of palmitoylation, the mechanism of the palmitoylation was remainded elusive due to the lack of potential candidate proteins. Based on biochemical studies two mechanisms have been proposed. The first is nonenzymatic where spontaneous acylation of a protein in the presence of long-chain acyl-coenzyme As (CoAs) occurs. The second is an enzymatic process where the enzyme protein acyltransferase mediates acylation of the protein.

I.4.1 Autoacylation

In the absence of cellular factors, activated palmitate in the form of palmitoyl-CoA can spontaneously form a thioester linkage to cysteinyl thiols. This mode of non-enzymatic acylation has been reported in several proteins such as Gα subunits (Duncan and Gilman, 1996), SNAP-25 (Veit, 2000), myelin proteolipid (Bizzozero et al., 1987), Bet3 (Kummel et al., 2006) and Ykt6 (Veit, 2004). In Gα subunits, both N-terminal myristoylation and presence of $\beta\gamma$ subunits are required for the autoacylation of Cys3 at physiological condition. Similarly, *in vitro* palmitoylation of SNAP-25 requires the α-helical form and is stimulated upon binding to its physiological interacting partner syntaxin 1 (Veit, 2000). Autoacylation is dependent on time, temperature, concentration and pH, and native conditions of the substrates. In the cytoplasm acyl-CoA binding protein (ACBP) binds to the long-chain fatty acyl-CoA and regulates the uncontrolled effects of acyl-CoA on various cellular processes (Faergeman et al., 2004; Leventis et al., 1997).

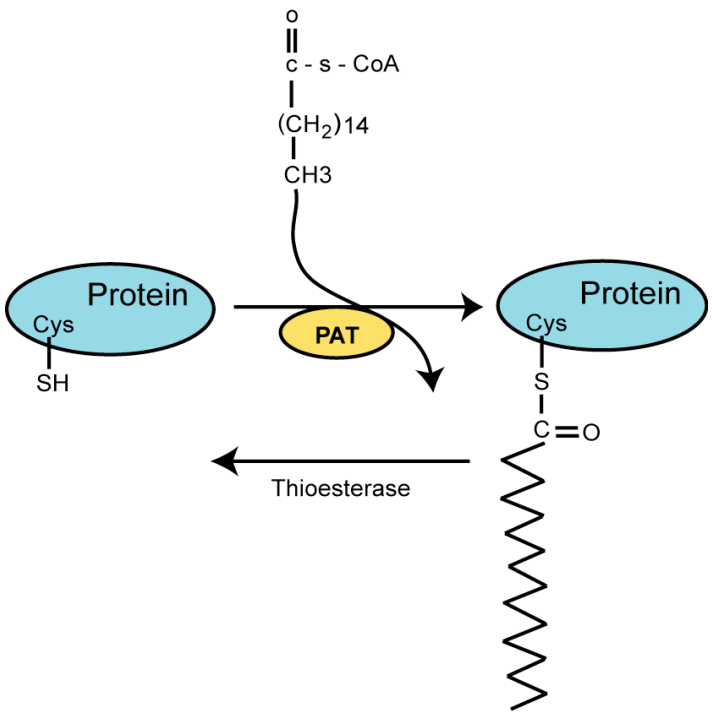

Figure 7. Mechanism of Palmitoylation

The fatty acid palmitate is transferred from the palmitoyl-CoA to a cysteine residue of the protein through the formation of a thioester linkage. This enzymatic addition of palmitate can be mediated by the protein acyltransferases (PAT). Palmitoylation is reversible; the palmitoyl thioesterase enzyme cleaves the thioester linkage of palmitate.

I.4.2 Enzyme-mediated acylation

The acylation of proteins with palmitate and related fatty acids has been known for over 30 years, but the molecular machinery that carries out palmitoylation remained elusive. The enzyme that mediates S-acylation was referred to as palmitoyl acyltransferases (PAT). Genetic and biochemical evidence for the PAT have been obtained in yeast *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Linder and Deschenes, 2004). This led to the identification of two

classes of PAT enzymes that modify the intracellular proteins and secreted proteins.

Palmitoylation of intracellular proteins

The enzymology of protein palmitoylation is poorly understood. The purification of the putative palmitoyl transferase remained unsuccessful due to the association of these activities with membranes (Berthiaume and Resh, 1995; Dunphy et al., 1996). However, the recent discovery of family of PATs, first in *S.cerevisiae* (Lobo et al., 2002; Roth et al., 2002) has opened the door for the finding of a family of PATs that mediate the palmitoylation of many cellular proteins.

The PATs belong to the family of proteins known as DHHC-Cysteine rich domain (CRD) proteins. The central DHHC-CRD domain is required for palmitoylation of proteins both *in vivo* and *in vitro*. Proteins with DHHC-CRD domains are conserved from yeast to mammals. Genetic and biochemical studies have identified the substrates that are palmitoylated by the DHHC proteins.

Topology of DHHC proteins

The DHHC-CRD motif was first described in an unique clone of human pancreatic cDNA library and a *Drosophila* open reading frame DNZ1 (Mesilaty-Gross et al., 1999; Putilina et al., 1999). The DHHC domain is similar to the C_2H_2 zinc finger motif based on the sequence studies, but there is no clear evidence of binding of zinc ions (Figure 8). All DHHC proteins are polytopic integral membrane proteins with four or more transmembrane domains (TM), with variable regions at both N and C terminal regions. The catalytic site of DHHC motif is generally located in between TM2 and TM3 at the cytosolic face of the membrane. The topology of the DHHC protein Akr1 has been shown experimentally, in which the DHHC domain is facing the cytoplasmic region of the membrane (Politis et al., 2005). Two conserved motifs: DPG (aspartate-proline-glycine) and TTxE (threonine-threonine-x-glutamate) motifs. A DPG motif is generally present next to TM2 whereas TTxE motif is found adjacent to TM4.

Both DPG and TTxE are predicted to be lie on the same side of the membrane along with the DHHC domain. The DHHC-CRD has also been referred to as the NEW1 domain and the zf-DHHC motif (Mesilaty-Gross et al., 1999; Putilina et al., 1999).

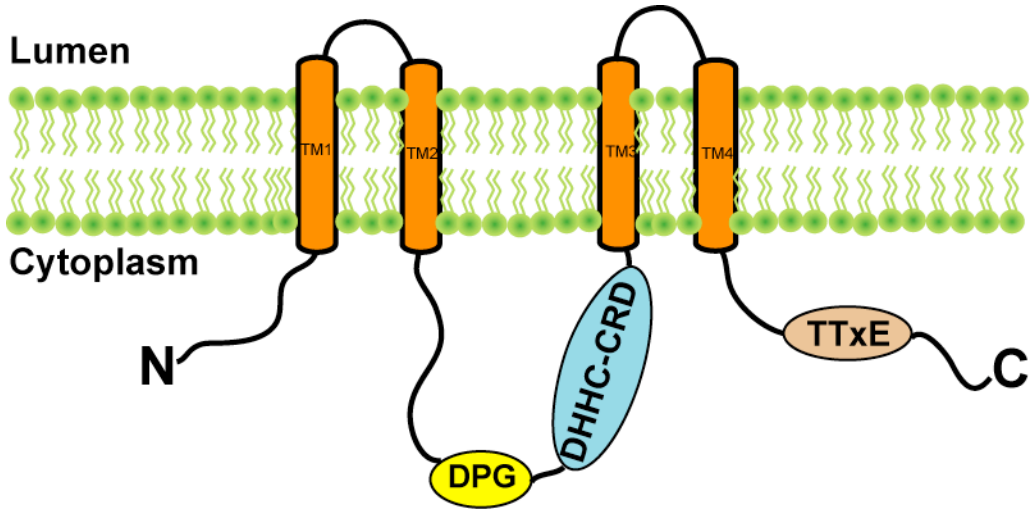

Figure 8. Topology of a DHHC domain protein

All DHHC proteins are polytopic integral membrane proteins with four to more transmembrane domains (TM). The putative catalytic site of the DHHC motif is generally located between TM2 and TM3, which are predicted to be at the cytosolic face of the membrane. In addition to the DHHC domain, two more conserved motifs are found on the same side of the membrane. A DPG (aspartate-proline-glycine) and TTxE (threonine-threonine-x-glutamate) are two conserved motifs, whose functional significance is not known.

DHHC proteins in yeast

There are seven DHHC proteins in yeast *Saccharomyces cerevisiae*, which are distributed along the endomembrane system (Figure 9). They mediate the transfer of palmitate to the specific substrate.

Erf2

Erf2 is a 42-kDa protein with four predicted membrane-spanning domains, acting as PATs for yeast Ras homologs Ras1 and Ras2. Plasma membrane localization of these Ras proteins requires a series of post-translational modifications beginning with the farnesylation of the cysteine residue of the CaaX box,

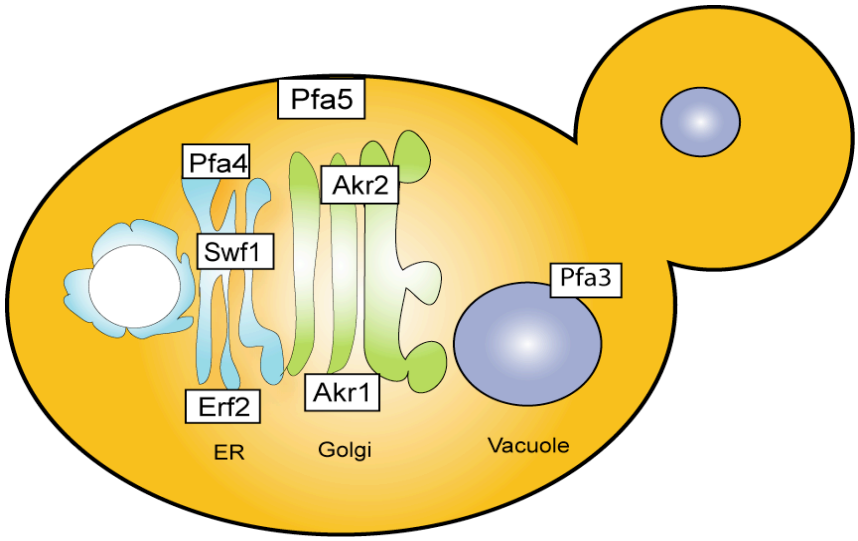

Figure 9. Distribution of DHHC proteins in the endomembrane system of yeast

Based on genetic and biochemical analysis in yeast *Saccharomyces cerevisiae*, there are seven DHHC proteins have been identified and they are distributed in the endomembrane system. The DHHC proteins Erf2, Swf1 and Pfa4 are localized in the endoplasmic reticulum (ER), and Akr1 and Akr2 at the Golgi complex. The DHHC protein Pfa3 is localized to the vacuole and pfa5 at the plasma membrane.

proteolytic cleavage of the –aaX residues, methylation of the resulting carboxyl group, followed by the palmitoylation of the cysteine. Deletion of *ERF2* causes a decrease in Ras palmitoylation and mislocalization of Ras2p from the plasma membrane to endomembranes (Bartels et al., 1999). Erf2 requires an additional protein. Erf4/Shr5 forms heterodimeric complex with Erf2 and mediates the

transfer of palmitate from palmitoyl-CoA to the cysteine residue of the Ras (Zhao et al., 2002). Erf4 is a novel 26-kDa protein that has no recognizable motifs. Neither Erf2 nor Erf4 alone is capable of carrying out Ras palmitoylation. Mutations in the conserved region of DHHC motif abolish PATs activity (Lobo et al., 2002).

Akr1

Akr1 is the 86-kDa size protein, localized in the Golgi complex. It is an ankyrin repeat containing protein with six predicted transmembrane domains. Akr1 is the putative protein acyltransferase for casein kinases. The yeast casein kinases Yck1 and Yck2 localize to the plasma membrane by palmitoylation at the C-terminal cysteine-cysteine motif (Babu et al., 2004; Feng and Davis, 2000; Sun et al., 2004). Deletion of *AKR1* prevents PM localization of Yck2 and mislocalized to cytosol. Mutation in the Akr1 DHYC domain abolishes the Akr1 autopalmitoylation and the palmitoylation of Yck2, indicating a direct participation of the DHYC domain in palmitate transfer (Roth et al., 2002). In addition to Yck1 and Yck2, an sphingoid long-chain base kinase, Lcb4 is palmitoylated by Akr1. Interestingly, palmitoylcysteines of Lcb4 are internal to the protein and not at the C terminus, as they are present in Yck1 and Yck2 (Kihara et al., 2005).

Recently, Davis and co-workers carried out a systematic characterization of palmitoylproteome in yeast. Here, they adapted the biotin-switch method (Drisdel and Green, 2004) for the mass spectroscopic analysis of palmitoylated proteins in *S.cerevisiae*. Applying this method, they identified 12 of the 15 known palmitoylproteins and 35 new proteins in yeast. Some of the new substrates, that are palmitoylated by Akr1, include Meh1, Ypl199c, and Ykl047w. In addition, two other palmitoylated proteins, Sna4 and Ypl236c, showed Akr1-dependent palmitoylation (Roth et al., 2006).

Swf1

Swf1 is a DHHC protein that palmitoylates the yeast SNAREs Snc1, Syn8, and Tgl1 at cysteine residues near the cytoplasmic side of their single transmembrane domain (Valdez-Taubas and Pelham, 2005). Deletion of *SWF1* gene abolishes the palmitoylation of Snc1, Syn8, and Tgl1 *in vivo*. Tgl1 is an endosomal SNARE involved in the recycling of proteins from the endosome to the Golgi. Normally, palmitoylated Tgl1 resides on endosomes. Nonacylated Tgl1 is being recognized by the ubiquitin ligase Tul1 and becomes ubiquitinated, a signal for entry into multivesicular bodies and is then targeted to vacuole for degradation. The study of Tgl1 and Swf1 suggests that palmitoylation appears to act as a stability factor, protecting Tgl1 from the cellular quality control mechanism. Other proteins include the three mannosyltransferases; Mnn1, Mnn10, and Mnn11, Sso1, as well as the yeast prion induction protein pin2 have been shown to be palmitoylated by Swf1 (Roth et al., 2006).

Pfa4

Pfa4 is a 46-kD protein present in the ER, with four predicted transmembrane domains, which appears to be a PAT for yeast chitin synthase Chs3 (Lam et al., 2006). Chs3 catalyzes the transfer of N-acetylglucosamine (GlcNAc) to chitin; it is required for synthesis of the majority of cell wall chitin, the chitin ring during bud emergence, and spore wall formation. Chs3 is synthesized at the ER, where Pfa4 mediates palmitoylation and Chs7 facilitate the export of Chs3 from the ER (Valdivia et al., 2002). At Golgi, Chs5 and Chs6 direct the transport of Chs3 to the PM, and Chs4 is required both for Chs3 activity at the cell surface and for its localization at the beck neck (Roncero, 2002). Deletion of *PFA4* gene abolishes the palmitoylation of Chs3, leads to the formation of high-molecular mass aggregates at the ER. Here, both palmitoylation and chaperone association are required for trafficking of Chs3 to the cell surface. In addition to Chs3, several amino acid permeases are palmitoylated in a Pfa4-dependent manner (Roth et al., 2006).

Pfa5

Pfa5 is a 43-kD protein, with four predicted transmembrane domains, localized to the plasma membrane (Ohno et al., 2006). Till now, no specific substrates have been identified.

Table 1. *Saccharomyces cerevisiae* DHHC proteins and selected substrates

DHHC Proteins	Selected substrates	Localization	Effects of DHHC deletion	References
Akr1	Yck2, Sphingolipid Lcb4	Golgi	Yck2, Lcb4 mislocalization	Roth et al; 2002, Roth et al; 2006, Kihara et al; 2005
Akr2	Unknown	Golgi	NA	Ohno et al; 2006
Erf2-Erf4*	Ras2	ER	Ras2 mislocalization	Lobo et al; 2002, Bartels et al 2002
Pfa3	Vac8	Vacuole	Vac8 mislocalization; altered vacuoles under stress	Smotrys et al; 2005, Hou et al; 2005
Pfa4	Amino-acid permease Chs3	ER	ER retention of Chs3	Ohno et al; 2006 Roth et al; 2006, Lam et al; 2006
Pfa5	Unknown	Plasm membrane	NA	Ohno et al; 2006
Swf1	SNARE protein Tlg1	ER	Missorting of Tlg to the vacuole for degradation	Roth et al 2006, Valdez-Taubas et al; 2005

* The DHHC protein Erf2 requires Erf4 for protein acyltransferase (PAT) activity in vivo

Akr2

Akr2 is a polypetide consisting of 749 amino acids with a deduced molecular mass of 85.2-kD. The PAT Akr1 shares high homology with Akr2 (37.9% identity

and 64.2% similarity). There is no evidence of selected substrates for this enzyme (Ohno et al., 2006).

Pfa3

Pfa3 is a vacuole localized PAT that palmitoylates the yeast vacuolar protein Vac8 (Smotrys et al., 2005). Membrane association of Vac8 requires myristoylation of the glycine residue and palmitoylation of up to three cysteines at the N-terminal region (Subramanian et al., 2006). Deletion of Pfa3, leads to the mislocalization of Vac8 (Hou et al., 2005). In latter part of my thesis, the regulatory role of Pfa3 in Vac8 function will be explained in detail.

Mammalian DHHC proteins

There are ~23 DHHC-CRD genes in the mouse and human genome database (Fukata et al., 2004). However, characterizing the activity and finding the specific substrates pose a great challenge. The following are the few examples of mammalian DHHC proteins.

Huntingtin-interacting protein-14 (HIP14, also known as DHHC17), shares a significant sequence identity with yeast Akr1, acts as PATs for huntingtin. HIP14 also possess PATs activity for SNAP-25, PSD-95, GAD65, and synaptotagamin I.

GODZ (DHHC3) is a Golgi-specific DHHC protein with four putative transmembrane domains. It interacts with GluRα1 glutamate receptor subunit and with the γ^2 subunit of the $GABA_A$ receptors. This interaction enhanced the palmitoylation in the heterologous cells (Keller et al., 2004). Like HIP14, GODZ shows a broad substrate specificity to SNAP-25, and G_α subunits (Fukata et al., 2004).

Abl-philin 2 (Aph2/DHHC16) is an Abl-associated protein containing a DHHC-CRD motif and predicted topology similar to that of Erf2. Abl is a non-receptor kinase implicated in DNA damage-induced cell death and in growth factor

receptor signaling. Aph2 mediates the palmitoylation in Abl, resulting in the regulation of its kinase activity. There are three cysteine residues in the N-terminal region of Abl that are being used as potential substrate for palmitoylation (Li et al., 2002).

DHHC 15 has PAT activity for the neuronal scaffold protein PSD-95 (Fukata et al., 2004). DHHC9 and Golgi-localized GCP16 forms a protein complex homologous to yeast Erf2/Erf4 complex constitute a protein acyltransferase activity for H- and N-Ras (Swarthout et al., 2005). DHHC21 resides in the Golgi apparatus palmitoylate and regulate the endothelial nitric oxide synthase (eNOS). The functional importance of DHHC proteins further extends to the plants as well.

In intestinal protozoan parasite *Giardia lambila*, a putative 50-kDa *Giardia* PAT (gPAT) contains DHHC motif and palmitoylates the two C-terminal cysteines of the surface protein VSPH7. Deletion of gPAT shifts the localization of VSPH7 to the non-raft region in the plasma membrane and abolish the cytotoxicity effect of the parasite against the anti-VSPH7 specific antibodies (Touz et al., 2005).

In *Arabidopsis thaliana* the TIP1 (Tip Growth Defect 1) gene regulates the root hair growth. Mutation in the gene, tip1-1 and tip1-2 shows wider, shorter, and irregularly root hairs. TIP1 protein has been predicted to contain six ankyrin repeats and DHHC motifs, similar to yeast Akr1 and human HIP14 proteins. Interestingly, overexpression of TIP1 rescues the phenotypes in *akr1Δ* cells, associated with slow growth, morphological defect, and mislocalization of Yck2 from the PM to the cytosol (Hemsley et al., 2005). The substrate for the TIP1 remains unknown.

Palmitoylation of secreted proteins

Secreted signaling molecules of families include Hedgehog (Hh) and Wingless (Wnt) are important for the development of metazoan organisms (Cadigan and Nusse, 1997; Hammerschmidt et al., 1997). Members of both families are

substrates for palmitoylation. Genetic studies in *D.melanogaster* identified the enzymes responsible for the palmitoylation of secreted morphogens of Hh and Wnt signaling families. Skinny hedgehog (also known as Rasp, Central missing, and Sightless) is required for palmitoylation of N-terminal cysteines present in Hedgehog (Chamoun et al., 2001) and Spitz (Miura et al., 2006). Porcupine is required for the palmitoylation of Wnt proteins (Zhai et al., 2004). Both Skinny hedgehog and porcupine share a significant sequence similarity to a super family of membrane-bound O-acyltransferases (MBOT) proteins (Hofmann, 2000). These proteins have multiple-membrane spanning regions (8-10) and share sequence similarity in a region that includes highly conserved histidine residue. Members of MBOT family proteins do not have sequence homology with DHHC family proteins and shows PATs activity to the secreted substrates.

I.5 Functions of palmitoylation

The covalent addition of palmitate moiety promotes the membrane association of the peripheral or cytosolic proteins. Palmitate acts along with other lipid modifications and protein motifs to facilitate targeting of proteins to the specific compartment (Bijlmakers and Marsh, 2003; Smotrys and Linder, 2004).

I.5.1 Membrane attachment

Palmitoylation confers the stable binding of proteins to the membranes. Other lipid modifications either myristoylation or farnesylation bind the protein weakly to the membrane. According to the 'two-signal hypothesis' either palmitate or polybasic domain provides a second signal for the myristoylated or farnesylated proteins (Schroeder et al., 1997) (Resh, 2006b). For example, the membrane binding of Src family kinases, Gα subunits use myristate and palmitate, whereas H-Ras and N-Ras use farnesyl and palmitate for stable binding. Another second signal, polybasic domain supports the binding of protein by electrostatic interaction with the membrane. The polybasic domain in Src kinase interact with the negatively charged phospholipids head groups that are enriched in the

cytoplasmic leaflet of the plasma membrane (Hancock et al., 1991; Hancock et al., 1990).

Proteins modified with myristate or farnesyl alone show nonspecific association with intracellular membranes. Palmitoylation can localize the myristatoylated or farnesylated proteins permanently to the specific membrane (Figure 10). This was explained by a "kinetic trapping mechanism". Here, palmitoylation increases the hydrophobicity and reduces the kinetic 'off' rate and protein becomes trapped to the membrane (Schroeder et al., 1997). Thus, the

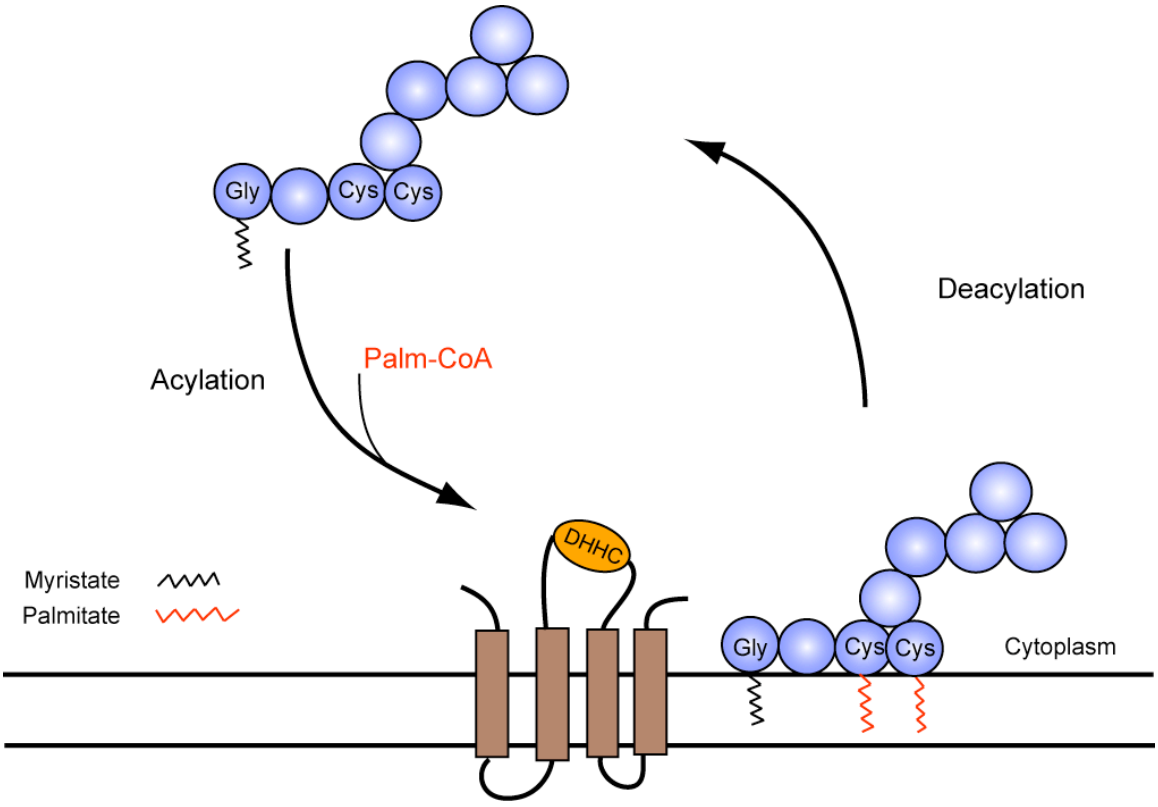

Figure 10. Schematic representation of protein acylation and deacylation

Myristoylated protein is targeted to the membrane, where it gets acylated for stable binding. The palmitate from active palmitoyl-CoA is attached to the cysteine residue of the protein. The membrane localized DHHC protein is a protein acyltransferase mediates the enzymatic acylation reaction. The membrane bound palmitoylated protein can be deacylated by the thioesterase enzyme.

site of palmitoylation (localization of PAT) determines the localization of the palmitoylated protein. The DHHC proteins have been described as palmitoyl acyltransferase for the protein palmitoylation (Mitchell et al., 2006; Roth et al., 2006). This mechanism accounts for the plasma membrane and Golgi localizations of H-Ras, N-Ras and yeast Ras2 (Dong et al., 2003; Goodwin et al., 2005; Rocks et al., 2005).

I.5.2 Targeting to lipid rafts

Many palmitoylated proteins are associated with the lipid rafts in the membrane. Lipid rafts are functionally defined as membrane subdomains that are enriched in cholesterol, glycosphingolipids and phospholipids containing saturated fatty acids. These lipids pack tightly into a 'liquid-ordered' subdomain leading to lateral segregation in the plane of the bilayer. Proteins containing saturated fatty acids segregate into the liquid-ordered domain of the membrane. For example, Src family kinases and $G_{i\alpha}$ subunits associate with the lipid rafts and are involved in signal transduction (Koegl et al., 1994). In contrast, proteins modified with bulkier lipids like unsaturated fatty acids or prenyl groups have low affinity for rafts (Webb et al., 2000).

I.6 Effects of palmitoylation on protein functions

The important role of palmitoylation is to localize protein to the membranes. Recent studies show that besides functioning as membrane anchor, palmitoylation has been implicated in various other functions of the protein (Resh, 2006a). The following are few examples of proteins, where palmitoylation play a vital role for the proteins function.

I.6.1 Protein trafficking

In mammalian cells, both H- and N-Ras requires palmitoylation for intracellular trafficking. Farnesylated H- and N-Ras are targeted to the Golgi

where they become palmitoylated and further target to the plasma membrane via the vesicle-mediated classical secretory pathway (Choy et al., 1999). At the plasma membrane H/N-Ras can be depalmitoylated by the action of a acylprotein thioesterase (APT), allowing the farnesylated H/N-Ras undergo rapid non-vesicular exchange with endomembranes including the Golgi and ER (Goodwin et al., 2005; Rocks et al., 2005). At ER or Golgi, Ras becomes repalmitoylated and once again cycle back to the plasma membrane. The half-life of palmitate ranges from 20 min for N-Ras to 2 h for H-Ras, suggesting fast rate of palmitate turnover depends on the nature of the lipidation (Baker et al., 2003). H-Ras consists of two palmitoylation cysteines whereas N-Ras has a single cysteine for palmitoylation (Rocks et al., 2005). This acylation and deacylation cycle appears to be essential for maintaining the subcellular localization and distribution of Ras in the endomembrane system.

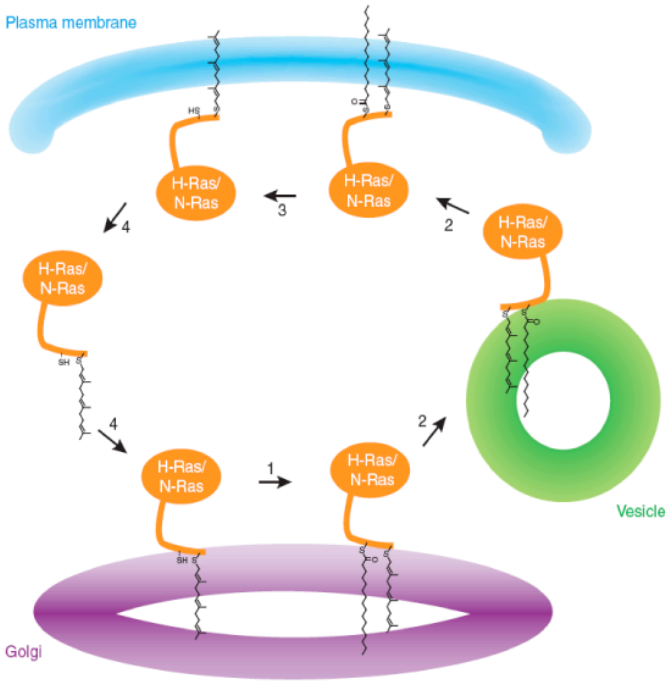

Figure 11. Trafficking of H-Ras and N-Ras in mammalian cells

(1) S-Palmitoylation of the two Ras isoforms occurs at the Golgi, (2) Palmitoylated Ras proteins are targeted to the plasma membrane by vesicular trafficking, (3) At the plasma membrane Ras isoforms are depalmitoylated by the thioesterase activity (4) Then the Ras proteins are

transported back to the Golgi via non-vesicular pathway. In mammalian cells, DHHC9 and GCP16 proteins may be involved in palmitoylation of Ras at the Golgi. Taken from (Gelb et al., 2006).

I.6.2 Protein stability

A recent study in yeast has shown that palmitoylation protects the SNARE protein Tlg1 from degradation. Tlg1 is involved in the membrane trafficking between Golgi and late endosomes. When the palmitoylated is prevented, Tlg1 gets ubiquitinated by the ubiquitin ligase Tul1 and mislocalizes to the vacuole, where it is subsequently degraded by vacuolar hydrolase (Valdez-Taubas and Pelham, 2005).

I.6.3 Protein aggregation

The yeast chitin synthase Chs3 is palmitoylated at the ER and targets to the cell surface for cell wall formation. Preventing the palmitoylation of Chs3 leads to the accumulation and exhibit the increased level of aggregation in the ER (Lam et al., 2006). Recent study has shown that the neuronal protein huntingtin requires palmitoylation for its function. Unpalmitoylated huntingtin becomes aggregated intracellularly and leads to Huntington's disease. Palmitoylation of huntingtin prevents the formation of neurotoxic aggregates (Huang et al., 2004; Yanai et al., 2006).

I.7 The study of protein palmitoylation at the yeast vacuole

I.7.1 The yeast vacuole as a model system for organelle biogenesis

Vacuoles of the budding yeast *Saccharomyces cerevisiae* are lysosome-like organelles. Vacuoles are generally large, low copy organelle, and have an important role in homeostasis of the endomembrane system. The vacuoles function as the cell's digestive machinery and contain proteases and lipases in

their lumen for the degradation of proteins and lipids. Vacuoles offer lots of advantages for studying. Vacuoles are about 1-3 μm in diameter and can be readily visualized by the vital dyes under microscope.

Vacuole inheritance

In yeast, vacuoles are inherited from the mother cell. During the early stage in the cell cycle, the vacuole aligns along the actin cables, which are polarized towards the nascent bud site (Figure 12). A portion of the mother-cell vacuole moves to the nascent bud site and from there it moves into the growing bud via tubular-vesicular structure (Weisman, 2006). Vacuole inheritance is mediated by

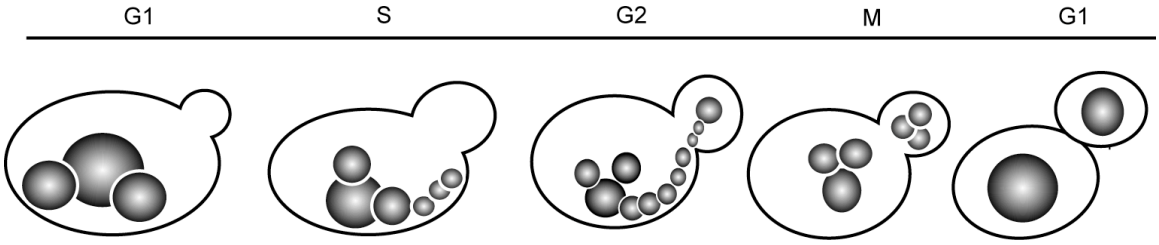

Figure 12. Schematic representation of vacuole inheritance in budding yeast

Vacuole inheritance begins in the early S phase of the cell cycle. Vacuoles from the mother cell move along the actin cables to the emerging bud site and from their moves into the bud through tubular-vesicular structure.

set of proteins including myosin-V motor Myo2, vacuolar protein Vac8 and Vac17. During vacuole inheritance, Vac17 attaches to the vacuole membrane by means of Vac8 and assembles the transport complex, Myo2-Vac17-Vac8. This transport complex moves the attached vacuole along actin cables into the bud and then the complex is disrupted by the turnover of Vac17 (Tang et al., 2003).

Homotypic vacuole fusion

Yeast vacuoles are dynamic in nature; they undergo cycles of fragmentation and fusion yielding one or three vacuoles per cell at steady state. Defects in vacuole fusion result in the fragmentation of vacuoles. The fusion of vacuoles has been a model for studying the membrane fusion. Homotypic fusion of yeast vacuoles occurs in ordered steps: Priming, Docking, and Fusion.

Priming

Yeast vacuole consists of three Q-SNAREs (Vti1, Vam7, Vam3) and two R-SNAREs (Nyv1 and Ykt6). In an ATP-dependent priming step, the NSF homologue Sec18 in the presence of the co-chaperone Sec17 (α-SNAP) disassembles the *cis*-SNARE complexes (Ungermann et al., 1999; Ungermann and Wickner, 1998). Vam7, a homologue of the neuronal SNARE SNAP 25 is transiently released from the vacuole (Boeddinghaus et al., 2002).

Docking

During docking step, unpaired SNAREs of the opposing membranes associate with each other and form *trans* complexes. Formation of stable *trans*-SNARE pairing is supported by Rab GTPase Ypt7 and its hexameric effector HOPS (homotypic fusion and vacuole protein sorting)/VPS class C complex (Wurmser et al., 2000). Vacuoles are tethered, drawn against each other and form a ring-shaped microdomain, termed the “vertex ring” (Wang et al., 2003). The proteins such as Ypt7, HOPS, and SNAREs (Wang et al., 2003) and the regulatory lipids like sterol, diacylglycerol, and phosphoinositides (Fratti et al., 2004) become concentrated at the vertex ring.

Fusion

The formation of *trans*-complexes will bring the opposing membranes close together and facilitate lipid mixing. Fusion requires the action of various factors like calmodulin, Ca^{2+} ions, and protein phosphatase 1, and the assembly of theproteolipid of the vacuolar ATPase, vacuolar transporter chaperone (Vtc)

complex (Muller et al., 2002; Peters et al., 2001; Peters and Mayer, 1998). In addition, fusion can be mediated by Vac8 (Wang et al., 2001), phosphoinositides (Mayer et al., 2000), actin (Eitzen et al., 2002), enolase (Decker and Wickner, 2006) and Bem1 (Xu and Wickner, 2006).

I.7.2 Vac8 is a model protein to study the palmitoylation at the vacuole

Vac8 is a yeast-specific vacuolar membrane protein (Fleckenstein et al., 1998; Pan and Goldfarb, 1998; Wang et al., 1998). It is about 64-kD size protein, composed of three domains. The N-terminal Src homology 4 (SH4 domain), 11 armadillo repeat motifs and aspargine rich sequence (Figure 13).

Vac8 is localized on the cytoplasmic region of the vacuole membrane by an N-terminal short peptide, a so called SH4 domain (Wang et al., 1998). This domain confers the site for fatty acid modifications. In Vac8, the N-terminal glycine residue gets myristoylated co-translationally after the proteolytic removal of N-terminal methionine. Then the myristoylated Vac8 gets palmitoylated on up to three cysteines as a prerequisite site for vacuole localization. An SH4 domain covers about 18 amino acids, characterized by a myristoylation motif (M-G-x-x-x-S/T-x) and a palmitoylation site (cysteine residue) or stretch of polybasic amino acids.

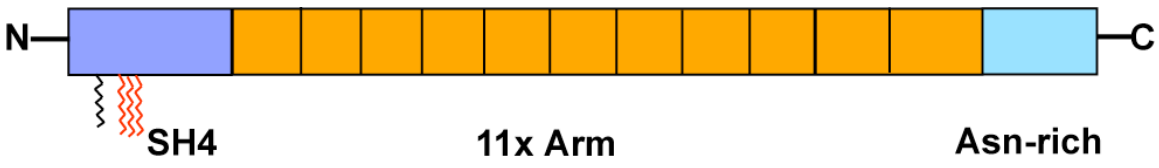

Figure 13. Schematic representation of Vac8 structure

Vac8 is composed of three domains. The N-terminal SH4 domain has site for myristoylation and palmitoylation for membrane anchoring, the middle eleven armadillo repeat motifs mediates protein-protein interactions, and aspargine rich regions with unknown function.

Vac8 is an armadillo (ARM) repeat-containing protein, closely related to β-catenin and plakoglobin (Peifer et al., 1992). ARM repeats protein contains tandem arrays of multiple imperfect repeats. Each ARM is about 42 amino acids and folds to form three alpha helixes separated by short loops and packed together to form super helical domain (Coates, 2003). These repeats have been proposed to serve as scaffolds for the assembly of multiprotein complexes (Peifer et al., 1994). There are eleven ARM repeat motifs in Vac8, which mediate the protein-protein interactions and thereby play essential roles for Vac8 functions. Each ARM motif binds the distinct binding partners and mediates the coordinate functions of Vac8. The ARM repeat motifs in Vac8 are overlapped with each other for specific functions. For example, deletion of ARM2 and ARM5 affects the vacuole inheritance (Tang et al., 2006). The ARM repeat motifs in Vac8 exhibit 45-49% similarity to the importin and catenin members of the armadillo repeat family (Fleckenstein et al., 1998).

The C-terminal extension of an armadillo repeat motifs has an aspargine rich region with unknown function.

Functions of Vac8

Vac8 is involved in vacuole inheritance, vacuole fusion, nuclear-vacuole junctions and cytosol-to-vacuole transport, an autophagy like process (Pan et al., 2000; Roberts et al., 2003; Wang et al., 1998) (Figure 14). For cytosol-to-vacuole transport, the palmitoylation of Vac8 is not required, where as it is mandatory for rest of the Vac8 functions (Scott et al., 2000; Wang et al., 1998).

Vacuole inheritance

Vac8 is required for the inheritance of vacuoles from mother cell to the bud during cell division. Vac8 interacts with the class V myosin protein, Myo2 through the adaptor protein Vac17 and moves the vacuoles to the developing bud (Tang et al., 2003). Vac17 was found on the vacuole membrane functions as the vacuole-specific receptor for Myo2. Vac8 binds to the distinct region of the Vac17

via the ARM repeats. Deletion of ARM2 and ARM5 in Vac8 prevents the interaction of Vac17 results in inheritance defect (Tang et al., 2006). Palmitoylation of Vac8 is essential for vacuole inheritance. Mutation of the palmitoylated cysteines affects the vacuole inheritance (Wang et al., 1998).

Nucleus-Vacuole junctions

Vac8 is required for the formation of nucleus-vacuole junction. Vac8 binds to the nuclear integral membrane protein Nvj1 and form a stable junction between the nucleus and vacuoles (Kvam and Goldfarb, 2004; Roberts et al., 2003). Nucleus-vacuole junctions mediate a unique microautophagic process that degrades the portions of the nuclear components through a process called 'piecemeal microautophagy of the nucleus' (PMN) (Roberts et al., 2003). Palmitoylation of Vac8 is required for the maintenance of nucleus-vacuole junctions.

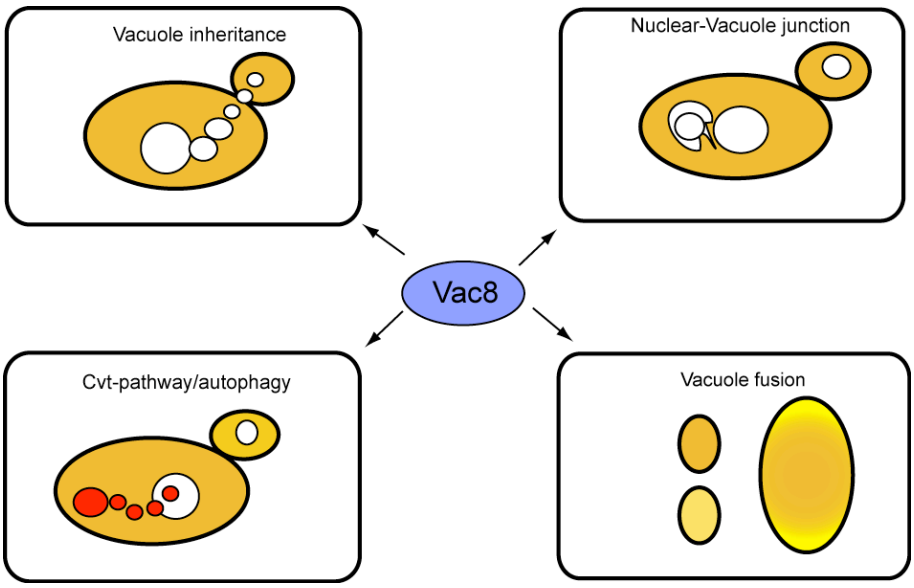

Figure 14. Functions of Vac8

Vac8 is required for vacuole inheritance, formation of nuclear-vacuole junction, cytosol-to-vacuole transport (Cvt)-pathway and vacuole fusion. Palmitoylation of Vac8 is not required for the Cvt pathway. See text for more details.

Mutations of all the three palmitoylated cysteines in Vac8 prevent the formation of nucleus-vacuole junctions and Nvj1 disperse throughout the outer nuclear membrane (Peng et al., 2006). The ARM2-6 and ARM10-11 motifs in Vac8 are required for the proper localization of Nvj1 to the nucleus-vacuole junctions (Tang et al., 2006). Nucleus-vacuole junctions serve as a site for the accumulation of proteins involved in lipid biosynthesis and trafficking (Kvam et al., 2005; Kvam and Goldfarb, 2004).

Cytoplasm-to-vacuole targeting pathway

In the cytoplasm to vacuole targeting (Cvt) pathway, the vacuolar hydrolase aminopeptidase 1 (AP1) is packaged into cytosolic vesicles and targeted to the vacuole. AP1 is synthesized as a precursor in the cytosol and transported to the vacuole by a membrane-bound complex called the Cvt complex. Vac8 binds directly to Apg1 and form protein complexes that mediate the Cvt pathway (Scott et al., 2000). Under starvation conditions, most of the precursor AP1 seems to be targeted to vacuole by autophagosomes (Shintani and Klionsky, 2004). However, the palmitoylation of Vac8 is not essential for this pathway.

Vacuole fusion

Vac8 is required for homotypic vacuole fusion. It is not necessary during priming and docking step but acts in the late stage of fusion (Wang et al., 2001). Deletion of Vac8 affects the vacuole fusion, and results in fragmented vacuoles. Earlier studies described that the activated fatty acids stimulate the Golgi and the vacuole fusion assay (Haas and Wickner, 1996; Pfanner et al., 1990). The acyl-CoAs may acylate the target protein at the vacuole and leads to the fusion of vacuoles. Vac8 is palmitoylated during priming step and is needed for vacuole fusion (Veit et al., 2001; Wang et al., 2001). Addition of antibodies to Vac8 or palmitoylation inhibitors (2-bromopalmitate, hydroxylamine) blocks the *in vitro* vacuole fusion assay (Veit et al., 2001). Besides palmitoylation, deletion of ARM2 and ARM5 motifs in Vac8 affects the vacuole fusion (Tang et al., 2006). The molecular function of Vac8 in vacuole fusion is not known clearly.

Caffeine resistance

A recent study from Weisman group has showed that Vac8 is required for caffeine resistance. Deletion of Vac8 inhibits the growth of cells in the presence of 0.2% caffeine. Vac8 interacts with Tco89 and mediate the caffeine resistance function (Tang et al., 2006). Tco89 is a subunit of the Tor (target of rapamycin) complex 1 and is required for the maintenance of cell wall integrity (Reinke et al., 2004). The ARM2 and ARM5 motifs of Vac8 are essential for caffeine resistance. However, the palmitoylation of Vac8 is not necessary for caffeine resistance.

Palmitoylation is required for Vac8 functions. Mutating the palmitoylated cysteines abolish the functions of Vac8. Palmitoylation of Vac8 can occur either by a non-enzymatic reaction or enzyme mediated acylation. Under in vitro condition, the *longin* domain of the SNARE Ykt6 mediates the palmitoylation of Vac8 (Dietrich et al., 2004). The *longin* domain has binding affinity for palmitoyl-CoA or palmitic acid and promotes Vac8 palmitoylation. Antibodies to the *longin* or Ykt6 block the transfer of palmitate to the target cysteines in Vac8 (Dietrich et al., 2004). Recently, it has been described that the DHHC protein Pfa3 palmitoylates Vac8 at the vacuole (Smotrys et al., 2005). The palmitoylation of Vac8 is also regulated by acetyl-CoA carboxylase enzyme (Acc1), a subunit of fatty acid synthase in cold-sensitive mutant cells. It affects the morphology of the vacuole through palmitoylation of Vac8 (Schneiter et al., 2000).

Palmitoylation of Vac8 occurs during priming step in the vacuole fusion (Veit et al., 2001). In vitro studies showed that Vac8 gets palmitoylated in an ATP-independent manner (Dietrich et al., 2005). The palmitoylation of Vac8 is required for vacuole fusion. However, the role of Vac8 palmitoylation in vacuole is not known so far. In this thesis, I addressed the importance of palmitoylation for Vac8 functions.

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II. Materials and Methods

Reagents

All biochemical reagents were purchased from Sigma (Steinheim, Germany) or Roth (Karlsruhe, Germany), unless indicated.

Yeast strains

The *Saccharomyces cerevisiae* strains used in this study are listed in the Table 2 and 3. Different clones of Vac8 were expressed in the *vac8Δ* background strain. GFP was tagged at the C-terminus genomically by using pYM29 as a template for PCR and selection was made on YPD plus geneticin plate. BY4741 yeast strains (MATa his3Δ leu2Δ met15Δ uraΔ) with deletions in the DHHC genes (*erf2*Δ, *akr1*Δ, *akr2*Δ, *pfa3*Δ, *pfa4*Δ, *pfa5*Δ, *swf1*Δ) were purchased from EUROSCARF (Frankfurt). For screening of Yck3, a pRS416-NOP1pr-GFP-Yck3 plasmid was transformed into the BY471 deletion strains. Pfa3 was tagged with the TAP tag at the C-terminus by using pYM13 as a template for PCR and selected on YPD plus geneticin plate.

Construction of integrative plasmids expressing Vac8

Vac8 mutants were expressed under the endogenous *VAC8* promoter. To construct *VAC8* promoter, 800 nucleotides upstream of the *VAC8* start codon were PCR amplified from the genomic DNA and ligated into *Hin*dIII/*Eco*R1 digested *pRS406* vector. *VAC8* and *vac8* mutants were amplified with the primers listed in the Table 4 (5' Vac8 primer sequence encoding wild type or N-terminal mutant amino acids). All the amplified PCR products were inserted into *pRS406-VAC8 pro* at the *Eco*R1/*Bam*H1 site. Plasmids were digested with *Xma*1 (a unique restriction enzyme in the *VAC8* promoter) and inserted into the *VAC8* promoter region of BJ3505 and DKY 6281 strains lacking the *VAC8* open reading frame (CUY72 and CU73). Expression levels of Vac8 mutants were compared with wild-type protein by Western blotting.

Construction of 2μ plasmids expressing Vac8-GFP

Wild type and mutants of Vac8 were expressed under *NOP1* promoter. To construct *NOP1* promoter, 750 nucleotides upstream of the *NOP1* start codon are amplified and ligated into *pRS426* vector at *Eco*R1/*Bam*H1 site. GFP was amplified from pGL (a generous gift from Sean Munro) and inserted with VAC8 at *Eco*R1/*Sac*1 and inserted the *VAC8-GFP* into the *pRS426-NOP1* promoter at *Bam*H1/*Sac*1 regions. Further mutations in the sequence coding for the first 18 amino acids of Vac8 were introduced into the 5' primer sequence (see Table 5) and amplified using *pRS426-NOP1pr-Vac8*$^{1-16}$*-GFP* as template. All plasmids were expressed in the CU72 strain.

Biotin-Switch Assay

To check the palmitoylation status of endogenously expressed protein, we used the method as described in the literature (Drisdel et al., 2006; Drisdel and Green, 2004; Valdez-Taubas and Pelham, 2005). Yeast cells were grown to a logarithmic phase and 20 OD_{600} units cells were pelleted and resuspended in 300 μl of lysis buffer (PBS, 1x protease inhibitor mixture from Roche and 5 mM EDTA). Cells were lysed with 100 μl of 0.5 mm glass beads in the cell disruptor for 4 min at 4°C. Lysed samples were precleared at 300 x g for 5 min, supernatants were removed, and the pellet was reextracted. One milligram of pooled supernatants was resuspended in 600 μl of lysis buffer containing 1% Triton X-100 and 25 mM *N*-ethylmaleimide to quench the freely available cysteines for 30 min at 4°C, and proteins were precipitated by methanol-chloroform precipitation. The pellets were air-dried and resupended in 100 μl of resuspension buffer (2% SDS, 8 M urea, 100 mM NaCl, 50 mM Tris HCl, pH 7.4) by sonication. Diluted the lysed cells with 600 μl of 1 M Hydroxylamine pH 7.4 and 300 μM biotin-BMCC (pierce) cross linker, and rotated for 2 h at 4°C. As a control, hydroxylamine was replaced by PBS. Then samples were precipitated with methanol-chloroform, dried, and resuspended by sonication as mentioned before. The lysed samples were again diluted with 1 ml of lysis buffer containing 0.1% Triton X-100. Aliquots of 60 μl were taken as loading control and

remainining fractions were incubated with 100 μl of neutravidin–agarose beads (Pierce) for 1 h at room temperature on a nutator. Neutravidin beads were washed with PBS containing 0.5 M NaCl and 0.1% Triton X-100 and then once with PBS. Proteins were eluted by heating for 5 min at 95°C with 20 μl resuspension buffer and 40 μl 4 X SDS sample buffer lacking 2-mercaptoethanol or dithiothreitol. Samples were analyzed by SDS-PAGE and Western blotting.

Microscopy

For GFP microscopy, cells were grown to mid-log phase in SD-Ura medium. Cells were washed and resuspended in fresh medium, an aliquot was placed onto a glass slide, covered with a cover slip, and immediately analyzed at RT. Images were acquired with a Zeiss Axiovert 35 microscope equipped with AxioCam, with filter set 10 or phase contrast, with a 100x objective. Color images were acquired with Zeiss Axio Vision 3.1 software, and processed using Adobe Photoshop 7.0. Confocal fluorescence microscopy was done using LSM 510 Meta from Carl Zeiss, Jena, Germany.

To visualize vacuole morphology and inheritance *in vivo* cells were stained with the vital, lipophilic dye N-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl)-pyridinium dibromide (FM4-64) as previously described (Vida and Emr, 1995). Yeast cells were grown to OD_{600} <0.5 in YPD. A 500 μl aliquot cell were pelleted down, resuspended in 30 μl of YPD and incubated with 50 μM FM4-64 for 1 hour (pulse). Cells were washed and resuspended in 1 ml YPD, grown for 3 hours (chase), and vacuoles were visualized by phase contrast and fluorescence microscopy from Carl Zeiss as described above, using the filter set 23. To quantify vacuole inheritance, photos of at least 10 random fields were taken. Images were also acquired with a Leica DM5500 Pursuit camera using GFP, FM4-64 or phase contrast filters. All the images were processed using Adope photoshop 7.0

Vacuole purification

Vacuoles were isolated as described in the reference (Haas, 1995). Yeast cells were grown in YPD medium to a density of OD_{600} =1.0. 1000 OD_{600} units cells were pelleted, resuspended in 50 ml DTT/ Tris buffer (100 mM Tris, pH 7.4, 10 mM DTT), and incubated at 30°C for 10 min. Cells were then collected and spheroplasted for 20 min at 30°C in 15 ml buffer (0.6 M D-sorbitol, 0.16 x YPD, and 50 mM Kpi, pH 7.5) containing 1 mg oxalyticase. Spheroplasts were lysed in 2.5 ml 15 % ficoll solution (10 mM PIPES/KOH, pH6.8, and 20 mM D-sorbitol) containing 40 μg DEAE-dextran and incubated on ice for 5 min, followed by 3 min incubation at 30°C and transferred the centrifuge tube. Then, the density gradient was poured sequentially by layering 2.5 ml of 8% ficoll solution on top of bottom layer, on top of this 2.5 ml 4% ficoll, and then the rest of the tube was filled with 0% ficoll. Vacuoles were recovered from the 4% and 0% ficoll interface after centrifugation at 30,000 rpm for 90 min at 4°C. Vacuolar protein concentration was determined with the Bradford reagents (Bio-Rad Laboratories)

Vacuole fusion

Standard fusion reactions contain 3 μg of vacuoles each isolated from BJ3505 and DKY6281 strains in 30 μl of reaction buffer (125 mM KCl, 5 mM $MgCl_2$, 20 mM PIPES-KOH pH 6.8, 200 mM sorbitol), a protease inhibitor mixture (7.5 μM Pefbloc SC, 7,5 ng/ml pepstatin, 3.75 μM *o*-phenanthroline), and an ATP-regenerating system. Vacuole fusion reaction was set up at 26°C for 90 min. Alkaline phosphatase activity was determined according to (Haas, 1995). PHO8 substrate solution (470 μl of 250 mM TrisCl, pH 9.0, 0.4% Triton X-100, 10 mM $MgCl_2$, 1 mM *p*NPP) was added to each sample at 30°C and incubated for 5 min, and then stopped by adding 500 μl of 1 M glycine-NaOH, pH 11.5. Para-Nitrophenol was determined by measuring the absorbance at 400 nm. One unit of fusion activity equals 1 μmol of *p*-nitrophenol phosphate hydrolyzed per min per μg *pep14Δ* vacuoles at 30°C. Vacuole fusion was analyzed for different Vac8 mutants expressed endogenously in *vac8Δ* strains.

Subcellular fractionation

Cultures (20 ml) were grown to OD_{600} = 0.8-1.0 in YPD. Until lysis, cells were treated exactly as for the vacuole purification, adjusted relative to the smaller culture size. Spheroplasts were resuspended in 1 ml of lysis buffer containing 200 mM sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM HEPES-KOH pH 6.8, 1X protease inhibitor cocktail, 1 mM PMSF and 1 mM DTT. Cells were then osmotically lysed in 2 μg/ml DEAE-dextran. The total extract was centrifuged at 300 x g in a microfuge for 5 minutes at 4° C and the supernatant (S3) was then centrifuged for 15 min at 13,000 x g. The pellet "P13" and supernatant "S13" fractions were collected. Equal volumes of samples were analyzed by SDS-PAGE and Western blotting.

IgG-pull down

The total extract of the cell was prepared as above. The total extract was lysed with 1% Triton X-100 detergent at 4°C for 30 min. A cleared detergent extract was obtained by centrifugation at 13,000 rpm at 4°C for 10 min. A fraction of 2% was removed and used as a loading control. The rest of the cleared lysate was incubated with 25 μl of prewashed IgG beads (GE health care). The samples were incubated in the rotating wheel for 2 hrs at 4°C. The IgG beads were washed three times with wash buffer (300 mM NaCl, 50 mM HEPES, pH7.5, 1.5 mM $MgCl_2$, 0.1% Triton X-100) and proteins were eluted with 0.1 M glycine pH 2.6. Samples were TCA-precipitated and analyzed by SDS-PAGE and Western blotting.

Triton X-114 partitioning

Yeast cells were grown to OD_{600} 0.8-1.0 in YPD. 20 ml of cultures were fractionated into P13 and S13 fractions. To the equal volume (~500 μl) of each fractions, 500 μl of phosphate-buffered saline (PBS) containing 2% Triton X-114 with protease inhibitors (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 10 μg pepstatin A) were added and mixed thoroughly. The samples were incubated for 20 min at 4°C to solubilize membrane proteins.

Then, samples were incubated for 3 minutes at 30°C, followed by centrifugation (10 minutes, 20,000 x g, room temperature) to separate the detergent-enriched and aqueous phases. The detergent phase was washed twice with PBS containing 0.05% Triton X-114 and the supernatant was discarded. The aqueous phase was washed with 2% Triton X-114 and transferred the supernatant to a fresh tube. Each phase proteins were precipitated with 13% TCA and resuspended in 100 μl of 2 X sample buffer containing 2-mercaptoethanol. Equal amounts of samples were loaded in SDS-PAGE and analyzed by Western blotting.

Vac8 pull down and mass spectrometry analysis

Vacuoles were isolated from the large-scale volume cultures. Yeast cells grown to a logarithmic phase around OD_{600}=1.0-1.5 and 12 liters of cultures were processed for vacuole preparation. Protocol was similar to the normal vacuole isolation; buffers were adjusted according to the volume. Spheroplast was prepared and incubated with 0.4 mg/ml Dextran for 3 min at 30°C. Then samples were overlaid with 4% ficoll and centrifuged at 45,000 rpm for 1 hr. Floated vacuoles were collected and diluted 1:10 with cold 0% ficoll and centrifuged at 11,000 x g to pellet down the vacuoles. The pelleted vacuoles were resuspended in 0% ficoll and measured the protein concentration by Bio-Rad. Six mg of vacuoles were lysed with 2% non-ionic detergent n-octylglucoside in 0% ficoll solution for 30 min at 4°C and centrifuged for 11,000 x g for 15 min at 4°C. Then the detergent lysed vacuole extract was incubated with anti-Vac8 IgG beads for overnight at 4°C and washed three times with wash buffer. Proteins were eluted with 0.1 M glycine pH 2.6, precipitated with TCA. As a negative control, six mg of vacuoles were processed similarly and incubated in anti-N' Nyv1 beads. Samples were loaded in 4-12% gradient gel (invitrogen) and analyzed by mass spectrometry.

Table 2. *Sachromyces cerevisiae* strains used for Vac8 study

Strain	Genotype	References
CUY72	MATα pep4Δ::HIS3 prb1-Δ 1.6R HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can vac8Δ::TRP1	Veit et al; 2000
CUY73	MATα leu2-3 leu2-112 ura3-52 his3-Δ200 trp1-Δ101 lys2-801 suc2-Δ9 pho8::TRP vac8Δ::HIS3	Veit et al; 2000
CUY363a	CUY72; VAC8pr::pRS406-VAC8pr-VAC8	
CUY363b	CUY73; VAC8pr::pRS406-VAC8pr-VAC8	
CUY861a	CUY72; VAC8pr::pRS406-VAC8pr-VAC8^{Cys4}	
CUY861b	CUY73; VAC8pr::pRS406-VAC8pr-VAC8^{Cys4}	
CUY367a	CUY72; VAC8pr::pRS406-VAC8pr-VAC8^{Cys5}	
CUY366a	CUY72; VAC8pr::pRS406-VAC8pr-VAC8^{Cys7}	
CUY366b	CUY73; VAC8pr::pRS406-VAC8pr-VAC8^{Cys7}	
CUY352a	CUY72; VAC8pr::pRS406-VAC8pr-VAC8^{Cys4,}	
CUY352b	CUY73; VAC8pr::pRS406-VAC8pr-VAC8^{Cys4,5}	
CUY365a	CUY72; VAC8pr::pRS406-VAC8pr-VAC8^{Cys4,7}	
CUY365b	CUY73; VAC8pr::pRS406-VAC8pr-VAC8^{Cys4,}	
CUY364a	CUY72; VAC8pr::pRS406-VAC8pr-VAC8^{Cys5,7}	
CUY364b	CUY73; VAC8pr::pRS406-VAC8pr-VAC8^{Cys5,7}	
CUY369a	CUY72; VAC8pr::pRS406-VAC8pr-VAC8^{Cys}	
CUY369b	CUY73; VAC8pr::pRS406-VAC8pr-VAC8^{Cys}	
CUY1092a	CUY72; VAC8pr::pRS406-VAC8pr-Src$^{1-16}$-Vac8$^{\Delta 1-1}$	
CUY1092b	CUY73; VAC8pr::pRS406-VAC8pr-Src$^{1-16}$-Vac8$^{\Delta 1-18}$	
CUY1615	CUY72; VAC8pr::pRS406-VAC8pr-Src$^{1-16(G2A)}$-Vac8$^{\Delta 1-18}$	
CUY940	CUY72; pRS426-NOP1pr-VAC8-GFP	
CUY973	CUY72; pRS426-NOP1pr-VAC8^{Cys4}-GFP	
CUY1042	CUY72; pRS426-NOP1pr-VAC8^{Cys5}-GFP	
CUY1063	CUY72; pRS426-NOP1pr-VAC8^{Cys7}-GFP	
CUY948	CUY72; pRS426-NOP1pr-VAC8$^{Cys—}$GFP	
CUY1549	CUY72; pRS426-NOP1pr-VAC8^{CysG2A}-GF	
CUY987	CUY72; pRS426-NOP1pr-Src$^{1-16}$-GFP	
CUY942	CUY72; pRS426-NOP1pr-VAC8$^{1-18}$-GFP	
CUY1006	CUY72; pRS426-NOP1pr-Src$^{1-16}$-VAC8 $^{\Delta 1-18}$-GFP	
CUY1229	CUY73; pRS426-NOP1pr-VAC8 $^{1-18Cys5}$-GFP	
CUY1230	CUY72; pRS426-NOP1pr-VAC8 $^{1-18Cys7}$-GFP	
CUY1909	CUY73; VAC8pr::pRS406-VAC8pr-Src$^{1-16;S4C}$-VAC8 $^{\Delta 1-18}$	

Table 3. *Saachromyces cerevisiae* strains used for SH4 domain study

Strain	Genotype	References
CU72	MATα pep4Δ::HIS3 prb1-Δ 1.6R HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can vac8Δ::TRP1	
CUY940a	CU72; ; pRS426-NOP1pr-VAC8-GFP	
CUY949	CU72; ; pRS426-NOP1pr-HB-GFP	
CUY944	CU72; ; pRS426-NOP1pr-VAC8$^{1-18}$-HB$^{\Delta 1-18}$-GFP	
CUY941	CU72; ; pRS426-NOP1pr-HB$^{1-18}$-Vac8$^{\Delta 1-18}$- GFP	
CUY1205	CU72; ; pRS426-NOP1pr-Vac8$^{1-11}$-HB$^{10-18}$-GFP	
CUY1312	CU72; ; pRS426-NOP1pr-HB$^{1-9}$-Vac8$^{10-18}$-GFP	
CUY1528	CU72; ; pRS426-NOP1pr-Vac8$^{S6T1-18}$-GFP	
CUY1530	CU72; ; pRS426-NOP1pr-HB$^{T6S\ 1-18}$-GFP	
CUY2148	CU72; ; pRS406-VAC8pr-HB$^{T6S\ 1-18}$-Vac8-GFP	
CUY943	CU72; ; pRS426-NOP1pr-HB$^{1-18}$-GFP	

Table 4. Primers used for *pRS406-VAC8pr-Vac8* constructs

Vac8	5´primer	3’ primer
WT	ccgGGATCCatgggttcatgttgtagt	cgcGGATCCtcaatgtaaaaattgtaaa
Cys4	ccgGGTCCatgggttcatgtGCtagtGCcttgaaagattc	
Cys5	ccgGGATCCatgggttcaGCttgtagtGCcttgaaagattc	
Cys7	ccgGGATCCatgggttcaGCtGCtagttgcttg	
Cys4,5	ccgGGATCC atgggttcaGCtGCtagttgcttg	
Cys4,7	ccgGGATCCatgggttcatgtGCTagttgcttg	
Cys5,7	ccgGGATCCatgggttcaGCTtgtagttgc	
Cys	ccgGGATCCatgggttcaGCTGCTagtGCCttgaaag attc	
G2A	ccgGGATCCatg GCCtctgttgtagttgcttg	
Src	ccgGGATCCatgggaagctctaagtcgaagcccaaggac cccagtcagcgtcgccgaatgtctaaaggagaag	
SrcG2A	ccgGGATCCatgGCCagctctaagtcgaagcccaag	
SrcS4C	ccgGGATCCatgggaagCTGtaagtcgaagcccaagga ccc cag	

Table 5. Primers used for *pRS426-NOP1pr-x-GFP* constructs

WT	5' primer	3' primer
Vac8	atgcGGATCCatgggttcattgttagt	ccgGAATTCatgtaaaaattgtaaaaatctgttg
Cys4	cgcGGATCCatgggttcatgtGCtagtGCcttgaaagattc	ccgGAATTCatgtaaaaattgtaaaaatctgttg
Cys5	cgcGGATCCatgggttcaGCttgttgtGCcttgaaagattc	ccgGAATTCatgtaaaaattgtaaaaatctgttg
Cys7	cgcGGATCCatgggttcaGCtGCtagttgcttgaaa	ccgGAATTCatgtaaaaattgtaaaaatctgttg
Cys-Src$^{1-16}$	cgcGGATCCatgggttcaGCtGCtagttgcttgaaa cgcGGATCCatgggaagctctaagtcgaagcccaaggacc ccagtcagcgtcgccgaCCAATTGCCGATAATGAAG	ccgGAATTCatgtaaaaattgtaaaaatctgttg ctagGAGCTCctacttgtatagttcatccatgc
Vac8$^{1-16}$	GATCCatgggtttcatgttgtagttgcttgaaagattcttcaga cgaggccagtgtccaG	AATTCtgagacactggcctcgtctgaagaatcttttc aagcaactacaacatgaaccatG

III. Rationale

The studies presented in this thesis have emerged from the following questions: What defines the palmitoylation of the protein? How does palmitoylation regulate the function of protein? How is palmitoylation regulated? My studies focused on the importance of palmitoylation for protein function at the yeast vacuole.

Is palmitoylation required for protein function?

Proteins modified with the fatty acid palmitate are targeted and anchored to the specific cellular membranes (Dunphy and Linder, 1998). Besides functioning as a membrane anchor, palmitate influences the protein functions (Linder and Deschenes, 2007). In the first part of my thesis, I will address the regulatory role of protein palmitoylation for the vacuolar protein Vac8. I characterize the three potential palmitoylated cysteine residues in Vac8. Earlier studies reported that the individual palmitate residues contribute distinct function in H-Ras protein (Roy et al., 2005). Here, I will address the individual functions of cysteine residues for Vac8.

How is palmitoylation regulated?

In the second part, I studied the palmitoylation machinery of the protein at the vacuole. Palmitoylation can occur either by autoacylation or enzyme-mediated reaction. Studying the enzymology of the protein palmitoylation pose a great challenge for many years. Recent studies have described that the proteins belong to DHHC family have palmitoyl acyltransferase activity. I analyzed the functionality of the vacuole-localized DHHC protein Pfa3 and its effect on various palmitoylated proteins at the vacuole.

How does palmitoylation target protein to the membrane?

Finally, I characterized the minimal membrane targeting motifs. The mechanism of membrane targeting is poorly understood. In mammalian cells, it has been shown that SH4 domain is targeted to endosomes and plasma

membrane. Here, I analyzed the targeting mechanism of SH4 domains from Vac8 and protozoan parasite HASPB (*h*ydrophilic *a*cylated *s*urface *p*rotein B) in yeast.

These studies will give an insight to understand the targeting and regulatory role of protein palmitoylation at the vacuole. In addition, the role of the vacuolar DHHC protein Pfa3 for Vac8 palmitoylation will be addressed.

IV. Results

The purpose of this thesis is to understand the mechanism and regulatory role of protein palmitoylation at the yeast vacuole. The results are arranged into three parts. The first part deals with the localization and functions of Vac8 mediated by protein palmitoylation. The second part focuses on the vacuole-localized DHHC protein Pfa3 and its role in Vac8 palmitoylation and functions. The third part describes analysis and membrane targeting of the SH4 domains. Taken all together, these studies give insight to the role of palmitoylation for protein functions.

IV.1 Role of palmitoylation for Vac8 localization and function

SH4 domain- a membrane-targeting motif

Initially, I analyzed the sequence homology of Vac8 with other proteins. Vac8 SH4 domain sequence homologues were found in three proteins: OSCPK2, the rice protein kinase (Martin and Busconi, 2000), HASPB an unconventionally secreted protein from *Leishmania major* (Denny et al., 2000), and AKAP18, which targets cAMP-dependent protein kinase A to the plasma membrane (Fraser et al., 1998). The two features are conserved among these proteins in the SH4 region: a myristoylation motif and a palmitoylated cysteine at the position 4 or 5 that seems to be necessary and sufficient for protein activity at the membranes (Figure 15). Vac8 is special in that it consists of three cysteine residues that act as the potential site for palmitoylation. These cysteines in the Vac8 are required for the localization at the vacuole.

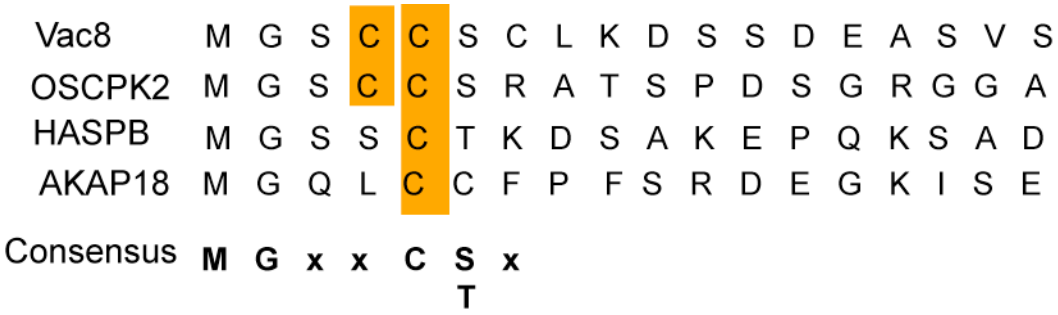

Figure 15. Amino acid sequence alignment of SH4 domains

Amino acid sequence alignment of Vac8 SH4 domain with other proteins: rice OSCKP2, *Leishmania* HASPB, and signaling protein AKAP18. The conserved cysteine at position 4 or 5 is highlighted in orange and M-G-x-x-C-S/T-x represents the consensus motif.

Palmitoylation-dependent localization of Vac8

Vac8 is a vacuole membrane protein. It consists of three potentially palmitoylated cysteine residues at the N-terminal region of the SH4 domain. The glycine residue is modified by myristate, which is the prerequisite for the palmitoylation. I asked whether these three cysteines residues are redundant or if they are differentially involved in the function of Vac8. In order to study this, the three cysteine residues were mutated to alanine individually or in combination of two cysteines. The Vac8 mutants were named according to their non-mutated cysteines.

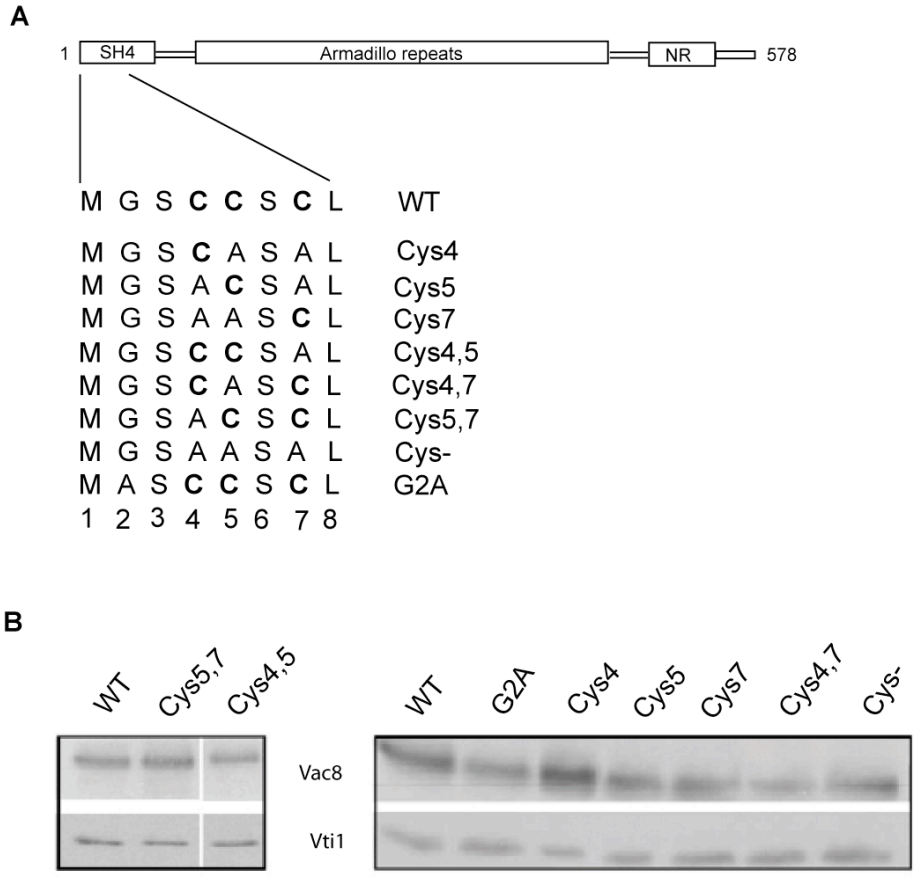

Figure 16. SH4 domain alignments and expression of Vac8 mutants

(A) Structure of the Vac8 domains and Vac8 mutants used in this study. Cysteines in the N-terminal region are mutated to alanine. The schematic diagram of the Vac8 structure with three

domains (SH4, Src homology sequence 4; NR, aspargine rich domain). Cysteine residues are highlighted in black. Constructs were named according to their non-mutated cysteines. Numbers represents the position of amino acid residues in the SH4 domain. (B) Expression levels of the endogenous Vac8 mutants. Proteins were extracted from the BJ3505 cells, expressing the indicated mutants and analyzed by SDS-PAGE and Western blotting with Vac8 and Vti1 antibodies.

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As a control, the glycine at position 2, and three cysteine residues were mutated to alanine in Vac8 (Figure 16A). All the Vac8 mutants were expressed endogenously in the *vac8Δ* background strains and studied their functions in vacuole fusion, inheritance, and morphology (Figure 16B). The reporter protein, GFP was tagged at the C-terminal region of the Vac8 and analyzed the *in vivo* localization by fluorescence microscopy. Wild type Vac8 is localized on the vacuole membrane and also the nuclear-vacuole junctions (Wang et al., 2001). Mutations of all the cysteines to alanine or G2A mutant lacking the myristoylation site showed the cytosolic staining (Figure 17A). Further, inorder to confirm the localization of Vac8 mutants, cells were fractionated into supernatant and pellet fractions. Vacuoles are enriched in the pellet fractions. Wild type Vac8 is more in the pellet fraction, contributed by the effective localization by palmitoylation, Vac8 mutants G2A or Cys- is in the supernatant fractions. Vac8 Cys- is weakly associated with the membrane fraction due to myristoylation (Figure 17B).

According to a previous study, the position of a cysteine near the N-terminal myristoylation site is not critical for membrane association (Navarro-Lerida et al., 2002). To investigate, whether the position of cysteine in Vac8 has same feature of localization; the Vac8-GFP constructs having single cysteine were expressed in *vac8Δ* cells. Surprisingly, each cysteine confers different binding efficiency of Vac8 on the vacuole membrane. Vac8 with either Cys4 or Cys5 is localized on the vacuole and cytoplasm, whereas the Cys7 mutant showed cystosolic staining (Figure 18A). Subcellular fractionation studies confirmed that Vac8 with single cysteine at position 4 or 5 is equally distributed in the supernatant and pellet fractions whereas Cys7 mutant is in the supernatant, similar to the Cys- (Figure

18C). The same result was obtained whether or not Vac8 was GFP tagged. From these data, it is apparent, that the position of cysteine residues is crucial for the palmitoylation of Vac8.

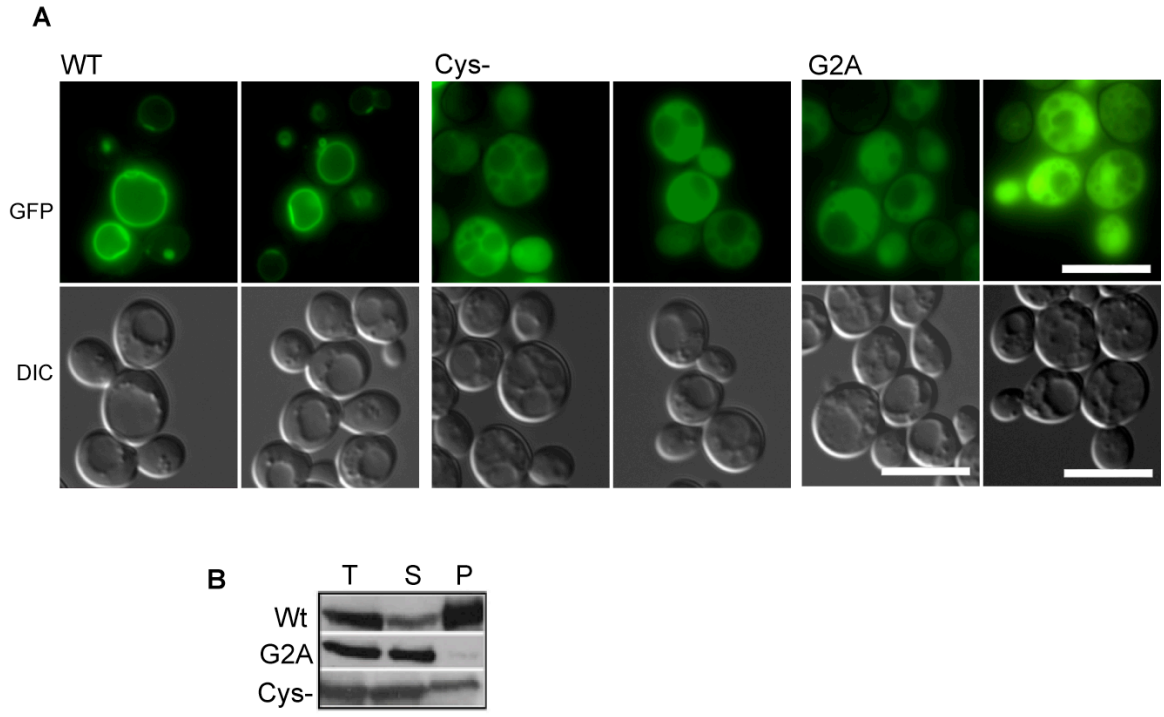

Figure 17. Localization of Vac8 on the vacuole membrane

(A) Vac8 and Vac8 mutants (Vac8 Cys-, Vac8 G2A) are tagged with GFP at the C-terminal region and expressed in BJ3505 *vac8Δ* cells. Localization of the indicated mutants of the Vac8 was observed by fluorescence microscopy. Bars 10 μm (B) Subcellular fractionation of Vac8 mutants. Cells expressing the respective mutants of Vac8 were fractionated as by the vacuole preparation. The cell lysates were cleared at low-speed centrifugation (300 x g, 4°C) and half the volume of the sample was saved as total (T), and rest of it was fractionated into supernatant (S) and pellet (P) fractions. Total and pellet fractions were precipitated by tricholoroacetic acid. Fractionated samples were analyzed by SDS-PAGE and Western blotting with antibodies to Vac8.

In order to extend our further studies, the Vac8 mutants were expressed endogenously without GFP in the *vac8Δ* background strains and estimated the amount of Vac8 on the vacuole membrane (Figure 20A). Similar to the

microscopy results, Vac8 with Cys4 or Cys5 is present about 40-50% on the membrane and Cys7 is poorly localized on the membrane same as the Cys-, approximately 10%. I asked whether any two cysteines within the SH4 domain contribute to the stable attachment like wild type Vac8. For this, Vac8-GFP with either Cys4,5 or Cys4,7 were expressed in *vac8Δ* cells and observed for vacuolar localization like that of wild-type Vac8 (Figure 18B). From these results it was inferred that Vac8 needs at least two cysteines for stable localization.

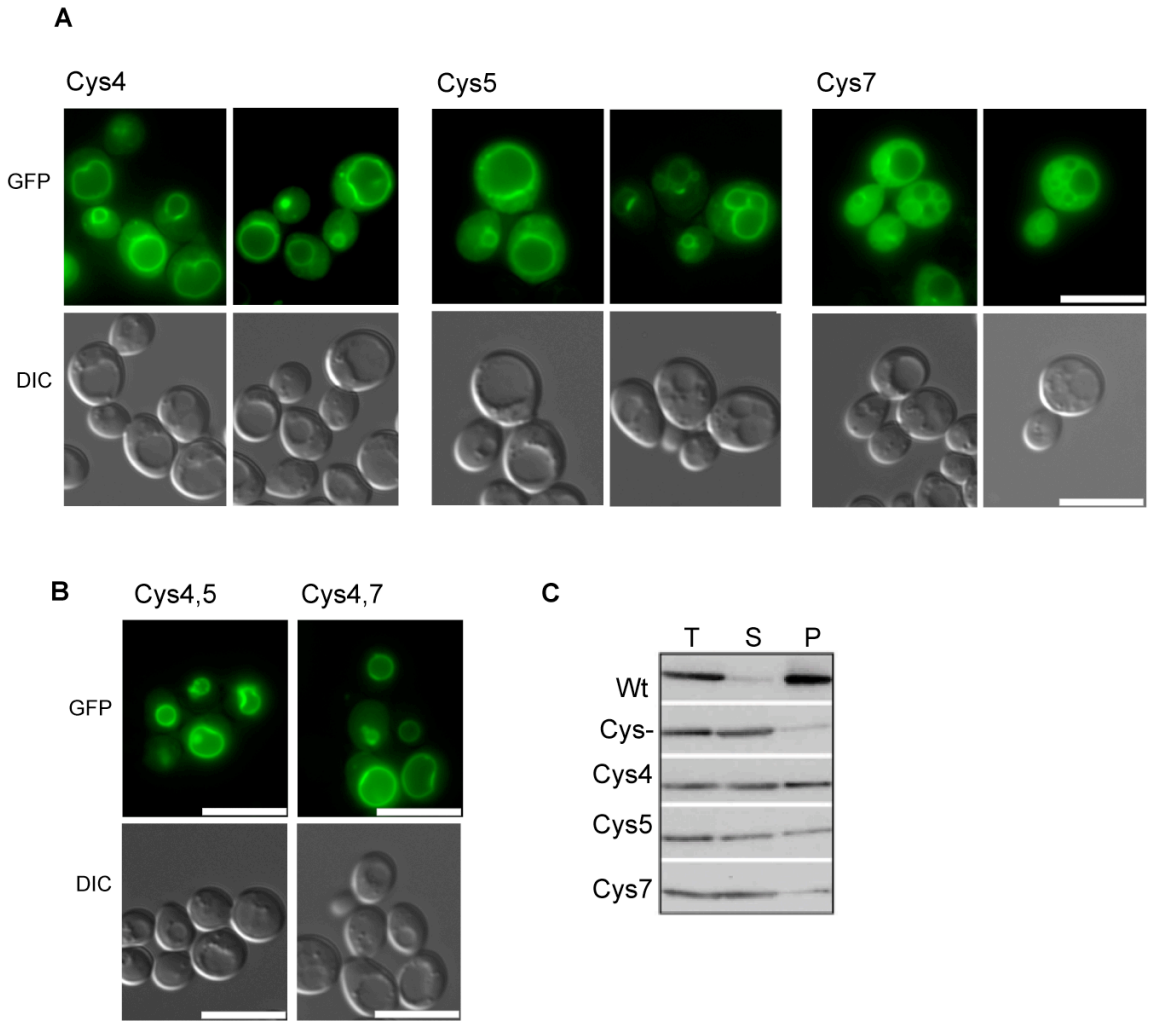

Figure 18. Localization of Vac8 mutants

(A) *In vivo* localization of Vac8-GFP mutants with single cysteine. Fusion protein encoding Vac8-Cys4-GFP, Vac8-Cys5-GFP, and Vac8-Cys7-GFP were expressed in BJ3505 *vac8Δ* cells, and

analyzed by fluorescence microscopy. (B) Localization of Vac8 with two cysteines, Cys4,5 and Cys4,7 were analyzed as described in A. (C) Subcellular fractionation. Cells with indicated Vac8 cysteine mutations were fractionated and analyzed as described in Fig. 17B. Western blots were probed with antibodies to Vac8.

Then I analyzed the membrane binding of the Vac8 mutants were due to palmitoylation. To detect the palmitoylation, I adopted a technique recently developed to label the palmitoylated protein by Biotin-crosslinker (Drisdel and Green, 2004). As an initial step, the free cysteines in the Vac8 were blocked with *N*-ethylmaleimide, then the thioester linkage between the cysteine and palmitate was cleaved with hydroxylamine reagent and cross-linked the free sulfhdryl group with Biotin-maleimide conjugate BMCC, followed by the pull-down of the biotinated protein by Neutravidin beads. First, the palmitoylation of the wild-type Vac8 protein was detected in the presence and absence of BMCC crosslinker. In the presence of BMCC crosslinker, hydroxylamine treated Vac8 was efficiently pulled down by Neutravidin beads. As a control, the SNARE protein Vti1 was not recovered. This means that cysteines in the SH4 are modified by palmitate (Figure 19A). Further, I subjected this method to analyze the palmitoylation of different Vac8 mutants. Vac8 constructs that localized partially or completely on the membrane were pulled down efficiently by the Neutravidin beads, whereas cystosolic Cys7 or Cys- was not (Figure 19B). Vac8 mutants with two cysteines (Cys4,7 or Cys5,7) were palmitoylated and localized to the vacuole membrane. I speculate that Cys7 is palmitoylated only if the proximal cysteine at position 4 or 5 has already been modified. This suggests that the palmitoylation of Vac8 is related to their membrane localization.

Regulation of Vac8 function

Vac8 is involved in various multi-vacuolar processes, including vacuole inheritance, cytoplasm-to-vacuole protein targeting pathway (autophagy), maintenance of nuclear-vacuole junction, vacuole-vacuole fusion. For my studies, I analyzed the effect of SH4 domain in Vac8 function with respect to inheritance, morphology and fusion. The *in vitro* vacuole fusion assay showed

that vacuoles carrying Vac8 Cys4 or 5 fused like the wild-type vacuoles, whereas Cys7 had a reduced fusion similar to that of as Cys- (Figure 20B). Vac8 with two cysteines showed a normal fusion similar to the wild type (data not shown).

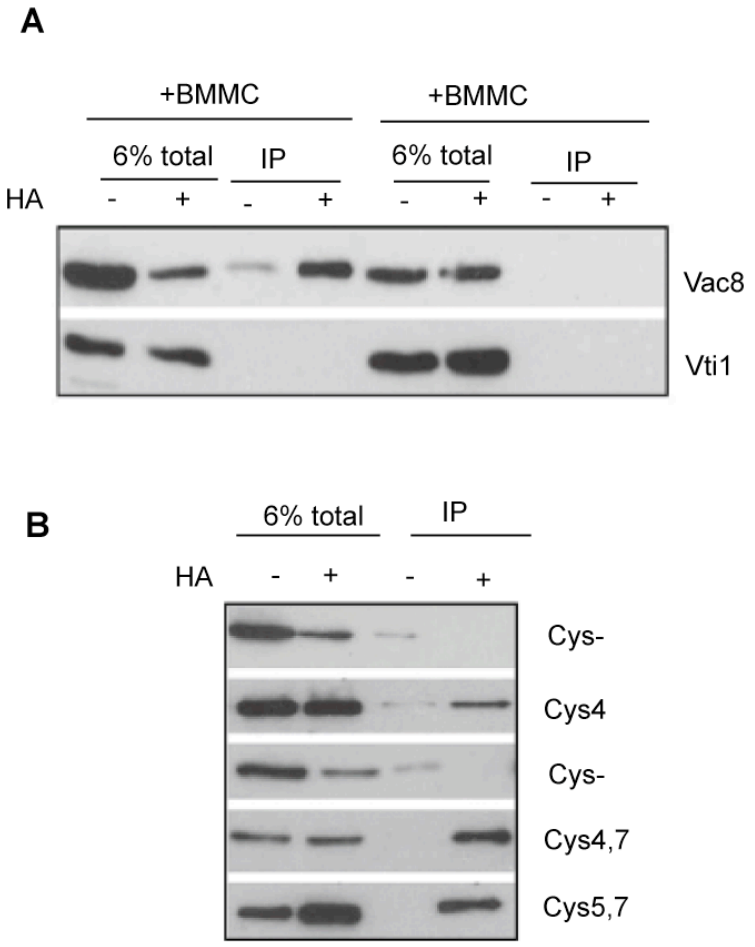

Figure 19. Detection of Vac8 palmitoylation by Biotin-Switch Assay

(A) To detect palmitoylation of wild type Vac8, cell lysate was prepared and treated as the following: quench the free cysteines with *N*-ethylmaleimide (NEM), cleavage of thioester linkage with 1 M hydroxylamine (HA) pH 7.4, crosslinking with Biotin-BMCC. A fraction of 6% total was removed as a loading control and precipitated by TCA. The rest of the modified proteins were pull-down by Neutravidin beads. As a negative control, assay was performed in the absence of BMCC crosslinker. Palmitoyalated proteins were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. (B) Yeast cells expressing indicated Vac8 mutants were analyzed for palmitoylation as described in (A).

To analyze the vacuole inheritance and morphology, we performed the FM4-64 staining by subjecting the cells to pulse for an hour and chase for 3 hours. Cells carrying Vac8 wild type showed round vacuoles and inheritance, and same phenotype was observed for cells with Vac8Cys4 or Cys5. In contrast, the Cys7 mutant cells showed fragmented vacuoles and an inheritance defect similar to the Cys- (Figure 20C). Thus, the membrane localization of Vac8 correlates with the functionality of Vac8. Mutants of Vac8 that were poorly localized to vacuoles were defective in their function.

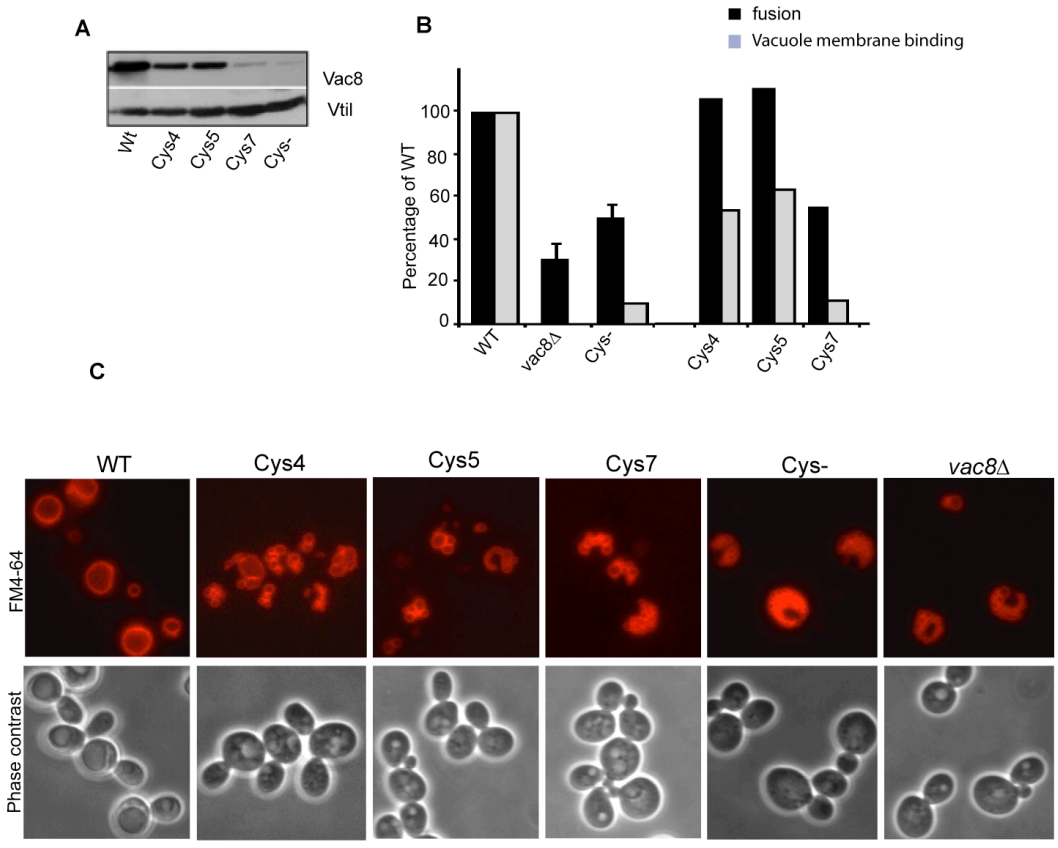

Figure 20. Effect of SH4 domains in Vac8 function

(A) Localization of Vac8 mutants to the purified vacuoles. Vacuoles were isolated from strains carrying the respective mutant in Vac8 and then washed with 20 mM PIPES (pH 6.8), 150 mM

KCl (Refer Materials and Methods). The isolated vacuoles of 30 μg were loaded in the SDS-PAGE and analyzed by western blotting with indicated antibodies. (B) Effect of SH4 mutations in Vac8 for vacuolar localization and vacuole fusion. Vac8 mutants on the purified vacuoles as in Figure 19A was analyzed by antibody against Vac8 and the bands were quantified by laser densitometry (grey bars). For vacuole fusion (black bars), vacuoles were purified from two tester strains BJ3505 and DKY6281 carrying their respective Vac8 mutants and measured their fusion activity under the standard in vitro fusion assay. Fusion values are the average of three independent experiments and error bars represent the standard deviation. (C) Role of SH4 in Vac8 for vacuole morphology/inheritance. To analyze the vacuole morphology/inheritance, strains with indicated Vac8 mutants were stained with lipophilic dye FM4-64 for an hour and chased for 3 hours and visualized by fluorescence microscopy.

Targeting of GFP with minimal SH4 motif

Based on my results, it was inferred that Vac8 needs at least two cysteines for the stable localization and also for its function. Therefore, I asked whether Vac8 SH4 domain alone confers sufficient targeting of the fusion protein to membranes. In order to analyze the localization, fusion protein of GFP and the membrane targeting Vac8 SH4 domain was expressed in *vac8Δ* strains. The fusion protein Vac8(1-18)-GFP was localized on the intracellular membranes and the plasma membranes. Studies in mammalian cells demonstrated that the SH4 domains are targeted to the endosomes and the plasma membrane (McCabe and Berthiaume, 1999). Here, I asked whether targeting of GFP fusion protein also requires two cysteines like Vac8. To analyze the membrane targeting, SH4-GFP chimera with single cysteines, Cys4, Cys5, Cys7 were expressed in *vac8Δ* background strain. In contrast to full-length Vac8 with single cysteine mutants, the SH4 domain confers more stable localization as observed in the fluorescence microscopy (Figure 21A). This was confirmed by the subcellular fractionation, where the SH4-GFP with single cysteines were enriched in the pellet fraction same as the wild type, whereas G2A and Cys- are distributed more in the supernatant fraction. Cys- showed weak distribution on the membrane, due to myristoylation (Figure 21B).

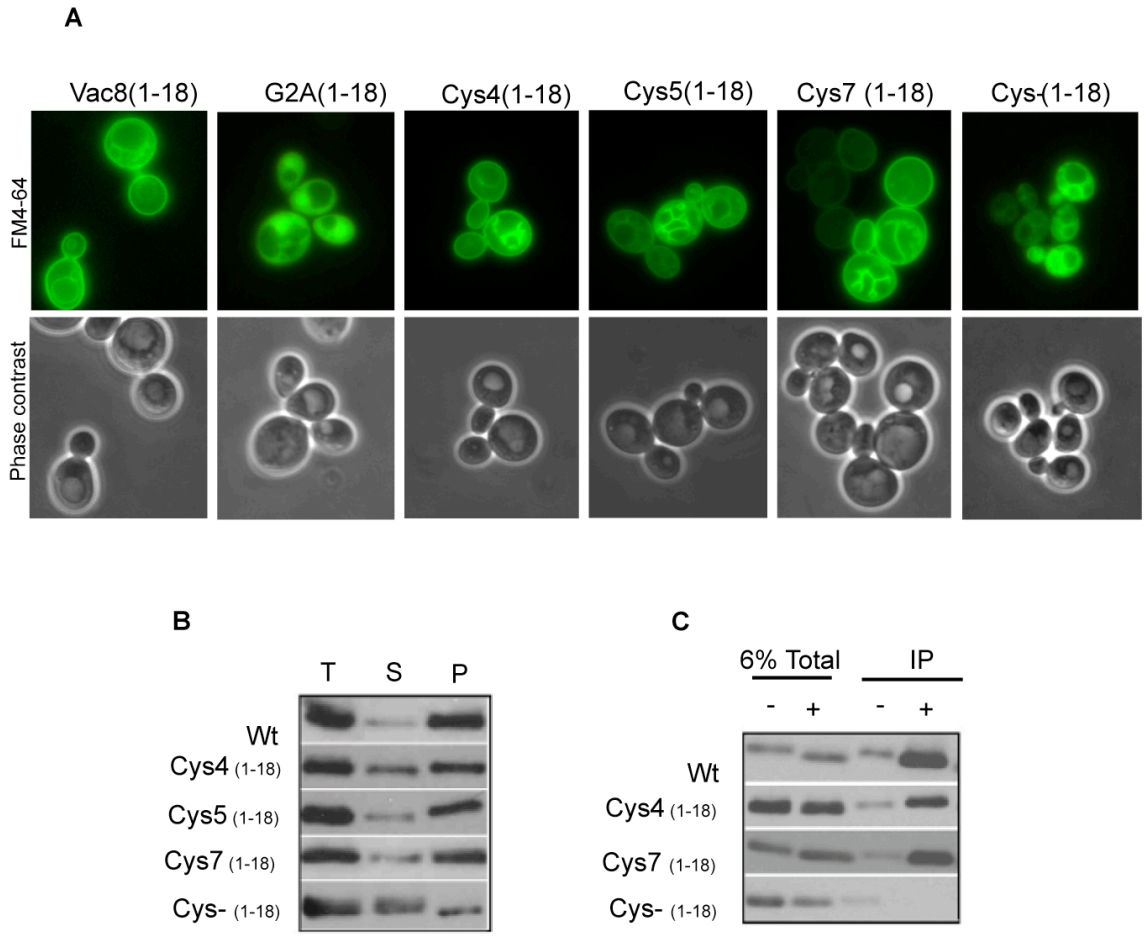

Figure 21. Targeting of SH4 domains of wild type and Vac8 mutants with GFP

(A) The first 18 amino acids at the N-terminal region (SH4 domain) of Vac8 and indicated Vac8 mutants were tagged with GFP, expressed in BJ3505 *vac8Δ* cells, their localization was analyzed by fluorescence microscopy. (B) Membrane association of SH4 domain mutants. Fractionated the cells having respective SH4 mutants into pellet and supernatant fractions and proteins were analyzed by SDS-PAGE and Western blotting with anti-GFP antibodies. (C) Palmitoylation of the SH4 mutant constructs. Biotin-switch assay was performed here and as described in Figure 19. Western blots were decorated with GFP antibodies.

In addition, I detected the palmitoylation of the SH4 domains by Biotin-switch assay, where Cys4, Cys7 were palmitoylated, similar to the wild type SH4 domain (Figure 21C). It is apparent that the SH4 domain requires a single

cysteine for membrane localization, whereas the full-length Vac8 protein needs multiple cysteines for stable membrane localization. Also, the SH4 domain is independent of position of cysteine residue for palmitoylation. Thus, we conclude that the SH4 domain is a universal membrane targeting motif that is recognized and palmitoylated at membranes and requires downstream sequences for membrane localization. In the case of Vac8, membrane localization requires at least two cysteines in the SH4 domain and targeting to the vacuole is regulated by the downstream armadillo repeat sequences.

Anchoring of Vac8 via polybasic motif

Vac8 requires two lipid modification, myristoylation and palmitoylation for localization and function. According to the “two-signal hypothesis” neither myristoylation nor farnesylation of a protein alone is sufficient for stable localization on the membrane. It requires an additional signal for effective membrane localization. This could be supported either by palmitoylation or positive charged residues (Resh, 1999). Here, I asked whether palmitoylation is required only for anchoring the proteins to the membrane or influences the various functions of protein. In order to study the functions, Vac8 was anchored in a palmitoylation-independent manner. For that I used the mammalian Src kinase domain (myristate/polybasic motif) for Vac8 anchoring (Figure 22A). In mammalian cells, full length Src kinase is localized on the plasma membrane and its SH4 domain on the endosomes and plasma membrane (McCabe and Berthiaume, 1999). I expressed the Src (1-16aa)-GFP chimera in yeast and it is localized to the internal organelle membranes and the plasma membrane, same as Vac8 (1-18)-GFP (Figure 21B). Next, the N-terminal 18 amino acids of Vac8 SH4 domain was replaced with Src (1-16aa), and expressed the Src-Vac8 chimera tagged with GFP at the C-terminal region in the *vac8Δ* strain. The Src-Vac8-GFP chimera was not localized on the plasma membrane and targeted to the vacuole membrane (Figure 22C). Then the subcellular fractionation was performed to confirm the distribution; Src-Vac8 chimera is localized in the pellet

fraction (Figure 22D). These data clearly indicates that Vac8 is targeted to the vacuole membrane, as long as it has a functional SH4 domain.

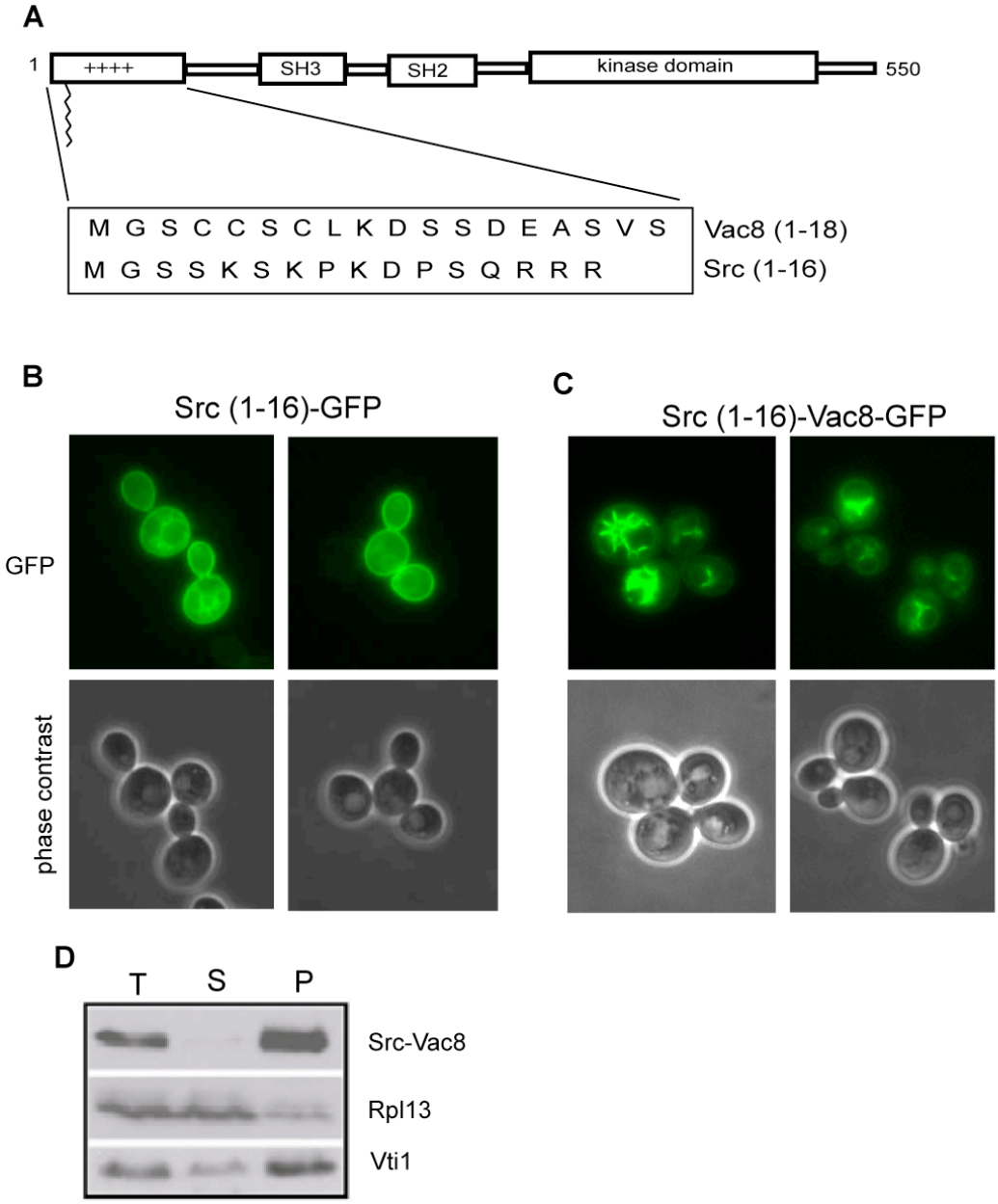

Figure 22. Palmitoylation-independent localization of Vac8

(A) Comparison of the SH4 domain of Vac8 and Src kinase sequence. Alignment of Vac8 and Src SH4 domain is shown. (B, C) Targeting of Vac8 via Src SH4 domain. The GFP-fusion protein of Src SH4 domain and Src-Vac8 chimera were expressed in *vac8Δ* cells and observed their

localization by fluorescence microscopy. (D) Subcellular fractionation of Src-Vac8. Fractionation of cells into pellet and supernatant fractions as described in Figure 13B. Pellets and TCA-precipitated proteins were analyzed by SDS-PAGE and Western blotting with anti-Vac8, anti-Rpl13 and anti-Vti1 antibodies. Rpl13 is a ribosomal subunit (cytosolic marker) and Vti1 is present on vacuoles (Membrane marker).

Palmitoylation-dependent function of Vac8

Once the localization of Src-Vac8 chimera on the vacuole membrane was established, I asked whether this construct was able to complement the function of Vac8. Firstly, the *in vitro* fusion of vacuoles was analyzed. Vacuoles were purified from two tester strains and measured the *in vitro* content mixing of vacuoles. Src-Vac8 showed a reduced fusion, similar to the fusion of cys- mutant. This clearly indicates that Src-Vac8 does not support vacuole fusion (Figure 23A). Secondly, vacuole morphology and inheritance was observed using the lipophilic dye FM4-64. Wild-type strain have one to three large round vacuoles and showed vacuole inheritance from mother to daughter cells. Src-Vac8 has multilobed vacuoles and showed the inheritance defect, similar to the *vac8Δ* and Cys- mutants (Figure 23B). Thirdly, Src-Vac8 is functional in the cytosol-to-vacuole transport pathway, an autophagy like process in yeast that depends on a palmitoylation-independent function of Vac8. Here, the inactive precursor aminopeptidase 1 (prApe1) is matured into active aminopeptidase 1 (Ape1) at the vacuole in cells expressing Src-Vac8 (Figure 24C). These results indicate that Src SH4 domain localizes the Vac8 on the vacuole but does not support Vac8 fusion and inheritance.

The inability of the Src-Vac8 chimera to complement Vac8 function could be due to its membrane binding efficiency. To assess the physical interaction of Src-Vac8 on the membrane, I used the partitioning experiment (Figure 24D). Cells were fractionated into soluble and membrane fractions and each fraction were subjected to TX-114 detergents. Upon warming to 30°C and centrifugation at room temperature, separates into aqueous phase and detergent phase.

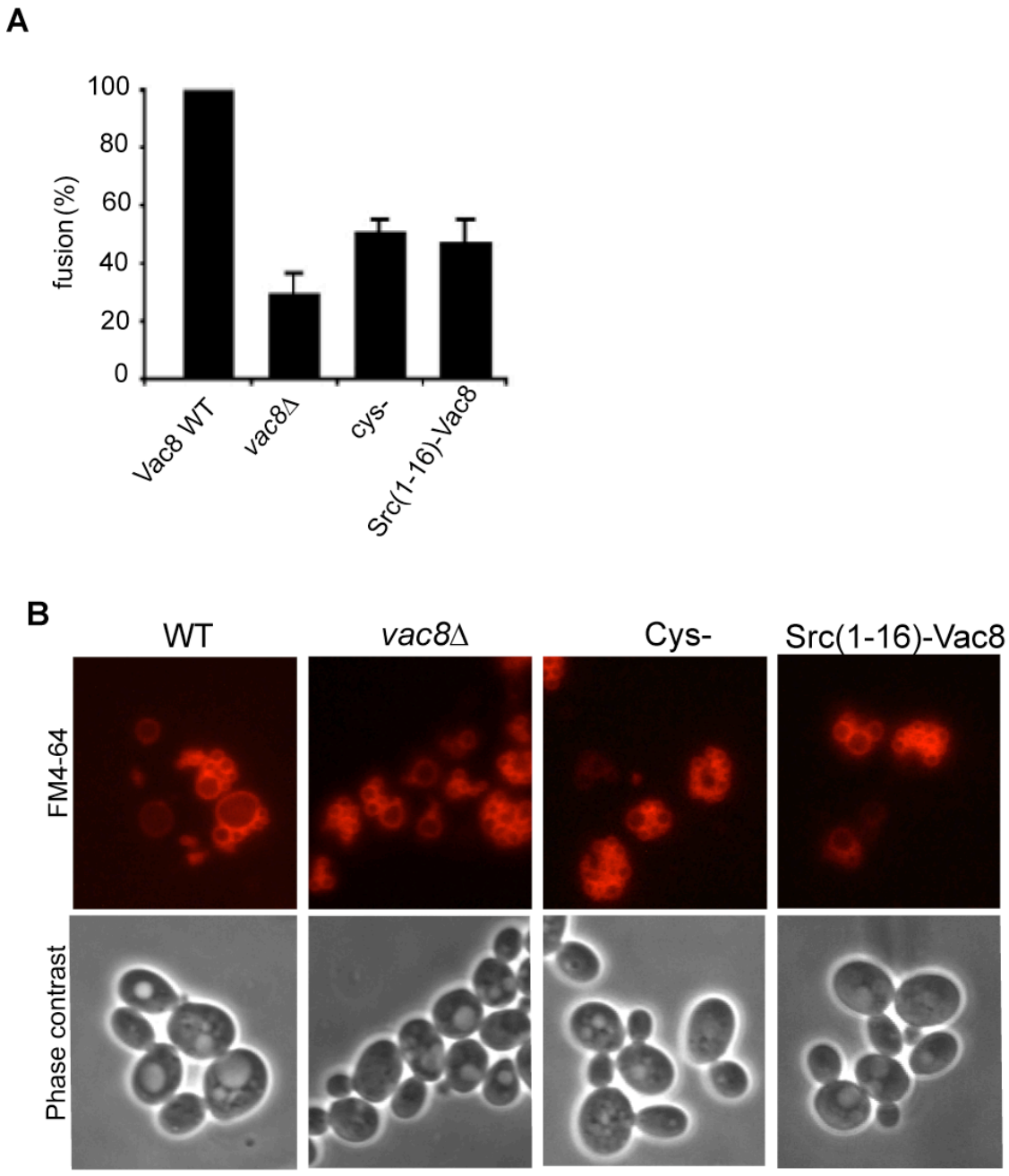

Figure 23. Palmitoylation-dependent function of Vac8

(A) Vacuole fusion. In vitro fusion assay of vacuoles were performed on the indicated mutant strains as described in Figure 20B. Fusion values are based on the average of four different experiments and error bars represent the standard deviations. (B) Vacuole inheritance and morphology. Cells carrying their respective Vac8 mutants in the *vac8Δ* were stained with FM4-64 and visualized by fluorescence microscopy.

Vac8 is predominately found in the detergent phase, whereas Src-Vac8 is equally distributed between the aqueous and detergent phase (Figure 24D). However, myristate group adds relatively less hydrophobicity to the Src-Vac8 protein. Mutation of glycine to alanine in Src-Vac8 shifts its localization towards the aqueous phase in the supernatant. This partitioning result showed that Vac8 is more hydrophobic due to its palmitate groups, whereas Src-Vac8 is less hydrophobic and binds electrostatically to membranes.

Furthermore, I asked whether cysteine in the SH4 domain of Src-Vac8 could rescue the function of Vac8. Single cysteine was introduced at position 4 and the construct Src (1-16; S4C)-Vac8 was expressed in the *vac8Δ* strain. Interestingly, Src (1-16; S4C)-Vac8 rescues the wild type vacuole morphology and inheritance (Figure 24A). Vacuole inheritance was quantified and compared with wild type and different Vac8 mutants (Figure 24B). From these data, I conclude that palmitoylation not only anchors Vac8 but also determines the functions of Vac8 at the vacuole.

Vac8 function depends on its binding partners

Vac8 needs to interact with specific proteins for vacuole fusion, inheritance, and the formation of nuclear-vacuole junction. For vacuole inheritance, Vac8 binds to the myosin protein Myo2 through vacuole-specific myosin receptor Vac17 and drives the vacuoles to the bud (Tang et al., 2003). Vac8 interacts with nuclear protein Nvj1 and maintains the nuclear-junction (Pan et al., 2000). Vac8 is required for vacuole fusion, but only limited knowledge is known at the molecular level. In order to investigate the interacting partners, Vac8 was purified via pull down from vacuoles and the proteins were analyzed by mass spectrometry (Figure 25). I found all the known Vac8 interacting proteins along with vacuolar

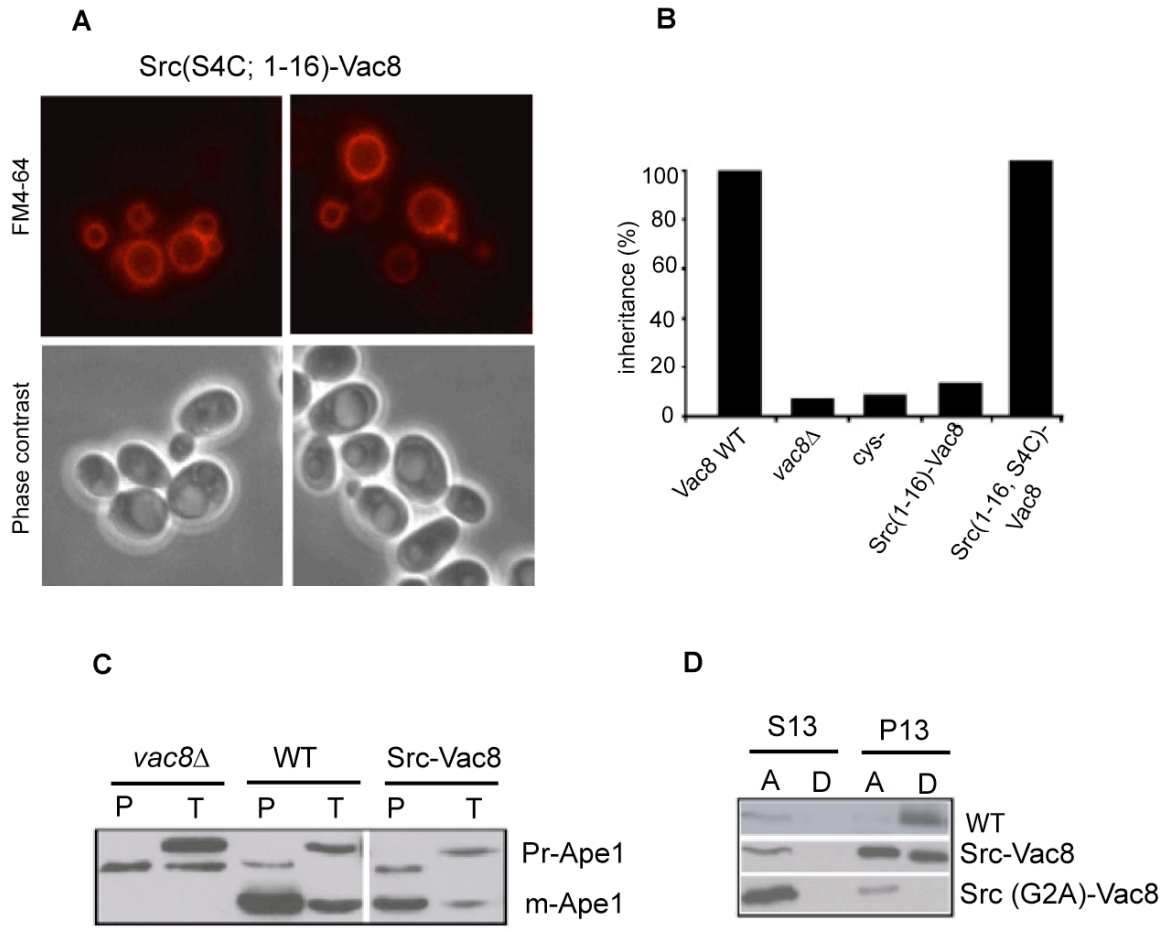

Figure 24. Membrane association and inheritance of Src-Vac8 mutants

(A) Vacuole inheritance and morphology of Src (1-16; S4C)-Vac8 mutant. (B) Vacuole inheritance of the respective mutants of Vac8 were quantified and compared with wild-type cells. Inheritance of wild-type control was on average 68%, which was set to 100% in the figure. (C) AP-1 maturation assay. Subcellular fractionation of the indicated cells, into total (T) and pellet (P) fractions and analyzed the proteins in SDS-PAGE and Western blotting with Ape 1 antibodies. Pr- precursor form protein; m- mature protein. (D) TX-114 partitioning of Vac8 and Src-Vac8 cells. To analyze the hydrophobicity of the proteins, Vac8 and Src-Vac8 cells were fractionated into supernatant (S) and pellet (P) fractions and treated with Triton X-114 detergent to separate into aqueous phase (A) and detergent phase (D). Proteins were analyzed by SDS-PAGE and Western blotting with Vac8 antibodies.

sorting proteins Vps30, Vps38 and enolase Eno1 and Eno2. Enolase is a cytosolic glycolytic enzyme, but small portion of enolase is bound to vacuoles. Deletion of *ENO1* or diminished expression of *ENO2* causes vacuole fragmentation, indicating that the protein is required for homotypic vacuole fusion (Decker and Wickner, 2006). However, a detailed study of Vac8 is required to understand the function of Vac8 in vacuole fusion.

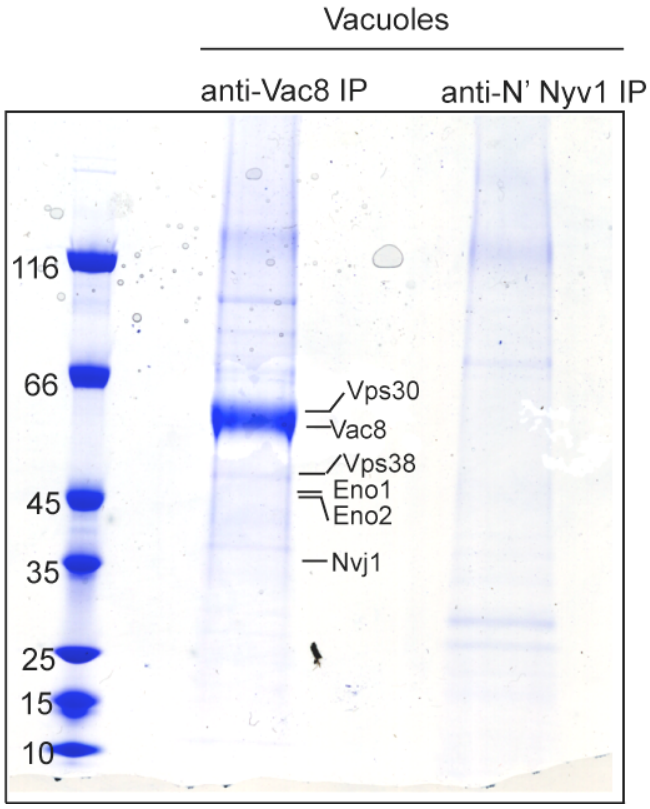

Figure 25. Mass spectrometry analysis of Vac8

To analyze the interacting partners of Vac8, vacuoles were isolated from BJ3505 wild-type strain in large scale and lysed the vacuoles with 1% octoglucoside and centrifuged at 14,000 rpm for 15 min. Lysed vacuoles were incubated with Protein A-agarose beads coupled with anti-Vac8 antibodies and eluted with 0.1 M glycine pH 2.6 and precipitated the proteins with tricholoroacetic acid. As a negative control, same amount of lysed vacuoles were incubated with Protein A-agarose beads coupled to an antibody to the N-terminal region of Nyv1. Proteins were loaded in the 4-12% gradient SDS-PAGE, stained with colloidal staining solution and proteins were analyzed by Mass spectrophotometry.

Table 6. Summary of data from this study

	Binding				
	in vitro	in vivo	Fusion	Inheritance	Wild-type morphology
WT	+	+	+	+	+
Cys-	–	–	-/+	–	–
Cys4 or 5	-/+	-/+	+	+	+
Cys7	–	–	-/+	–	–
Cys 4,5*	+	+	+	+	+
Src(1-16)	+	+	-/+	–	–
Src (S4C)	ND	+	ND	+	+

IV.2 The DHHC protein Pfa3 regulates Vac8 palmitoylation and function

Since the discovery of palmitoylation, the mechanism of the reaction is remained elusive due to the lack of candidate proteins. The palmitoylation can occur either by autoacylation or mediated by protein acyltransferases. Earlier studies showed that the cysteinyl-containing proteins and peptides get autoacylated *in vitro* in the presence of palmitoyl-CoA. This led to the hypothesis that the acylation in cells was spontaneous and was driven by local acyl-CoA concentrations (Bano et al., 1998). In addition, further studies demonstrated the palmitoylation of proteins that occurs non-enzymatically *in vitro*, through direct chemical reaction of acceptor thiols with palmitoyl-CoA (Veit, 2000). However, recent studies in yeast *S.cerevisiae* has provided compelling evidence that the palmitoylation of many proteins is mediated by the family of proteins (Lobo et al., 2002).

The family of protein acyltransferase consists of cysteine-rich domain with a characteristic DHHC motif. Proteins with DHHC-CRD motif are conserved from yeast to mammals. In yeast there are seven DHHC proteins (Akr1, Akr2, Erf2, Pfa3, Pfa4, Pfa5, and Swf1) that appear to be distributed throughout in the endomembrane system (Mitchell et al., 2006). In mammalian cells, 23 DHHC proteins have been identified and they overlap with each other in their functions (Fukata et al., 2004).

Here, I studied the effect of DHHC proteins on the protein palmitoylation at the yeast vacuole.

Results

Localization of Vac8 depends on vacuolar DHHC protein Pfa3

The DHHC protein Pfa3 is localized on the vacuole membrane and its catalytic DHHC motif is at the cytoplasmic phase of the membrane. I asked whether vacuolar palmitoylated proteins depend on any DHHC proteins for their localization. For my studies, I analyzed the localization of Vac8, Yck3 and Meh1 by subcellular fractionation or fluorescence microscopy. Vac8 is palmitoylated up to three cysteines at the N-terminal SH4 region. Palmitoylation is required for

Vac8 localization and function at the vacuole (Wang et al., 1998). The CK1 isoform Yck3 is anchored through the palmitoylation of a cysteine string-like motif is involved in the regulation of vacuole fusion (Sun et al., 2004). Recently, a vacuolar protein Meh1/Ego1 has been identified as a palmitoylated protein, involved in microautophagy (Dubouloz et al., 2005; Gao et al., 2005). The SNARE protein, Ykt6 is palmitoylated at the C-terminal region of the CaaX motif mediated by its own palmitoltransferase activity (Dietrich et al., 2004; Fukasawa et al., 2004).

Cells were fractionated into supernatant and pellet fractions. In wild type cells, Vac8 is localized in the pellet fraction, indicating vacuolar localization, whereas in *pfa3Δ*, Vac8 is found in the supernatant fractions (Figure 26B). GFP fusion protein of Yck3 is localized on the vacuole in all DHHC deletion strains and showed no defect in its localization (Figure 26C,D). Similarly vacuolar protein Meh1/Ego3 has no effect in localization (data not shown).

In our fractionation studies, we observed that Vac8 showed a stronger localization defect in the EUROSCARF background strain (BY4741). We deleted the Pfa3 in the *pep4Δ* (protease-deficient) background strain in which Vac8 is partially localized on the membrane (Figure 26A). A similar localization of Vac8 was also observed in another tester strain DKY6281 (data not shown). For our further studies, we used the protease deficient BJ strain for Vac8 localization, fusion and inheritance.

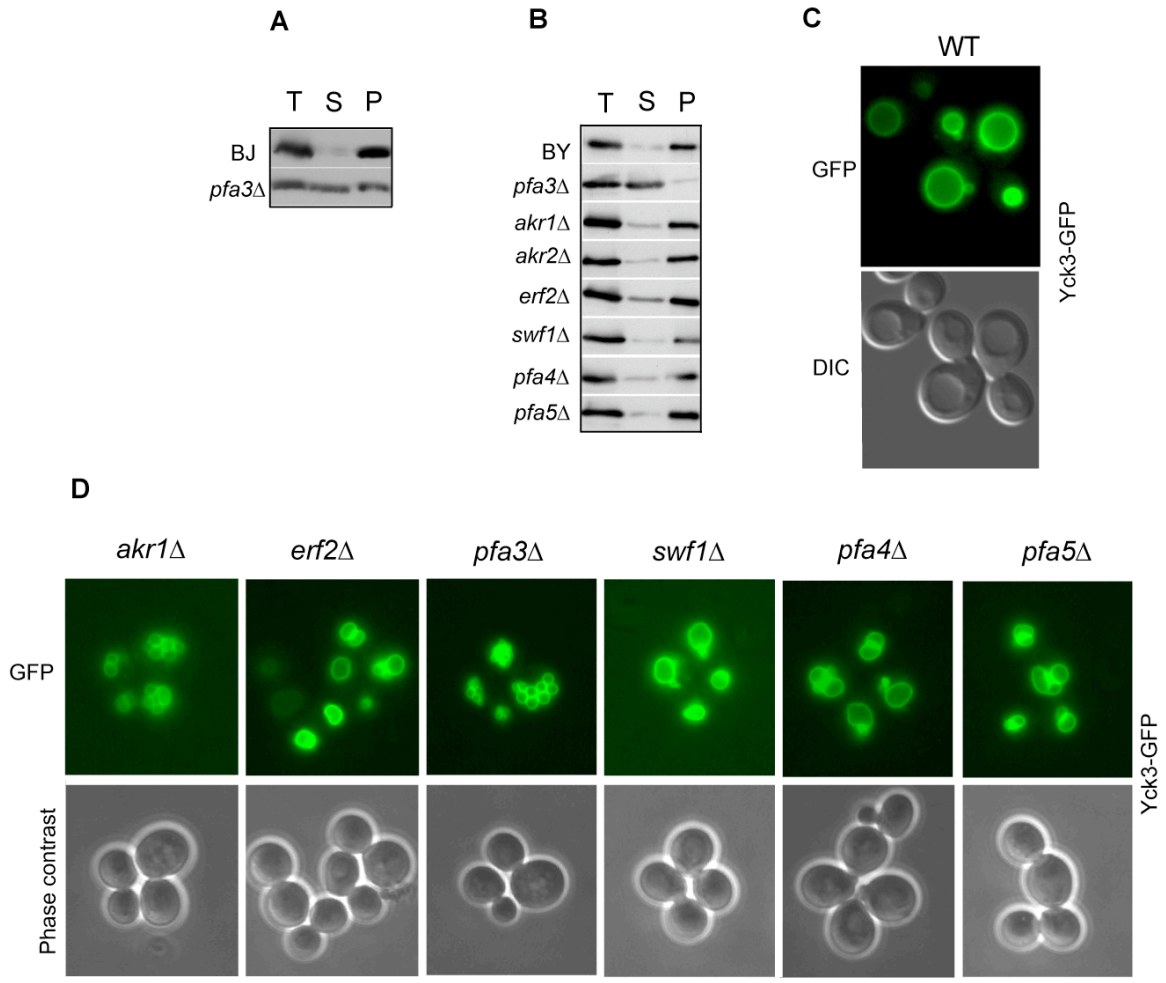

Figure 26. Analysis of DHHC mutants for Vac8 and Yck3 localization

(A) Subcellular localization. Cells were fractionated from wild-type BJ3505 strain and BJ3505 *pfa3Δ* cells into supernatants (S) and pellet (P) fractions and analyzed by SDS-PAGE and western blotting with Vac8 antibodies. (B) The DHHC mutant strains from the deletion library EUROSCARF were fractionated as described in A. (C,D) Localization of Yck3 in DHHC mutant strains. Fusion protein of Yck3-GFP is expressed in all DHHC mutant strains and observed by fluorescence microscopy.

I observed the localization of Vac8 in both BJ3505 wild type and *pfa3Δ* cells under fluorescence microscopy. Fusion protein of Vac8 with GFP is localized to the vacuole in wild-type cells but mislocalizes in *pfa3Δ* cells (Figure 27A, B)

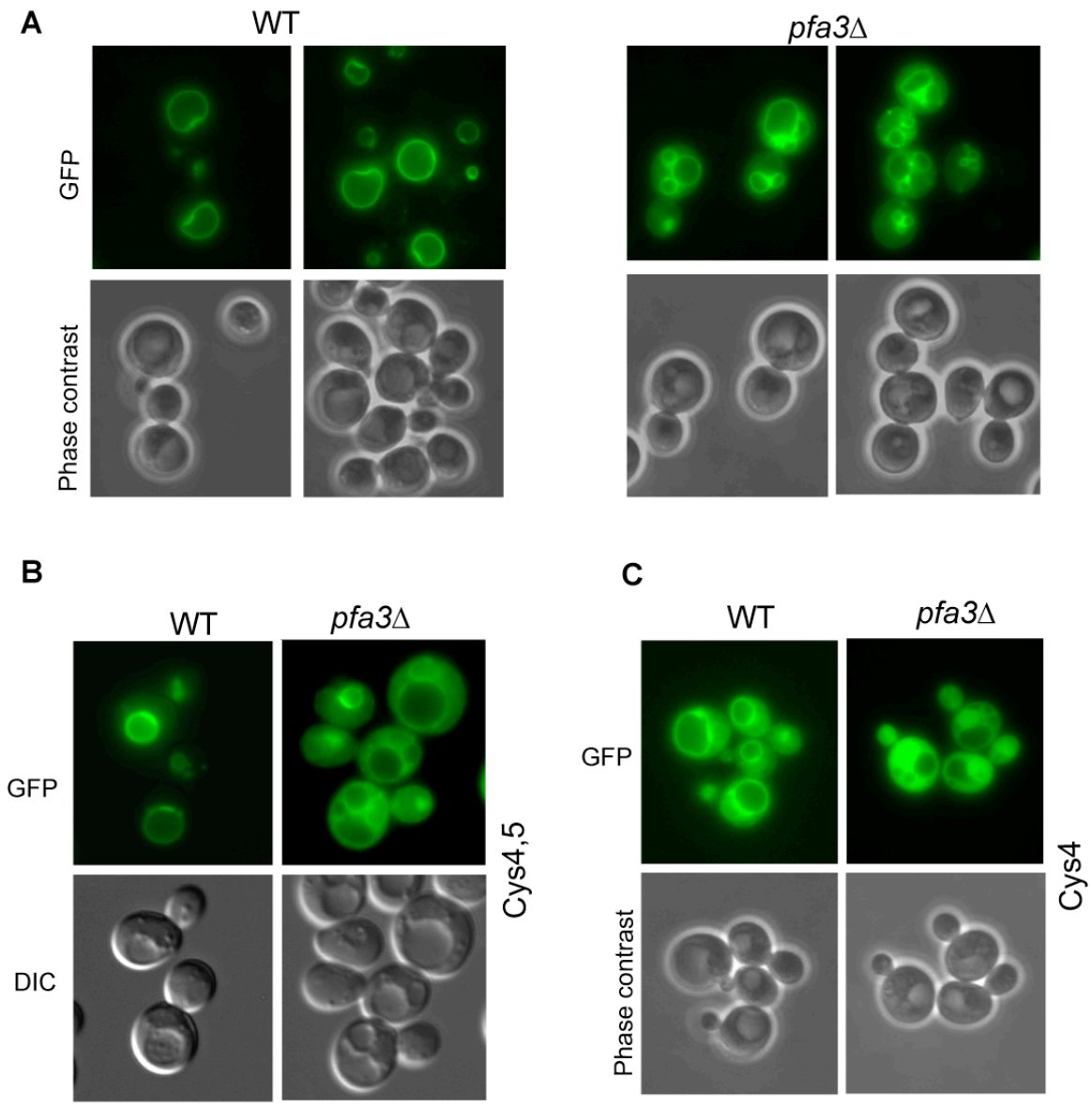

Figure 27. Effect of Pfa3 in Vac8 localization

(A) In vivo localization of Vac8. GFP fusion of Vac8 is expressed in BJ3505 wild type and *pfa3Δ* cells and analyzed by the fluorescence microscopy. (B, C) Localization of Vac8 mutants Cys4 and Cys4,5 were also observed by fluorescence microscopy.

According to the study from Linder and colleagues, Pfa3 is a protein acyltransferase for the palmitoylation of Vac8. Mutation of the cysteine residue in the catalytic DHHC motif abolishes the protein acyltransferase activity of Pfa3, therefore no palmitoylation of Vac8 is observed (Smotrys et al., 2005).

Further, I analyzed the localization of Vac8 mutants expressing single and double cysteines in the SH4 domain. The single cysteine Vac8 Cys4-GFP is partially localized on the wild-type cells but shows completely cytosolic staining in *pfa3Δ* strains (Figure 27C). Vac8 with two cysteines (Vac8Cys4,5-GFP) is mislocalized like wild-type Vac8 in *pfa3Δ* cells (Figure 27B). This result suggests that the DHHC protein Pfa3 affects only the localization of Vac8, but no other palmitoylated proteins at the vacuole.

Pfa3 affects the palmitoylation of Vac8

My study confirmed the mislocalization of Vac8 in *pfa3Δ* cells. Next, the palmitoylation of endogenous Vac8 was detected in *pfa3Δ* cells using biotin-switch method (Figure 28A). I observed that Vac8 was pulled down more efficiently in the wild-type cells, whereas in the absence of Pfa3, the Vac8 was not palmitoylated. However, the result contradicts the fractionation or fluorescence data in which a significant portion of Vac8 is localized in *pfa3Δ* cells. It may be due to protein-protein interactions or by palmitoylation mediated by other DHHC proteins. Another possibility is that the thioesterase enzyme may become active and depalmitoylate during cell lysis in the biotin-switch method. Membrane extraction of Vac8 from both wild type and *pfa3Δ* cells with carbonate, urea, or salt showed that 50% of Vac8 was associated with the membrane. Vac8 is released upon treatment with Triton X-100 detergent; it behaves like hydrophobic and palmitoylated protein in *pfa3Δ* cells (Hou et al., 2005).

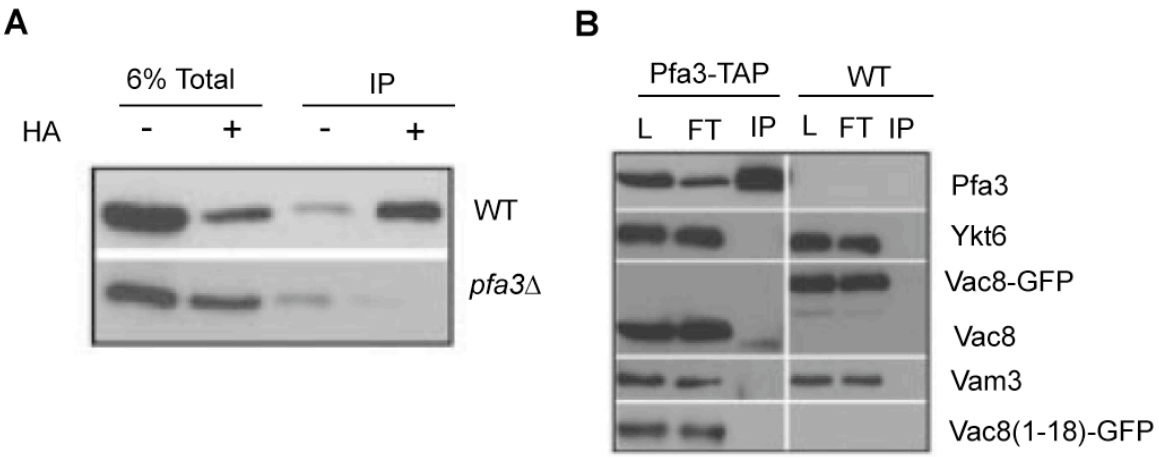

Figure 28. Pfa3 is required for Vac8 palmitoylation

(A) Palmitoylation of Vac8 is detected by Biotin-Switch assay. To detect palmitoylation, cells were lysed, free cysteines were quenched with *N*-ethylmaleimide, cleaved the thioester linked palmitate with hydroxylamine (HA), and crosslinked with BMCC crosslinker. The biotinylated proteins were captured by Neutravidin beads. A 6% total was removed, before incubated the modified proteins with the Neutravidin beads (pierce). Proteins were eluted by boiling at 95° C for 5 min and analyzed by SDS-PAGE and western blotting with anti-Vac8 antibodies. (B) Physical interactions of Pfa3. BJ Pfa3-TAP strain expressing Vac8 (1-18)-GFP of 20 OD cells were lysed with glass beads and precleared the lysates at 300 x g for 5 min. Around one milligram of cell lysate was added with equal volume of lysis buffer (20 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.5% Triton X-100, 1X PIC), insoluble material was removed by centrifugation (20,000 X g for 10 min at 4°C), the detergent extract was incubated with IgG beads overnight at 4°C. Load (L) and flow-through (FT) (1% each) were removed, and proteins were TCA precipitated. Washed the beads two times with lysis buffer and eluted by boiling and analyzed by SDS-PAGE and western blotting. BJ *vac8Δ* cells expressing Vac8-GFP was used as negative control. The lower band in the Vac8 is due to the Pfa3-TAP signal, which runs at a similar molecular weight.

Role of pfa3 in vacuole inheritance and fusion

Vac8 is essential for vacuole fusion and inheritance. Deletion of Vac8 or mutating all the cysteines in the SH4 domain leads to the fragmentation of vacuoles and inheritance defect. In the absence of Pfa3 vacuoles were round and showed only 10-20% defect in the inheritance, compared to the wild type cell (Hou et al., 2005). Vac8 needs palmitoylation for vacuole inheritance. How could Vac8

support vacuole inheritance in the absence of Pfa3? The palmitoylation of Vac8 can be mediated by other DHHC proteins or the SNARE Ykt6 that compensate the absence of the Pfa3 at the vacuole.

In vitro vacuole fusion assay showed the reduction in fusion to 50% equivalent to the Cys- mutant of Vac8. However, a Vac8 mutant with a single cysteine (Vac8Cys4 or Cys5) is partially localized on the vacuole, is functional and supports the vacuole fusion (Subramanian et al., 2006). It has been shown that deletion of both Pfa3 and Swf1 or stressed *pfa3Δ* cells have phenotype of fragmented vacuoles, due to the vacuole fusion defect (Smotrys et al., 2005). This suggests that the deletion of Pfa3 may likely affect other proteins involved in vacuole fusion (Hou et al., 2005).

Pfa3 does not interact with Vac8

To analyze the physical interaction of Pfa3, protein-A tagged Pfa3 was expressed in Vac8 SH4-GFP strains (Figure 24B). Immunoprecipitation was performed from the detergent lysed cell lysate. Western blotting of Pfa3-TAP preparation demonstrated that Pfa3 did not show any interaction with any vacuolar proteins Ykt6, Vam3, and Vac8. Thus, modification of Vac8 or regulation of Pfa3 may be mediated by the transient interactions of vacuolar proteins. Also, suggesting that palmitoylation of Vac8 occurred independently of Ykt6.

Role of pfa3 in sorting of SH4 domain to the vacuole

Having established that Pfa3 is an important factor for Vac8 palmitoylation and function, I asked whether it regulates the localization or palmitoylation machinery of SH4 domain. The SH4 domain is the minimal targeting motif to the membranes. Studies in mammalian cells revealed that the SH4 is localized to the plasma membrane and endosomes (McCabe and Berthiaume, 1999).

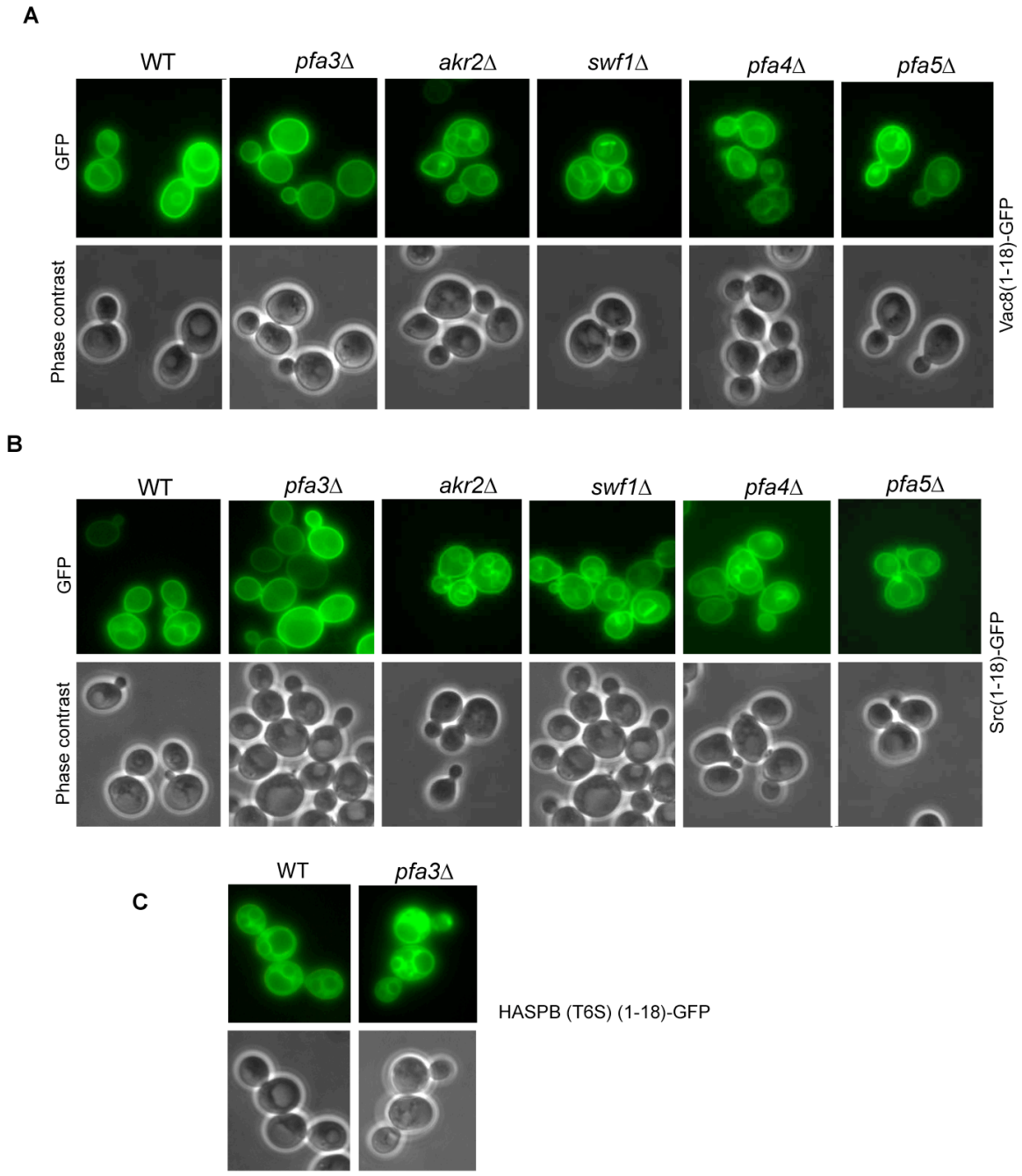

Figure 29. Localization of GFP with minimal targeting constructs in yeast

(A) Vac8 (1-18)-GFP (B) Src (1-16)-GFP constructs were expressed in wild type or all DHHC deletion strains and observed their localization by fluorescence microscopy. (C) HASPB (T6S; 1-18)-GFP is the SH4 domain protein from *Leishmania* served as a negative control, which shows no defect in its localization.

The N-terminal region of Vac8 and Src kinase consists of a characteristic SH4 domain of 16-18 amino acids, which includes a myristoylation motif, linked to palmitoylated cysteines or polybasic residues. To analyze the effect of Pfa3, both Vac8 (myristate/palmitate) and Src (myristate/polybasic), SH4 domains were fused with GFP and expressed in all the DHHC mutant strains (Figure 29A, B). Both fusion proteins [Vac8(1-8)-GFP, Src(1-16)-GFP] localize to the plasma membrane and to the internal organelle membranes in wild type and other DHHC mutant strains. Interestingly in the absence of Pfa3, both fusion proteins were localized exclusively at the plasma membrane. This suggests that Pfa3 is required for localization of both fusion proteins to the vacuole and acts as a vacuole-sorting factor.

Another SH4 domain protein from *Leishmania major* highly acylated surface protein B (HASPB) consists of a myristoylation and palmitoylation sites. In mammalian cells, HASPB is palmitoylated and localized to the plasma membrane. I used the HASPB SH4 domain for my studies and found one of the mutant HB(T6S) (HASPB with a T6S mutation allows for myristoylation) showed intracellular and plasma membrane localization. HB(T6S) does not show any defect in the *pfa3Δ* mutant cells (Figure 29C).

All cysteine-containing SH4 domains are palmitoylated *in vivo* irrespective of their membrane localization (Figure 30A). Pfa3 recognizes specific amino acids or motifs in the Vac8 and Src sequences, which are absent in HASPB (Figure 30B). From this, I conclude that Pfa3 attributes the localization of the minimal sorting motif to the vacuole membrane

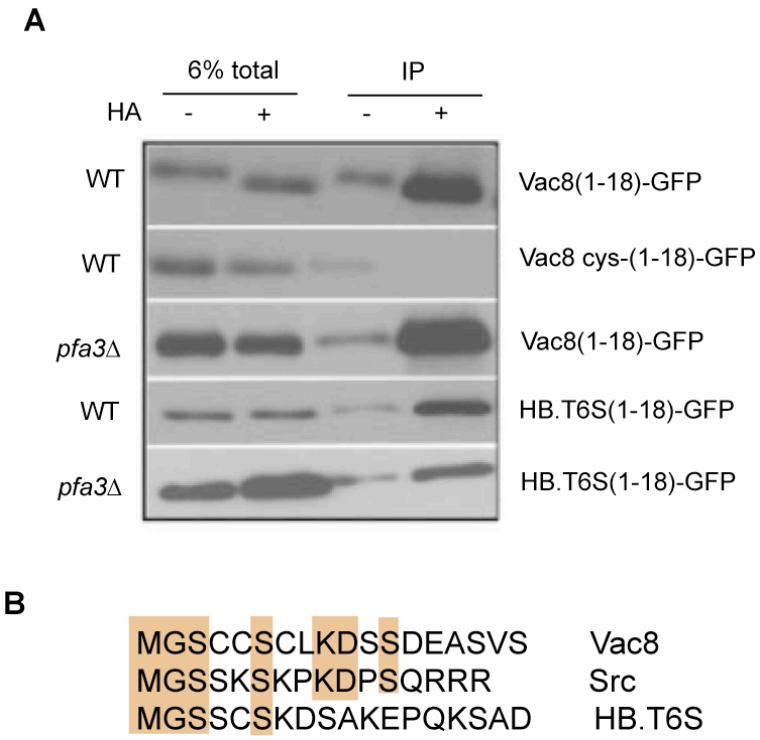

Figure 30. Palmitoylation of SH4 domain constructs

(A) Biotin-switch assay was performed to detect the palmitoylation of the indicated SH4 domain constructs in WT and *pfa3Δ* cells. Total of 6% was saved as the loading control and analyzed by SDS-PAGE and western blotting with anti-GFP antibodies. Refer Methods and Materials. (B) Alignment of the N-terminal 15 amino acids of the Vac8, Src, and HASP (T6S) sequence.

IV.3 Analysis of SH4 domains and membrane targeting

Lipidation allows the anchoring of proteins to the membrane. Most of the proteins involved in the signaling cascade are modified by the combinations of covalently attached fatty acids or polybasic amino acids at their N-terminal region. For example, Src-related protein tyrosine kinases (Koegl et al., 1994), G protein α subunits (Resh, 1999), endothelial nitric oxide synthase (eNOS), and A-kinase-anchoring protein AKAP18 (Fraser et al., 1998) are modified by myristate and palmitate at their N-terminal region. Some proteins contain myristate and a polybasic domain that have been described in protein tyrosine kinase Src and Blk, MARCKS, and HIV gag proteins (Resh, 1999). In addition, two or more covalently linked palmitates at the N-terminal have been found in GAP-43, $G\alpha_q$, and PSD-95 (Dunphy and Linder, 1998; Resh, 1999). In all cases, the stable binding of proteins to the membrane is mediated by two signals. Either myristoylated or farnesylated signals are linked to the second signals for stable membrane association. The second signal could be provided by the palmitate or polybasic domain. The SH4 domain in the N-terminal region harbors the myristoylation and palmitoylation or polybasic residues for membrane attachment of the protein. (Resh, 1999). In eukaryotic cells, the SH4 domain is targeted to plasma membrane and endosomes (McCabe and Berthiaume, 1999). However, the localization of the SH4 domain in the context of a protein is often restricted to one compartment. For example, the SH4 domain in Vac8 is localized on the vacuole (Wang et al., 1998). The SH4 domain in Vac8 consists of a myristoylation and three palmitoylated cysteine residues.

In protozoan parasite *Leishmania major* a family of protein called HASPs (hydrophilic acylated surface proteins) is exported to the extracellular surface via “non-classical pathway”. The HASPB protein is anchored to the plasma membrane with the N-terminal SH4 domain. It has been shown that SH4 domain of HASPB is acylated *in vivo* and is targeted to the plasma membrane. Mutation in the acylation site leads to cytosolic localization (Denny et al., 2000).

In this part of my thesis, I compared the membrane targeting motifs (SH4 domain) of both Vac8 and HASPB in yeast. The membrane targeting mechanism of the SH4 domain is not known clearly. I asked whether the neighboring residues in the acylation motif involved in the targeting of SH4 domain to the membrane. I took advantage of the HASPB SH4 domain for my studies.

SH4 domain is the minimal targeting domain

I analyzed the SH4 domains of both Vac8 and HASPB proteins. Sequence comparisons reveal two common features between them (Figure 31). First is the myristoylation motif (M-G-x-x-C-S/T-x), where a glycine residue is myristoylated and second, the palmitoylation sites. HASPB has only one cysteine at position 5, whereas the Vac8 has three cysteines that are required for its localizations and functions. Here, I split the SH4 domain into acylation motif, followed by the linker region for my studies.

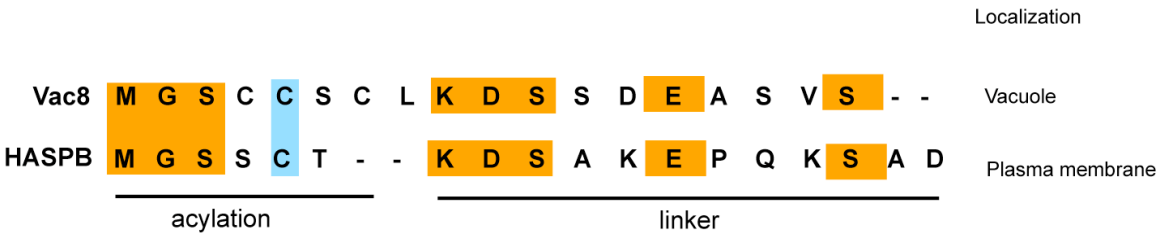

Figure 31. Sequence alignments of SH4 domains of Vac8 and HASPB

Both SH4 domains contain a myristoylation and palmitoylation site (acylation motif) and the linker region for membrane localization. In yeast, Vac8 is localized on the vacuole and HASPB in *Leishmania* localizes to the plasma membrane.

HASPB protein is mislocalized in yeast

SH4 domain is the universal membrane targeting motifs. It drives the protein to the cytoplasmic leaflet of the cellular membranes. First, I compared the localization of SH4 domains in the context of full-length Vac8 and HASPB. To analyze the *in vivo* localization, both Vac8 and HASPB proteins were expressed

in BJ3505 *vac8Δ* cells. GFP was added to the C-terminal region of both proteins. As expected, Vac8 is targeted and localized exclusively to the vacuole membrane. Surprisingly, the HASPB protein is completely mislocalized and shows cytosolic staining (Figure 32A). It indicates that the HASPB SH4 domain is not functional, although it has Vac8 like SH4 domain.

To understand the mechanism of targeting, the SH4 domain of HASPB was replaced with the SH4 domain of Vac8. The chimera Vac8(1-18)-HASPB-GFP is targeted to intracellular membrane and the plasma membrane. Then, I analyzed the localization of Vac8 with HB SH4 domain.

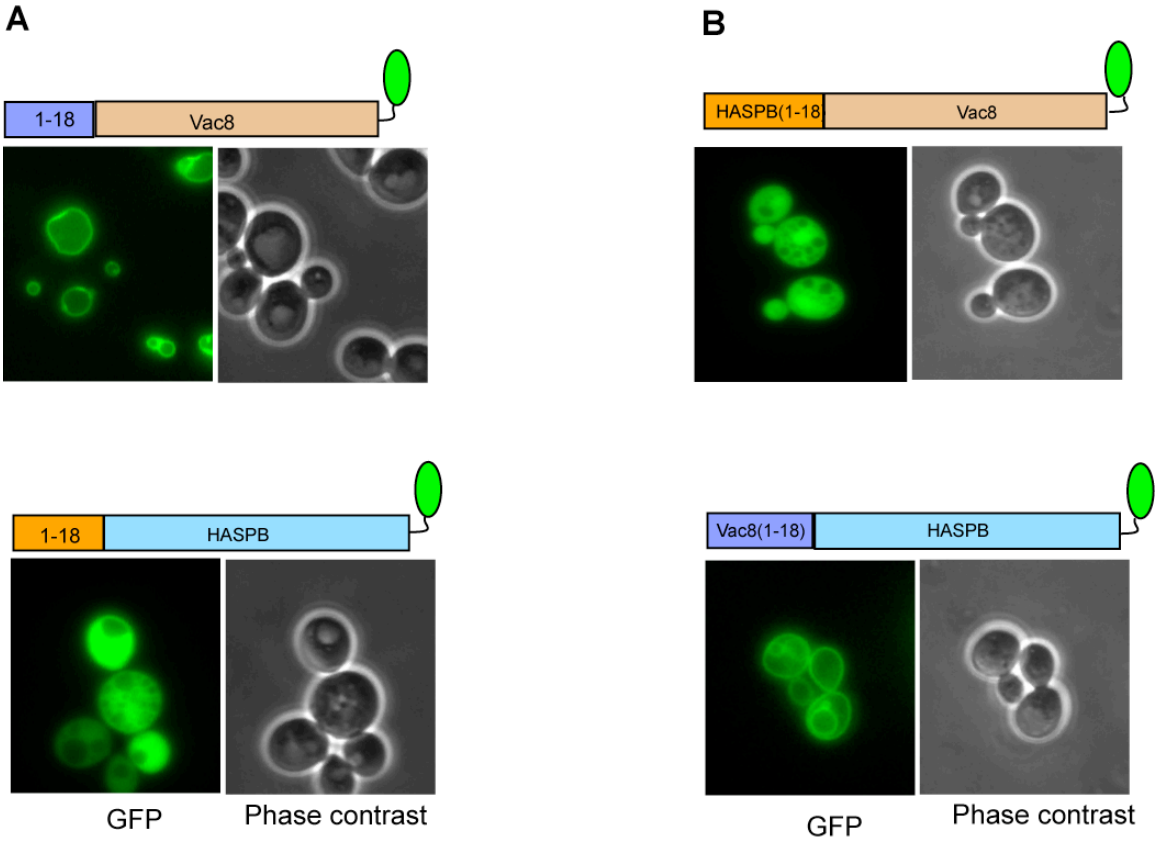

Figure 32. In vivo localization of Vac8 and HASPB fusion protein

(A) Both Vac8-GFP and HASPB-GFP were expressed in *vac8Δ* cells and observed under fluorescence microscopy. (B) The chimeras, HASPB(1-18)-Vac8-GFP and Vac8(1-18)-HASPB-GFP were expressed in *vac8Δ* cells and analyzed by fluorescence microscopy.

The construct HASPB(1-18)-Vac8-GFP is not targeted to the membrane and shows the cytosolic localization (Figure 32B). These result implicate that the HASPB SH4 domain is not recognized by the lipid modification system either myristoylation or palmitoylation in yeast.

Targeting of GFP with SH4 domain

In mammalian cells, the HASPB SH4 domain of 18 amino acids is targeted to the plasma membrane. Here, I analyzed the targeting of SH4 domains from Vac8 and HASPB. GFP fusion protein of both SH4 domains were expressed in BJ *vac8Δ* cells for in vivo localization and observed under the fluorescence microscopy. Vac8(1-18)-GFP is localized to intracellular membranes and the plasma membrane but the HASPB(1-18)-GFP is localized in the cytosol (Figure 33A).

The SH4 domain was split into acylation motif and the linker region. The acylation motif has site for myristoylation and palmitoylation, followed by the linker region. The acylation motif of HASPB(1-10) was replaced by acylation motif of Vac8(1-10) and generated the chimera Vac8(1-10)-HASPB(11-18) which localized to the membranes. The chimera HASPB(1-10)-Vac8(1-18) remained in the cytosol (Figure 33B).

Next I asked whether the number of cysteines in the SH4 domain is required for membrane association. Vac8 SH4 domains with single cysteine fusion proteins, Vac8C4(1-18), Vac8C5(1-18), Vac8C7(1-8) are targeted to the membranes. In this case, localization is independent of the position of the cysteine (see figure 21A). I introduced additional cysteines in the HASPB SH4 domain and observed their localization. Surprisingly, HASPB SH4 domain is still mislocalized to the cytosol (data not shown).

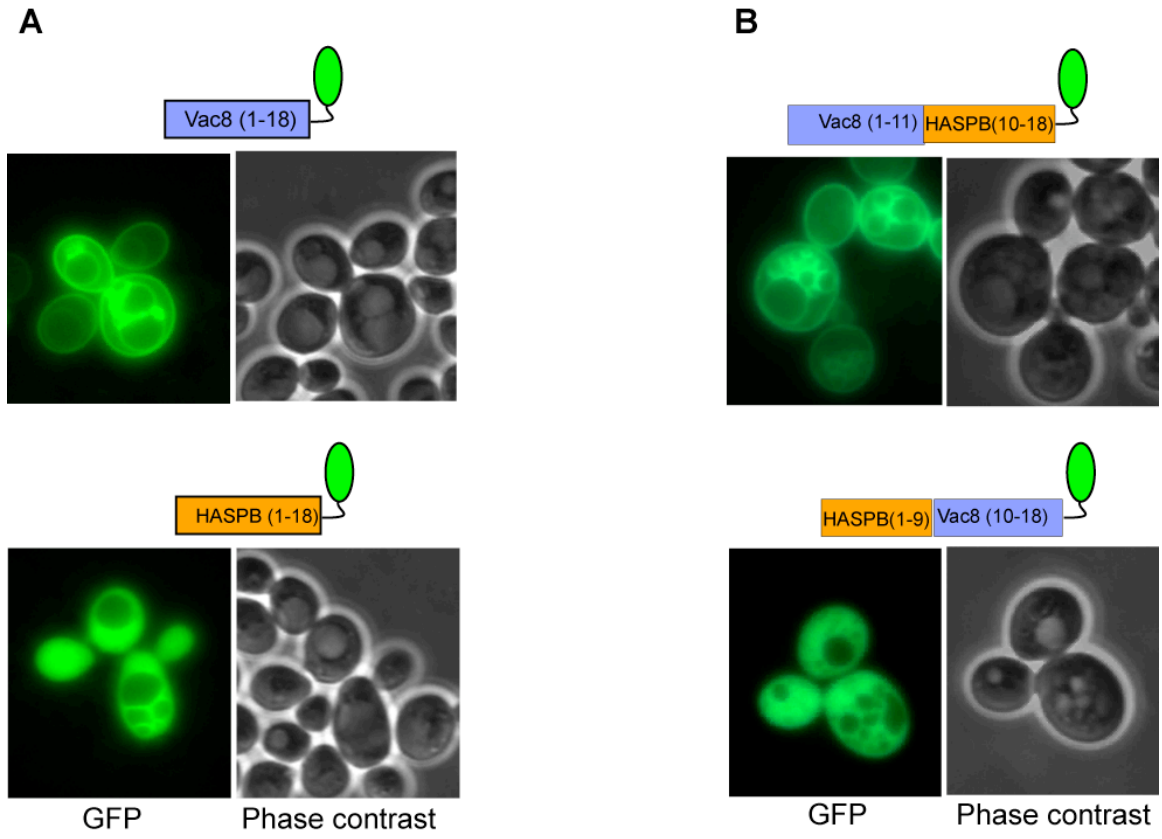

Figure 33. Localization of GFP with SH4 domain of Vac8 and HASPB

(A) GFP fusion protein of both Vac8 and HASPB SH4 domain was expressed in BJ *vac8Δ* cells and analyzed under fluorescence microscopy. (B) Acylation motif of Vac8 is exchanged for the HASPB acylation motif and vice versa and observed under fluorescence microscopy.

HASPB (T6S) is targeted to the membranes

Based on the mutational analysis in HASPB SH4 domain, I found a mutant HASPB(T6S; 1-18) that localized to intracellular membranes and the plasma membrane (Figure 34A). In general, serine or threonine is the important consensus amino acid for the myristoylation (Resh, 1999). In the HASPB (T6S; 1-18) mutant, serine is required for the myristoylation, which favors the palmitoylation of the cysteine amino acid. In the Vac8 SH4 domain, mutating the theronine to serine showed no defect in localization (Figure 34B). This result

indicates that a serine at position 6 is important for HASPB SH4 domain localization.

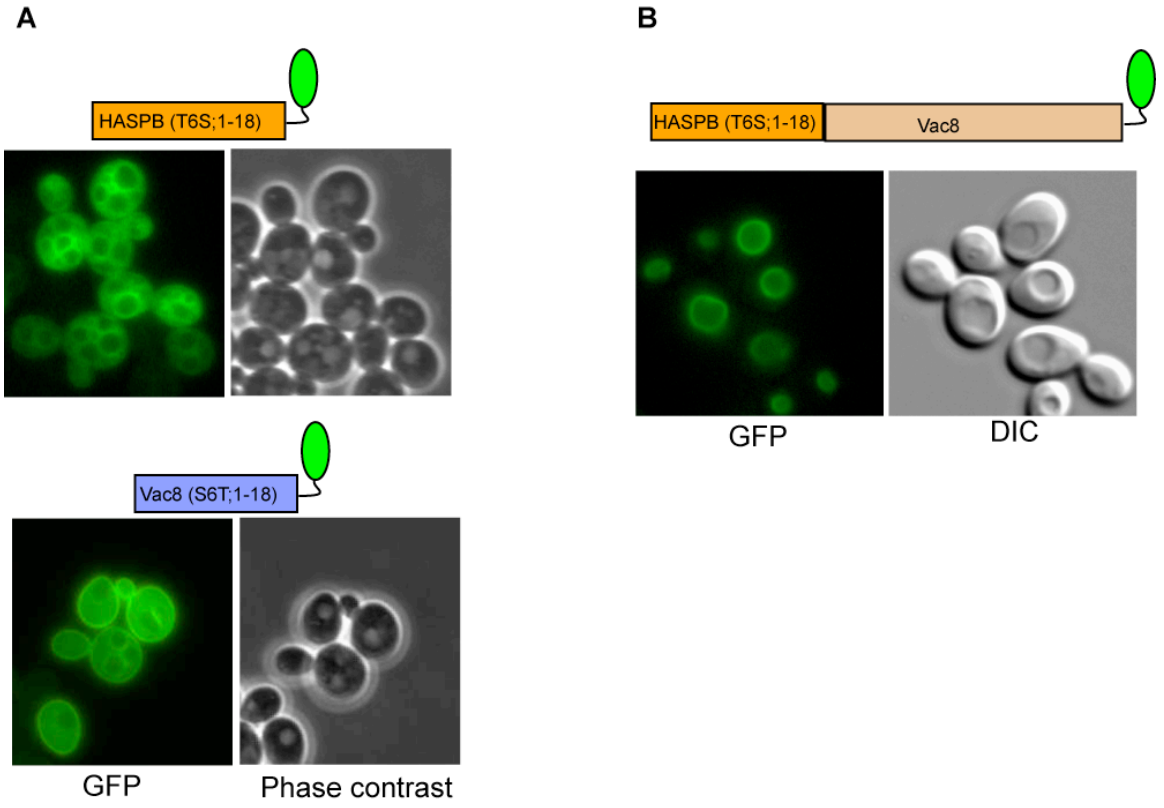

Figure 34. Targeting of HASPB(T6S) to the membrane

(A) Both Vac8 and HASPB SH4 domains were mutated at serine or threonine at position 6 and their localization was analyzed under the fluorescence microscopy. (B) HASPB(T6S; 1-18)-Vac8-GFP chimera was expressed in wild type cells and observed by fluorescence microscopy.

The SH4 domain HASPB(T6S) is palmitoylated

Having established the membrane attachment of HASPB(T6S) was due to palmitoylation. Biotin-switch assay was used to detect the palmitoylation. As expected, HASPB(T6S) mutant showed palmitoylation, consistent with its membrane localization (see Figure 30A). I asked whether the HASPB(T6S) mutant depended on any acyltransferase for its palmitoylation. The HASPB(T6S) SH4 domain was expressed in all DHHC mutant strains; it never showed any

localization defect. Surprisingly the Vac8 and Src kinase SH4 domains required the vacuolar DHHC protein Pfa3 for the vacuolar localization (see Figure 29). They are exclusively localized on the plasma membrane and showed sorting defect to the vacuole (Hou et al., 2005).

Targeting of Vac8 with HASPB(T6S; 1-18)

Vac8 requires at least two cysteines for the stable localization at the vacuole. Mutant Vac8 with single cysteine (Cys4 or Cys5) is partially localized on the vacuole membrane. For my studies, I replaced the SH4 domain of Vac8 with HASPB(T6S) SH4 domain and the chimera HASPB(T6S; 1-18)-Vac8-GFP was expressed in wild-type cells. Unlike Vac8 with single cysteine, the HASPB(T6S; 1-18)-Vac8-GFP construct localizes more efficiently to the vacuole membrane (Figure 35A). This result indicates that HB(T6S; 1-18) domain is functional and able to target the Vac8 to the vacuole.

Having established the localization of HASPB(T6S;1-18)-Vac8-GFP at the vacuole. I asked whether this chimera is dependent on any DHHC protein for its localization. Our earlier studies showed that the DHHC protein Pfa3 affects the palmitoylation and functions of Vac8 (Hou et al., 2005). Vac8 is the potential substrate for the protein acyltransferase activity of Pfa3. In *pfa3Δ* cells, the HASPB(T6S; 1-18)-Vac8-GFP is partially localized to the vacuole membrane (Figure 35B). It is consistent with the localization of Vac8 in *pfa3Δ* cells (Hou et al., 2005). In the strain deletion of four DHHC genes (*akr2Δ pfa3Δ pfa4Δ pfa5Δ*), HASPB(T6S; 1-18)-Vac8-GFP is still partially localized on the vacuole. Interestingly, the disruption of Golg-localized DHHC protein Akr1 in the 4-fold-deleted *akr2Δ pfa3Δ pfa4Δ pfa5Δ* strain affects the localization of HB(T6S; 1-18)-Vac8-GFP more severely and mislocalized from the vacuole. Endogeneous Vac8 also shows complete mislocalization in the five DHHC gene deletions strain (Hou unpublished results).

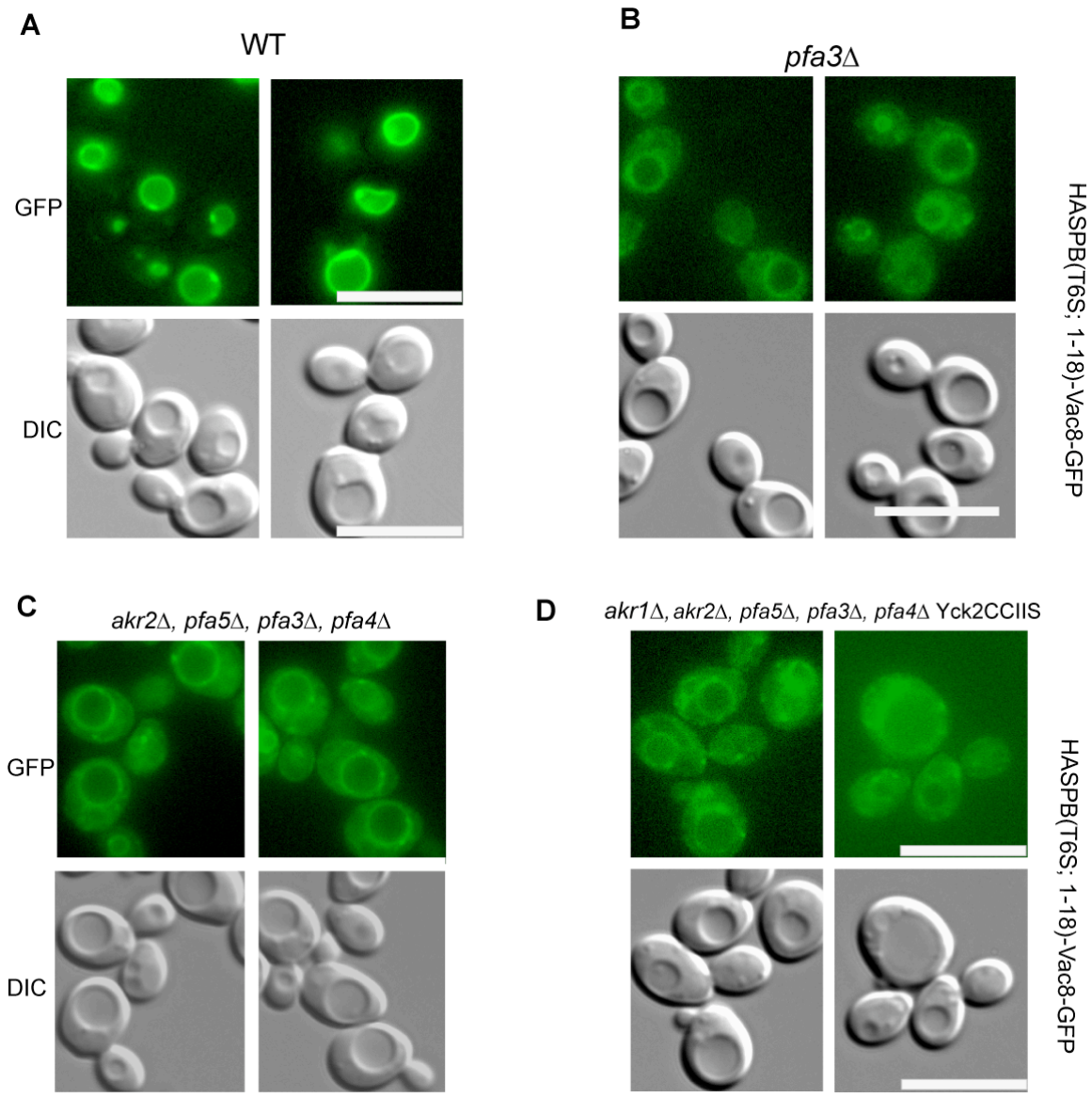

Figure 35. Targeting of Vac8 with HASPB(T6S; 1-18) SH4 domain

The chimera, HASPB(T6S; 1-18)-Vac8-GFP is expressed in wild-type (A) and DHHC mutant strains (B-D), and observed under fluorescence microscopy. Bars 10μm

V. Discussion

V.1 Palmitoylation determines the localization and function of Vac8.

The results show that palmitoylation is required for Vac8 localization and function at the vacuole. Several findings suggest that palmitoylation regulates the functions of the many proteins besides the membrane localization (Lam et al., 2006; Rocks et al., 2005; Valdez-Taubas and Pelham, 2005).

Localization, acylation of Vac8

The yeast vacuolar protein Vac8 is a multifunctional protein, required for vacuole inheritance, morphology, fusion, maintenance of nuclear-vacuole junction and cytosol-to-vacuole transport (CVT) pathway (Wang et al., 1998). All these functions depend on the palmitoylation of Vac8 except the CVT pathway. The N-terminal SH4 region contributes the site for a myristoylation and three palmitoylated cysteines for stable binding to the vacuole.

Why does Vac8 require multiple cysteines for its localization and function even though a single cysteine is sufficient for membrane association? Our mutational analyses demonstrate that the cysteines are differentially involved in the functional regulation of Vac8. Fluorescence microscopy and fractionation experiments revealed that Vac8 with single cysteine mutant (Cys4 or Cys5) binds partially to the vacuole whereas with double cysteines (Cys4,5 or Cys4,7) confers localization to the wild-type level. Surprisingly, we found a mutant; Vac8 Cys7 showed a complete mislocalization and is not palmitoylated. This localization is reminiscent of the localization of influenza hemagglutinin (HA) in that three cysteines are essential for targeting to the detergent resistant membrane (Melkonian, 1999). It is most likely that the Vac8 Cys7 mutant undergoes rapid palmitoylation and depalmitoylation cycles or is not recognized by the palmitoylation machinery mediated by the DHHC protein Pfa3 (Hou et al., 2005) or other protein SNARE Ykt6 (Dietrich et al., 2004). Studies from Weisman group also observed that Vac8 Cys7 was not functional at the vacuole (Peng et al.,

2006). Interestingly, Vac8 Cys7 is localized and palmitoylated, if the proximal cysteines are modified with palmitate.

In addition, my studies confirm the requirement of palmitoylation for Vac8 function. Replacement of the N-terminal SH4 domain of Vac8 (myristate/palmitate) with SH4 domain of Src kinase (myristate/polybasic amino acids) results in a chimera that localized to the vacuole membrane like that of wild-type Vac8. However, the chimera Src-Vac8 is targeted to the vacuole and allows Vac8 function in the CVT pathway but was rendered non-functional at the vacuole (*i.e*. defective in vacuole fusion and inheritance). Most importantly, the introduction of a single cysteine in the SH4 domain of Src-Vac8 chimera is able to rescue the vacuole inheritance and vacuole fusion. Addition of a palmitate may confer the stable localization of Src-Vac8 to the vacuole membrane, which would allow restoring the Vac8 functions. It has been shown that the saturated fatty acyl chains (palmitate) in the modified protein inserts deeply into liquid ordered raft domains at the plasma membrane (Koegl et al., 1994).

How could palmitoylation affect the Vac8 function?

Vac8 interacts with specific proteins for its function (Kvam and Goldfarb, 2004; Scott et al., 2000; Tang et al., 2003; Wang et al., 2001). For example, during vacuole inheritance the palmitoylated Vac8 interacts with vacuole-specific receptor Vac17 that in turn binds to myosin protein Myo2 and drives the vacuoles to the bud via tubular-segregation structure (Tang et al., 2003). Therefore, palmitoylation of Vac8 could affect these interactions, either by forming direct protein-lipid interactions or by inducing conformational changes that permit certain protein-protein interactions. Another possibility is that palmitoylation may bring Vac8 to specific lipid domains on the vacuole membrane. Ergosterol and low levels of sphingolipids could form raft-like regions on the vacuole membrane. It has been shown that ergosterol is an essential factor required for vacuole fusion (Fratti et al., 2004; Kato and Wickner, 2001). In contrast to Vac8, the Src-Vac8 chimera does not have such regulatory mechanism and shows the

phenotype of Vac8 Cys-. Although the Src-Vac8 is properly localized on the vacuole and is functional in the cytosol-to-vacuole transport, the altered SH4 domain might interfere the interaction of Vac8 with other proteins or lipid bilayer.

The Triton X-114 partitioning experiment demonstrated the hydrophobicity of the membrane bound Vac8. The Src-Vac8 chimera was found in both the aqueous and detergent phases, indicating that it interacts more weakly with the lipid layer than Vac8. The partitioning of Src-Vac8 into the aqueous phase could reflect the ability of the Vac8 to accommodate the hydrophobic myristoyl tail within the protein. Such a switch-like mechanism has been discussed for other myristoylated proteins like HIV-Gag (Resh, 2004)

Previous studies showed that Vac8 becomes palmitoylated in the early stage of fusion reaction. During the priming step, SNAREs are released from the vacuole and Vac8 gets palmitoylated and anchors on the vacuole membrane and functions as an important factor for vacuole fusion (Veit et al., 2001; Wang et al., 2001). Interaction of Vac8 with the SNARE protein Vam3 in a complex is required for fusion (Veit et al., 2001). In my studies, I observed that Vac8 interacts with known interacting partners along with Vps30, Vps38, and Enolase 1 and 2. Both Enolase 1 and 2 are involved in vacuole fusion (Decker and Wickner, 2006). However, the functional role of Vac8 in vacuole fusion has not been addressed.

Vac8 is palmitoylated by the novel function of SNARE Ykt6 at the vacuole. The regulatory longin domain at the N-terminal region of Ykt6 promotes the transfer of palmitate from palmitoyl-CoA by a non-enzymatic reaction. Acylation of Vac8 occurs in an ATP-independent, temporally controlled subreaction on vacuoles (Dietrich et al., 2004; Dietrich et al., 2005). The vacuolar DHHC protein Pfa3 has been identified as an important factor for Vac8 localization. Deletion of Pfa3 leads to the partial localization of Vac8 on the vacuole and affects the vacuole fusion reaction (Smotrys et al., 2005). Further studies will require

understanding the mechanism of Vac8 palmitoylation and the role of Vac8 in vacuole fusion.

V.2 The DHHC protein Pfa3 affects the Vac8 functions.

In this study, I show the function of the vacuole-localized DHHC protein Pfa3. Based on the subcellular fractionation and fluorescence microscopy, I found that Pfa3 affects the localization of palmitoylated Vac8 but does not show any effect on other palmitoylated proteins at the vacuole. Studies from Linder and colleagues showed that Pfa3 is the protein acyltransferase for the palmitoylation of Vac8 (Smotrys et al., 2005). In the absence of Pfa3, Vac8 is partially mislocalized to the cytosol. However, some Vac8 resides on the vacuole membrane in *pfa3Δ* cells, suggesting that other factors could mediate the palmitoylation of Vac8. It has been shown that the SNARE Ykt6 promotes Vac8 palmitoylation in a non-enzymatic reaction (Dietrich et al., 2004). The N-terminal *longin* domain of Ykt6 binds palm-CoA through non-covalent interaction and transfers it to the Vac8. Addition of α-Ykt6 or the N-terminal *longin* antibodies in vacuole fusion assays blocks both membrane fusion and Vac8 palmitoylation (Dietrich et al., 2004). However, there is no direct *in vivo* evidence for the palmitoylation of Vac8 by the SNARE Ykt6. Recent studies indicate that the palmitoylation of Vac8 could be mediated by other DHHC proteins (Roth et al., 2006)

Vacuole inheritance and fusion are the important functions of Vac8. These functions depend on the palmitoylation of Vac8 (Subramanian et al., 2006; Wang et al., 1998). In the absence of Pfa3, cells do not have a vacuole inheritance defect and showed normal wild type vacuole morphology. This suggests that a decreased amount of Vac8 palmitoylation does not affect vacuole inheritance (Hou et al., 2005). This result is consistent with the vacuolar inheritance of cells expressing Vac8 with single cysteine mutant (Cys4 or 5). These mutants are partially localized on the vacuole, able to support vacuole inheritance. Studies

from Weisman group showed that the Cys4 or Cys5 mutant of Vac8 was functional at the vacuole (Peng et al., 2006). The fragmented vacuole phenotype was observed in the *pfa3Δ* cells only under stressful conditions or in the *pfa3Δswf1Δ* cells (Smotrys et al., 2005). Swf1 is the ER localized DHHC protein required for the palmitoylation of the SNARE proteins Snc1, Syn8, and Tlg1 (Valdez-Taubas and Pelham, 2005). Vacuole fusion is another function of Vac8. The fusion of vacuoles in *pfa3Δ* cells is reduced to 50%, consistent with the defective localization of Vac8 on the vacuole (Hou et al., 2005). It is most likely that deletion of Pfa3 affects additional proteins required for vacuole fusion.

Do the DHHC proteins overlap functionally?

The occurrence of protein palmitoylation is not restricted to the final destination compartment. The proteins are palmitoylated at one compartment and targeted to the final compartment for their function. ER-localized Erf2 palmitoylates Ras2, which is found at the Golgi and the plasma membrane, Pfa4 palmitoylates the casein kinase Chs3 at ER and is targeted to plasma membrane, Golgi-localized Akr1 modifies the plasma membrane Yck2, Swf1 palmitoylates the endosomal SNARE Tlg2 at ER (Bartels et al., 1999; Lam et al., 2006; Roth et al., 2002; Valdez-Taubas and Pelham, 2005). The presence of DHHC proteins at multiple organelles provides protein acyltransferase activity for the specific substrate. For example, palmitoylation of amino acid permeases Tat1 is abolished when the gene encoding the ER-localized Pfa4 is deleted. In this case, palmitoylation of amino acid permeases is fully depend on Pfa4 (Roth et al., 2006). This is not true for all palmitoylated proteins. Some palmitoylated proteins might rely on the actions of multiple, overlapping functions of DHHC proteins. For example, the Erf2 substrate Ras2 show reduced palmitoylation and partial localization on the membrane in *erf2Δ* cells (Bartels et al., 1999). The localization of Ras2 is not further reduced even when five of the six other DHHC proteins are deleted (Roth et al., 2006). These findings imply that the residual palmitoylation of the protein is due to the following: (i) Enzymatic-redundancy exists in the DHHC-CRD family proteins; (ii) non-DHHC proteins may function as

PATs in yeast; and (iii) nonenzymatic palmitoylation may occur. In my studies I have observed that Vac8 is palmitoylated and partially localized on the vacuoles in *pfa3Δ* cells. This data is consistent with the detection of residual palmitoylation of Vac8 by metabolic labeling with [^{3}H] palmitate in *pfa3Δ* cells (Smotrys et al., 2005). I speculate that residual palmitoylation of Vac8 in *pfa3Δ* cells could be mediated by the SNARE Ykt6 in a non-enzymatic manner (Dietrich et al., 2004) or other DHHC proteins in the endomembrane system. In the absence of Pfa3 along with other DHHC proteins Akr1, Akr2, Pfa4, Pfa5; Vac8 is completely mislocalized, suggesting that the DHHC proteins have overlapping functions (Hou, unpublished data). Overexpression of the Golgi-localized DHHC protein Akr1 in the *pfa3Δ* cells promotes the effective localization of Vac8 to the vacuole (Hou, unpublished data).

In addition, I observed that Pfa3 regulates the sorting of SH4 domain to the vacuole. In the absence of Pfa3, the SH4 domains of Vac8 (myristate/palmitate) and Src (myristate/polybasic amino acids) are localized exclusively to the plasma membrane. This suggests that Pfa3 may act as a vacuole-sorting factor by recognizing certain amino acids or structural motif in the SH4 domain. Taken all together, my results show that Pfa3 is the key protein affects the palmitoylation and functions of Vac8 at the vacuole. Further studies will be needed to determine the specificity and overlapping functions of all DHHC proteins that regulate the palmitoylation of vacuolar proteins.

V.3 Targeting of SH4 domains to membranes

In this study, I characterized the SH4 domains of yeast vacuolar protein Vac8 and the HASPB SH4 domain in *Leishmania major* parasite. The SH4 domain is the minimal motif targeting the proteins to the membrane. My studies demonstrate that the SH4 domains of Vac8 and HASPB differ in their membrane localization. It has been shown in mammalian cells that the SH4 domain fused with GFP is targeted to the endosomes and plasma membrane (McCabe and Berthiaume, 1999). Myristoylated and nonpalmitoylated motifs localized to the

intracellular membranes, including Golgi and the endoplasmic reticulum and were absent from the plasma membrane (McCabe and Berthiaume, 1999).

In yeast, the Vac8 SH4 domain is localized to intracellular membranes and the plasma membrane, whereas the HASPB SH4 domain showed cytosolic distribution. In mammalian cells, HASPB SH4 domain was found to localize mainly to the plasma membrane (Denny et al., 2000). In contrast, in yeast the myristoylation or palmitoylation machinery is not recognizing the HASPB SH4 domain. Interestingly, the Src SH4 domain (myristate and polybasic domain) is functional and localizes to intracellular membranes and the plasma membrane (Figure 22B). What determines the difference in targeting of SH4 domains to the membrane? It is likely the myristoylation occurs in Src SH4 domain and confers membrane attachment along with polybasic residues. Based on these studies, I speculate that the HASPB SH4 domain is not recognized by the *N*-myristoyltransferase in yeast. Myristoylation is a prerequisite for the occurrence of palmitoylation in the SH4 domain. Interestingly, if I mutated the threonine to serine in the consensus sequence (M-G-x-x-C-x-T), HASPB SH4 domain is targeted to the intracellular membrane and also to the plasma membrane. This shows, that the serine at position 6 is important for the glycine modification (myristoylation), followed by the palmitoylation in HASPB proteins. However, myristoylation is not universally required to achieve palmitoylation of N-terminal cysteine residues. For example the α subunits of heterotrimeric G proteins ($G\alpha_s$, $G\alpha_q$, $G\alpha_{12}$) are not myristoylated but still undergo palmitoylation (Degtyarev et al., 1993; Veit et al., 1994; Wedegaertner et al., 1993). Another possible explanation for the cytosolic localization of HASPB SH4 domain is that the serines or threonines residues become phosphorylated and this has steric or electrostatic effects for membrane binding. Replacement of Vac8 SH4 domain in full-length protein with HASPB(T6S) SH4 domain, resulting in HASPB(T6S; 1-18)-Vac8 chimera is localized to the vacuole like wild-type Vac8. It shows that the HASPB(T6S) is functional in yeast (Figure 34C). HASPB(T6S;1-18)-Vac8 is localized more stably to the vacuole with single palmitoylated cysteine whereas the Vac8 with single cysteine (Cys4 or Cys5) is partially localized and may exhibit

palmitoylation or depalmitoylation cycle. However, there is no evidence for the existence of depalmitoylation cycle in Vac8.

Furthmore, I show the mislocalization of the HASPB(T6S;1-18)-Vac8 in *pfa3Δ* cells. It appears that Pfa3 palmitoylates the HASPB(T6S;1-18)-Vac8. However, HASPB(T6S; 1-18)-Vac8 is still localized partially to the vacuole. Other DHHC proteins or palmitoyl acyltransferse activity of the SNARE Ykt6 (Roth et al., 2006) (Dietrich et al., 2004) could support the residual palmitoylation of HB(T6S; 1-18). In the four or five fold-deleted DHHC strain, HASPB(T6S; 1-8)-Vac8 shows more localization defect, suggesting that its localization is dependent on other DHHC proteins. Further studies will be needed to address the targeting mechanism of the SH4 domain with repect to the DHHC proteins (protein acyltransferases).

VI. Conclusion

The results presented in this thesis demonstrate the palmitoylation of the vacuolar protein Vac8. Initially, palmitoylation was thought to be for membrane localization of proteins, and now its role is extended further for the regulation of protein function(s). In my studies, I observed that palmitoylation plays a vital role for Vac8 functions including vacuole fusion, inheritance and morphology. The vacuolar-localized DHHC protein Pfa3 exhibits the palmitoylacyltransferase for the palmitoylation of Vac8. Further understanding of the roles of protein palmitoylation will require the detailed knowledge of the mechanism of palmitoylation.

VII. References

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