Analyses of Vesicular Transport within the Endoplasmic Reticulum-Golgi Interface in *Saccharomyces cerevisiae*

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Table of Contents

1 Summary

A characteristic feature of every eukaryotic cell is its division into different compartments. This subdivision into different intracellular organelles like the endoplasmic reticulum (ER), the Golgi apparatus or the endosomal/lysosomal system enables cells to provide the appropriate environment for a great variety of biochemical processes. However, it also necessitates an elaborate machinery for the communication between these compartments or organelles. On one hand, material has to be exchanged between organelles, but on the other hand, their integrity with respect to their protein and lipid content, has to be maintained to fulfil their function. Transport processes between different organelles are mediate by intracellular traffic pathways. Proteins enter the secretory pathway at the ER, where they acquire first posttranslational modifications. From the ER, they are delivered to the Golgi, where they are further modified and sorted to their target compartments. In the secretory pathway, transport carriers, so-called vesicles, bud from one organelle (donor) and fuse with the next organelle (acceptor) along their trafficking route. Understanding the molecular mechanisms and regulations underlying vesicular transport is crucial and therefore has been a main topic of research over the last decades. The machinery required for budding and fusion of vesicles along their trafficking pathways is conserved from yeast to human. Therefore, the yeast *Saccharomyces cerevisiae* represents a suitable organism to study the secretory pathway.

In this thesis, we used *S. cerevisiae* to examine the regulation of vesicular traffic at the ER-Golgi interface, more specifically the fusion of vesicles with ER membranes. The consumption of a vesicle at its target membrane is mediated by the orchestrated action of various members of conserved protein families that act in a regulated manner. Main players involved in vesicular fusion are Rab GTPases, tethering factors and SNAREs. The tethering factors and the Rab GTPases mediate the first contact of an incoming vesicle with its acceptor organelle, whereas the SNARE proteins are responsible for the final fusion event between vesicles and target membranes.

Here, we identified the Rab GTPase Ypt1p as mediator of vesicle fusion with the ER. Moreover, Ypt1p was not only required for vesicle fusion at the ER, but also for the maintenance of the morphology and protein composition of the Golgi, and for vesicle formation at the Golgi. In addition, the tethering complex responsible for the docking of Golgi-derived vesicles with the ER, the Dsl1 tethering complex was analyzed. We found that this complex, apart from mediating the first contact of the incoming vesicles with the ER membrane, seems to play an additional role in proofreading and stabilization of SNARE complexes that are responsible for vesicle fusion at the ER.

2 Introduction

2.1 Intracellular transport

The subdivision into functionally distinct, membrane-enclosed compartments is a hallmark of all eukaryotic cells. Each of these compartments, or organelles, is uniquely equipped with a characteristic set of proteins, which are either embedded in the membrane or can be found in its aqueous inner space, the lumen. The basic functions performed by the diverse organelles are generally the same in all cell types, and the specific protein content is crucial for the compartments to fulfil their characteristic functions in the cell.

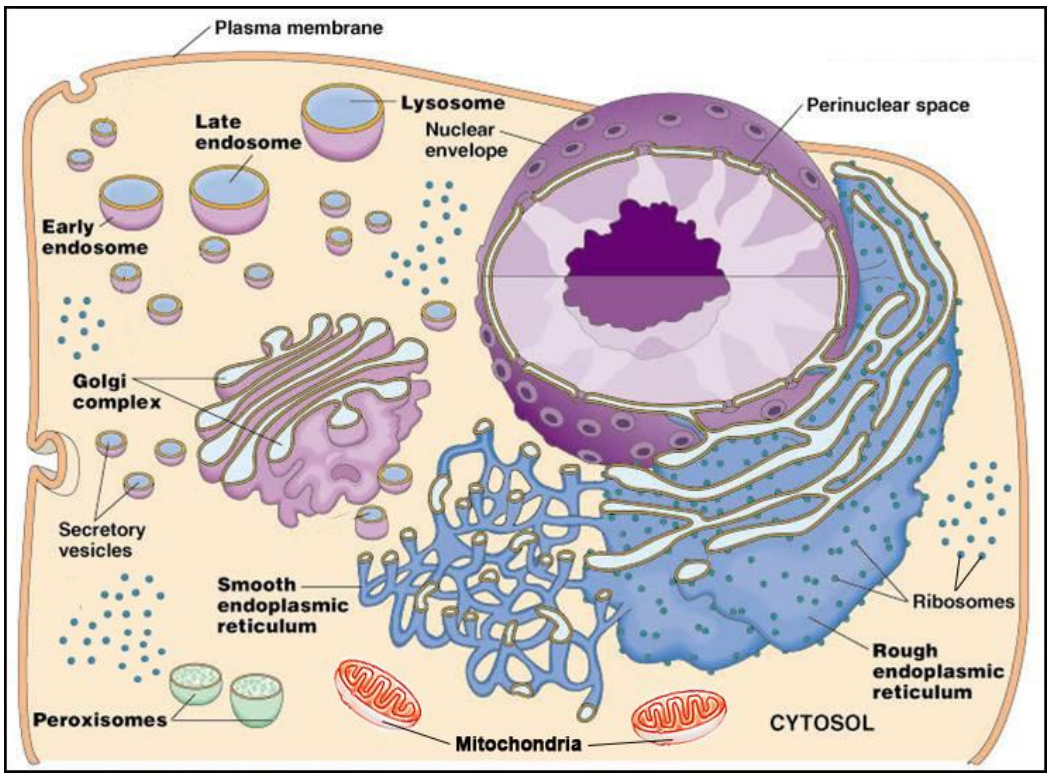

Fig. 1: ***Major intracellular compartments common to eukaryotic cells*** *(adapted from Campbell & Reece, 2000)*

In a eukaryotic cell, the main cellular organelles that are found embedded in the cytosol are the nucleus, the endoplasmic reticulum (ER), the Golgi apparatus, which is compartmentalized into cis-, medial- and trans-Golgi, the lysosomal/endosomal compartments, the peroxisomes and the mitochondria (Fig. 1). Proteins which function in the cytosol, the nucleus, the peroxisomes or the mitochondria are synthesized on ribosomes in the cytoplasm. Their fate is determined by organelle-specific targeting sequences within their amino acid sequence. Once their synthesis is completed these proteins are released into the cytosol and from there they can be imported into their target organelles. Proteins destined for membrane-bound organelles along the secretory pathway (ER, Golgi, lysosomal/endosomal

system or plasma membrane) (Palade, 1975) and for secretion at the cell surface contain special signal sequences. During synthesis these signal sequences are recognized, which leads to a binding of the translating ribosomes to the cytoplasmic face of the ER membrane. There, the newly synthesized proteins are co-translationally inserted into the membrane or released into the lumen of the ER. Once folding, assembly and initial modifications, like glycosylation, in the ER lumen are completed, proteins are transported to the Golgi apparatus, and from there sorted to the lysosomal/endosomal system or to the plasma membrane (Bonifacino & Glick, 2004). In addition to secreting proteins the cell also takes up material from the outside, in a process called endocytosis. It furthermore retrieves back proteins that have escaped from their resident organelles. The transport within the secretory and the endocytic pathways, as well as the retrieval of proteins is mediated at least in part by small vesicles (Fig. 2). These membrane-bound and protein-coated carriers bud from the membrane of one organelle (donor membrane) and fuse with the membrane of another organelle (acceptor membrane), thereby delivering proteins and lipids (Palade, 1975; Rothman & Wieland, 1996; Schekman & Orci, 1996). Due to the constant exchange of lipids and proteins among the cellular compartments, specific sorting and retrieval mechanisms are necessary to maintain organelle identity and integrity. Vesicular transport, therefore, requires a tight regulation. For example, a defect in the regulation of the delivery and removal rate, could severely interfere with organelle identity and function (Spang, 2008). The significance of intracellular trafficking and its proper regulation is also reflected by the observation that several diseases in humans are caused by mutations affecting the vesicular transport machinery (Huizing *et al.*, 2000; Kins *et al.*, 2006; Olkkonen & Ikonen, 2006; Fromme *et al.*, 2007; Jenkins *et al.*, 2007; Corbeel & Freson, 2008; Schonthaler *et al.*, 2008).

2.2 Molecular mechanism of vesicular traffic

2.2.1 Different vesicle types

As mentioned above, small vesicles are involved in the secretory and endocytic trafficking as well as in the retrieval of escaped proteins back to their resident organelles. These vesicles can be classified by their different proteinaceous coats into COPII, COPI and clathrin coated vesicles (Fig. 2). Additionally, other less well characterized potential vesicle coats have been described in the past years (Godi *et al.*, 2004; Seaman *et al.*, 1998; Trautwein *et al.*, 2006; Wang *et al.*, 2006). For example, the exomer complex might function as a coat that sorts specific cargo directly from the trans-Golgi to the plasma membrane in yeast (Trautwein *et al.*, 2006; Wang *et al.*, 2006). Coat proteins are multimeric proteins which polymerize at the site of vesicle formation. The coat polymerization will deform the membrane and thereby assists to pinch off the vesicle from the donor compartment. COPII vesicles are responsible

for the transport from the ER to the Golgi (Barlowe *et al.*, 1994; Bonifacino & Glick, 2004). Their coat is composed of the small GTPase Sar1p and two protein complexes Sec23/24p and Sec13/31p, respectively. COPI vesicles travel back from the cis-Golgi to the ER as well as between Golgi stacks (Letourneur *et al.*, 1994; Lee *et al.*, 2004), and are coated by the small GTPase Arf1p and the coatomer complex, which is comprised of seven subunits (α, ß, ß', γ, δ, ε, ζ). Most of the transport steps at the trans-Golgi-plasma membrane interface are mediated by clathrin-coated vesicles (Bonifacino & Glick, 2004; Owen *et al.*, 2004). The clathrin coat is heterogeneous, it contains the small GTPase Arf1p, clathrin, and in addition various adaptor complexes (AP1–4) and adaptor-like complexes (GGAs).

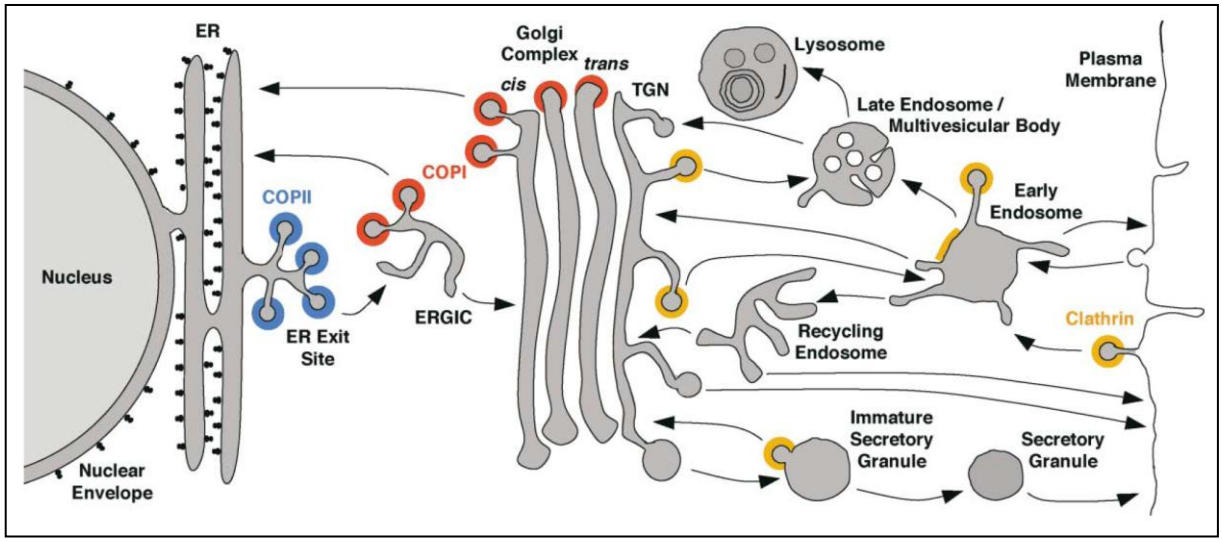

Fig. 2: ***Intracellular Transport Pathways*** *(taken from Bonifacino & Glick, 2004)*
Transport steps are indicated by arrows. Colors indicate the known or assumed locations of COPII (blue), COPI (red) and clathrin (yellow). In S. cerevisiae the ER-Golgi-intermediate compartment (ERGIC) does not exist and the vacuole has the function of the lysosome. Additional coats or coat-like complexes are not represented in this figure.

2.2.2 The life-cycle of a transport vesicle

Despite the different types of vesicles described above, the basic mechanisms involved in the budding and the fusion of vesicles are conserved among the different intracellular transport pathways and between different eukaryotic species (Fig. 3).

The first step in the life cycle of a vesicle is the recruitment of a small GTPase of the *ARF1*/*SAR1* family to the membrane of the donor organelle. The association of the small GTPase with the membrane is followed by a recruitment of coat components from the cytosol via an interaction with the small GTPase. The coat components incorporate cargo and membrane-anchored fusion factors, so-called vesicle SNAREs (v-SNAREs). The SNAREs are required for the consumption of the vesicles at the target membrane (Hanson *et al.*, 1997; Lin & Scheller, 1997; Nichols *et al.*, 1997; Weber *et al.*, 1998). Since COPII and COPI vesicles can be formed *in vitro* using only synthetic liposomes, guanine nucleotides, GTPases and coat components, the importance of cargo proteins for vesicle formation was

neglected for a long time. Recently, a significant contribution of cargo in coat recruitment and stabilization became apparent (Aoe *et al.*, 1998; Forster *et al.*, 2006; Pepperkok *et al.*, 2000; Spang, 2008; Springer *et al.*, 1999). A model was proposed in which a so-called primer complex that contains only the small GTPase, a v-SNARE or cargo, and coat components is formed (Springer *et al.*, 1999). If enough cargo for transport is available, more such complexes can be formed, thereby the coat is stabilized and can polymerize. When the growing vesicle has reached a certain size (determined by the coat), the vesicle is released by scission. In former times, it was believed that uncoating happens already during or right after vesicle release. More recent evidence suggest that the vesicles stays at least partially coated until they arrive at their target membrane (Spang, 2008).

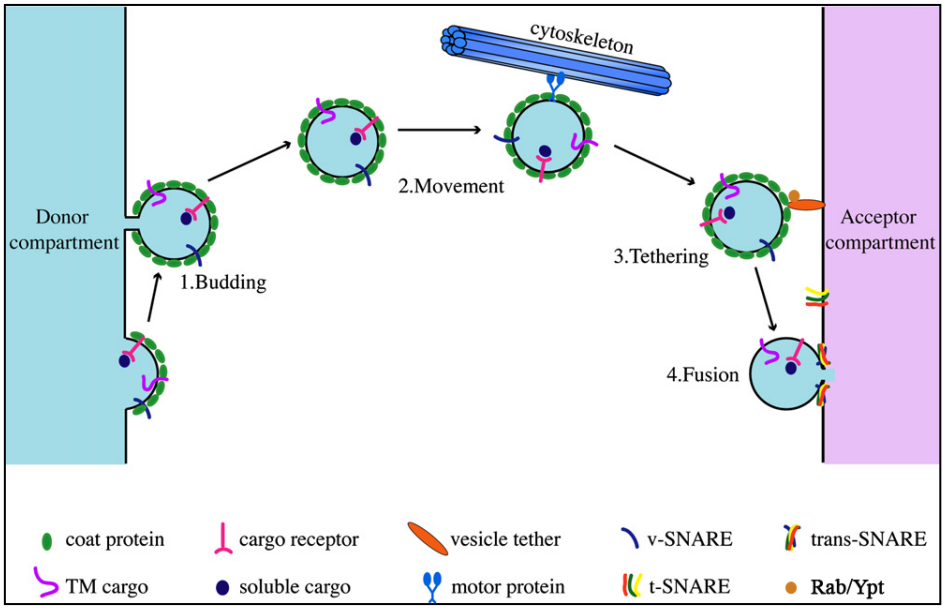

Fig. 3: ***Essential Steps in Vesicle Transport*** *(adapted from Cai et al., 2007a)*
(1) Budding: A small GTPases is recruited to the donor membrane. If enough cargo is available, coat proteins are recruited to induce the formation of a vesicle. Cargo and SNAREs are incorporated into the budding vesicle by binding to coat subunits. (2) Movement: the vesicle moves toward the acceptor compartment by diffusion or with the aid of a cytoskeletal track. (3) Tethering: tethering factors work in conjunction with Rab GTPases to tether the vesicle to their acceptor membrane. (4) Fusion: the vesicle-associated SNARE and the SNARE on the acceptor membrane assemble into a four-helix bundle (trans-SNARE complex), which drives membrane fusion and the delivery of cargo.

After budding, the vesicle is transported to its final destination by either diffusion or motor-mediated transport along cytoskeletal tracks. Components involved in vesicular trafficking have been reported to interact with molecular motors like kinesin, dynein and myosin (Cai *et al.*, 2007a; Hammer & Wu, 2002; Matanis *et al.*, 2002; Short *et al.*, 2002). In higher eukaryotes, transport along microtubules plays an important role in vesicular transport, e.g. in the trafficking of synaptic vesicles. In yeast, the actin cytoskeleton performs a similar function as microtubules in mammalian cells. It e.g. is involved in the transport of certain vesicles from the trans-Golgi to the bud tip. When the vesicle arrives at its target membrane,

a recognition process that involves tethering proteins, Rab/Ypt GTPases and probably coat proteins takes place. This leads to the docking of the vesicle to the acceptor membrane and possibly to its final uncoating. In the next step, the v-SNAREs and the SNAREs on the acceptor membrane (t-SNAREs) assemble into a four-helix bundle (trans-SNARE complex), which drives membrane fusion and thereby the delivery of cargo to the target compartment (Sollner *et al.*, 1993).

2.2.2.1 Vesicle formation

In the following section the generation of COPII-, COPI- and clathrin-coated vesicles is described in more detail.

2.2.2.1.1 COPII vesicle biogenesis

The first event in the formation of a COPII vesicle is the recruitment of the small GTPase Sar1p to the ER membrane. Small GTPases of the *ARF1/SAR1* family are molecular switches that exist in a GDP-bound (inactive) and in a GTP-bound (active) form. The exchange from GDP for GTP is mediated by a guanine nucleotide exchange factor (GEF). The GTP-bound form localizes to membranes. GTP hydrolysis to GDP is achieved by the help of a GTPase activating protein (GAP). GDP-bound small GTPases are released from the membrane. The recruitment of the small GTPase Sar1p is mediated by the GEF Sec12p. Sec12p is an ER-resident, transmembrane anchored protein and the only Sar1p GEF. Therefore, Sar1p specifically associates with the ER (Barlowe & Schekman, 1993; Spang, 2008). Moreover, COPII vesicles are formed at specific sites at the ER, the so-called ER exit sites. It seems that several types of exit sites exist, which may be responsible for different subclasses of cargo proteins (Castillon *et al.*, 2009; Spang, 2008; Spang, 2009). The peripheral membrane protein Sec16p, in addition to Sec12p, plays a major role in the organization and biogenesis of these exit sites (Connerly *et al.*, 2005; Supek *et al.*, 2002; Watson *et al.*, 2006). Upon GTP-binding, Sar1p exposes an N-terminal, 23 residues long, hydrophobic, α-helical membrane anchor, leading to membrane attachment. After binding of the small GTPase to the ER membrane, Sec23p, the GAP for Sar1p and Sec24p, which is responsible for cargo inclusion, are recruited as a dimer. During cargo recognition most transmembrane cargo binds directly to Sec24p via specific, cytosolicly exposed sorting signals (Barlowe, 2003; Peng *et al.*, 1999). A variety of such sorting signals can be recognized. Some examples are di-acidic, di-basic and short-hydrophobic sequences. Furthermore, in yeast and mammals three and four Sec24p orthologs, respectively, have been identified (Kurihara *et al.*, 2000; Shimoni *et al.*, 2000; Shimoni & Schekman, 2002; Tang *et al.*, 1999; Wendeler *et al.*, 2007). At least in mammalian cells it has been shown that they interact with different subsets of transmembrane cargo proteins (Wendeler *et al.*, 2007). Soluble cargo proteins and GPI-anchored proteins are in the ER lumen and cannot directly

bind to Sec24p. These proteins are recruited by export cargo receptors, which in turn bind to the Sec23/24p complex. The p24 family members Emp24p and Erv25p play a role in the export of GPI-anchored proteins from the ER (Muniz *et al.*, 2000; Takida *et al.*, 2008). In addition, the cargo receptor Erv29p mediates the recruitment of COPII cargo proteins like the yeast pheromone α-factor, the vacuolar carboxypeptidase Y and proteinase A (Belden & Barlowe, 2001; Malkus *et al.*, 2002). Furthermore, SNARE proteins are included into the forming vesicle. For this purpose GTP-bound Sar1p activates the Sec23/24p complex to bind SNARE proteins that are involved in the fusion of the vesicle with its acceptor membrane (Mancias & Goldberg, 2007; Mossessova *et al.*, 2003; Springer & Schekman, 1998). Three distinct binding sites (A-site, B-site and Arg342-site) for SNAREs involved in the ER-Golgi-shuttle have been identified, each recognizing specifically a SNARE (Miller *et al.*, 2003; Mossessova *et al.*, 2003). In the last step of the COPII vesicle generation, the tetrameric Sec13/31p complex binds to the membrane and the cargo-associated Sec23/24p complex, thereby building the outer layer of the vesicle coat. The Sec13/31p aids in deforming the membrane and stabilizes the polymerizing coat, providing the major bending force needed to allow the formation of a COPII vesicle (Spang, 2008; Spang, 2009; Stagg *et al.*, 2006). As soon as the nascent vesicle forms an almost complete sphere it pinches off from the membrane. The mechanism behind this release is so far not well understood. The finding that Sar1p, as well as Arf1p, possess membrane tubulation activity provides some insight into this process (Beck *et al.*, 2008; Bielli *et al.*, 2005; Lee *et al.*, 2005; Lundmark *et al.*, 2008). The main steps of COPII vesicle biogenesis are shown in Fig. 4.

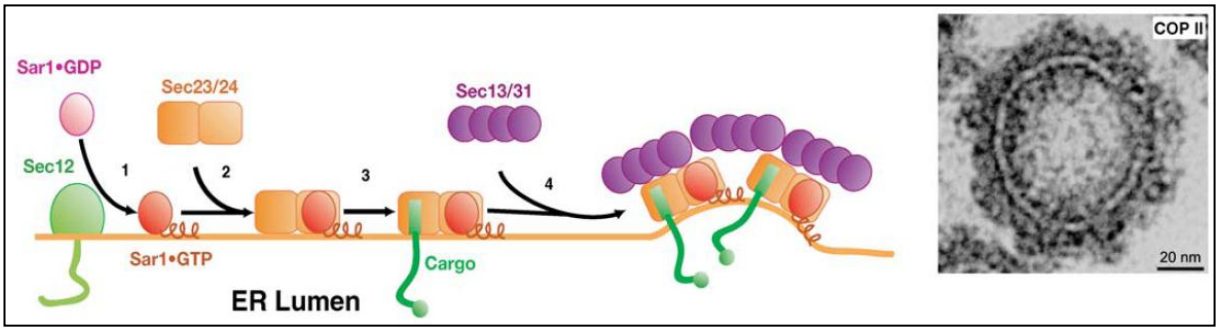

Fig. 4: ***COPII vesicle biogenesis*** *(adapted from Lee et al., 2004)*
COPII coat assembly is initiated by the ER resident, Sec12p, which serves as a guanine nucleotide exchange factor (GEF) for the small GTPase, Sar1p (1). GTP binding by Sar1p exposes a hydrophobic α-helix that facilitates association with the ER membrane. Membrane-associated Sar1p recruits the Sec23/24p heterodimer (2), and this complex interacts with cargo proteins via specific sorting signals (3). The Sar1p-Sec23/Sec24p complex then recruits the Sec13/31p heterotetramer (4), which is thought to drive polymerization of the coat and membrane deformation to yield a COPII vesicle. An EM picture of an COPII vesicle is shown (Schekman & Orci, 1996).

2.2.2.1.2 COPI vesicle biogenesis

The formation of COPI vesicles is organized by the small GTPase Arf1p. In contrast to Sar1p, Arf1p induces the formation of vesicles at different membranes and interacts not only with coatomer to form COPI vesicles, but also with adaptor complexes and clathrin to form clathrin-coated vesicles (Spang, 2008). The existence of several Arf GEFs, e.g. five (Gea1p, Gea2p, Sec7p, Syt1p, Yel1p) in yeast, reflects the involvement of Arf1p in multiple vesicle budding events in the cell (Donaldson & Jackson, 2000; Jackson & Casanova, 2000). After the recruitment of GDP-bound Arf1p to the membrane and the exchange of GDP for GTP, Arf1p exposes a 17 amino acid long, myristoylated, highly hydrophobic α-helix, which enables it to tightly associate with the Golgi membranes. This activation is followed by the recruitment of an ArfGAP (Lewis *et al.*, 2004; Yang *et al.*, 2002) and binding of the large, heptameric coatomer complex to the membrane from the cytosol. Two subcomplexes of coatomer have been identified, the membrane-proximal F-COP subcomplex, consisting of the β-, γ-, δ- and ζ- COP subunits (Sec26p, Sec21p, Ret2p and Ret3p in yeast) and the membrane-distal B-COP subcomplex composed of α-, β'- and ε-COP (Sec33p, Sec27p and Sec28p in yeast) (Eugster *et al.*, 2000; Gaynor & Emr, 1997; Hara-Kuge *et al.*, 1994; Waters *et al.*, 1991).

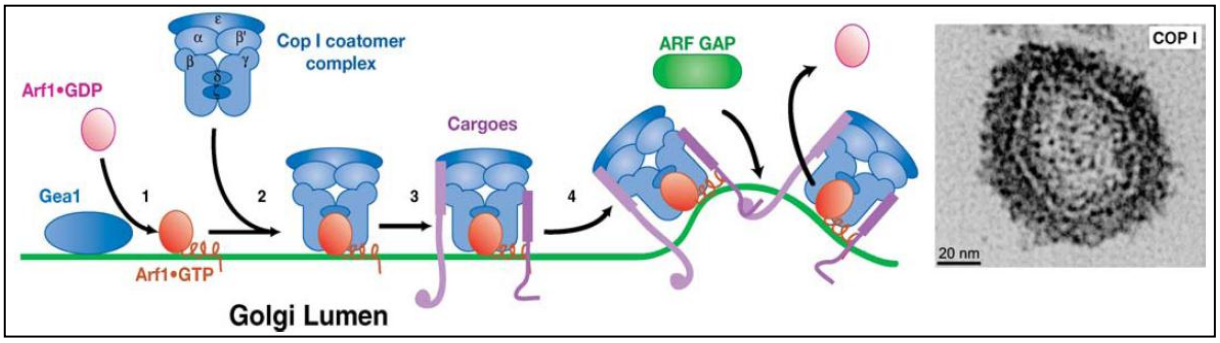

Fig. 5: ***COPI vesicle biogenesis*** *(adapted from Lee et al., 2004)*
In COPI coat assembly coat recruitment is initiated by GDP-GTP exchange on Arf1p, mediated by an ARF GEF (1). Membrane-bound Arf1p then recruits an ArfGAP and the preassembled coatomer complex, which contains seven subunits: the α/β/ε complex and the β/γ/δ/ζ complex (2). The coatomer complex contains multiple cargo recognition sites on separate subunits that mediate recruitment of cargo proteins (3). Ultimately, the coat polymerizes and subsequently the vesicle dissociates from the membrane (4). An EM picture of a purified COPI vesicle is shown (Schekman & Orci, 1996).

Arf1p interacts with the ß- and the γ subunit of the F-COP subcomplex (Zhao *et al.*, 1997; Zhao *et al.*, 1999). Recruitment of cargo into COPI vesicles is mainly mediated also by coatomer. The β-, γ-, δ- subunits have been shown to be involved in cargo recognition (Cosson *et al.*, 1998; Harter & Wieland, 1998; Michelsen *et al.*, 2007). The γ-subunit, for example, interacts with transmembrane cargo proteins bearing the ER-retrieval signal K(X)KXX in their cytosolic domain (Cosson & Letourneur, 1994; Harter *et al.*, 1996). The KDEL-receptor binds to soluble cargo in the Golgi-lumen that is carrying the ER-retrieval

sequence KDEL and interacts with ArfGAP (Aoe *et al.*, 1999). Furthermore, members of the p24 family of cargo receptor proteins described above are also involved in the generation of COPI vesicles (Aguilera-Romero *et al.*, 2008; Bethune *et al.*, 2006; Sohn *et al.*, 1996). Furthermore, SNAREs are included into COPI vesicles. Their incorporation is ensured by interaction with the ArfGAP which induces a conformational change on the SNAREs that promotes the direct interaction with Arf1p (Rein *et al.*, 2002; Schindler & Spang, 2007; Spang, 2002). Finally, the polymerization of coatomer complexes induces the deformation of the donor membranes and leads to the budding of the vesicle. The main steps of COPII vesicle biogenesis are shown in Fig. 5.

2.2.2.1.3 Biogenesis of clathrin-coated vesicles

Clathrin-coated vesicles can form at different compartments, e.g. the trans-Golgi, endosomes or the plasma membrane. The small GTPase Arf1p is required for their formation. It functions together with the adaptor complexes AP1, AP2, AP3, AP4 and the monomeric GGAs (Edeling *et al.*, 2006; Royle, 2006; Spang, 2008; Young, 2007) APs and GGAs are responsible for the recognition and recruitment of cargo (Boehm & Bonifacino, 2001; Bonifacino & Glick, 2004; Robinson, 2004; Spang, 2008). First an AP complex is recruited to the membrane, forming a membrane-proximal layer of the coat, later clathrin triskelions are bound forming a membrane-distal layer (Smythe *et al.*, 1992). Arf1p functions together with the adaptor complexes AP1, AP3, AP4 and the GGAs to generate clathrin-coated vesicles at the trans-Golgi. The AP2 adaptor complex is involved in receptor-mediated endocytosis at the plasma membrane. AP1 and AP3 also participate in the formation of clathrin-coated vesicles at endosomes (Robinson, 2004). The final fission of clathrin-coated vesicles from the donor membranes requires the GTPase dynamin (Sever, 2002).

2.2.2.1.4 The fate of the coat

For COPI and COPII coats, the GTPase activating proteins, are intrinsic components of the coat. As a result, GTP hydrolysis of the small GTPases Sar1p and Arf1p may already occur during vesicle formation. Moreover, it was shown that GTP hydrolysis by Arf1p during vesicle biogenesis is required for the efficient packaging of cargo into COPI vesicles (Lanoix *et al.*, 1999; Lanoix *et al.*, 2001; Malsam *et al.*, 1999; Weiss & Nilsson, 2003). Thus, if the stability of the coat would solely depend on the GTP-state of the small GTPase, it could come off even before the budding process is completed (Spang, 2008). In contrast, coated vesicles can be isolated from cells (Spang, 2008), and it was shown that tethering factors at the respective target membranes interact with components of the COPI and COPII coat (Andag *et al.*, 2001; Barlowe, 1997; Cai *et al.*, 2007b; Vanrheenen *et al.*, 2001). The current model to explain this apparent discrepancy is the existence of so-called metastable coats. In this scenario, most of the GTP hydrolysis occurs already during the budding process, and the small GTPase leaves the vesicle while the remaining coat components are still staying on the

vesicle (Antonny *et al.*, 2001; Spang, 2009). In this case the coat would be bound to the vesicle by coat-cargo, coat-coat and coat-lipid interactions (Spang, 2009). Upon arrival at the target membrane the coat then would be disassembled, via interaction with e.g. tethering factors. Such a link between tethering and uncoating has been described for COPI vesicles arriving at the ER (Zink *et al.*, 2009).

2.2.2.2 Vesicle consumption

As mentioned above, the consumption of a vesicle at the target membrane is mediated by orchestrated action of different conserved proteins that act in a regulated cascade leading to lipid bilayer mixing (Markgraf *et al.*, 2007). The main players involved in vesicular fusion are Rab GTPases, tethering factors and SNAREs. They will be described in more detail in the following section.

2.2.2.2.1 Rab GTPases

Rab GTPases (Rabs) are ubiquitously expressed proteins of the small monomeric Ras-like family of GTPases (Chavrier & Goud, 1999). To date eleven Rabs have been identified in yeast and over sixty in mammalian cells (Schultz *et al.*, 2000). Rab GTPases are (like *ARF1/SAR1* GTPases) molecular switches, cycling between GTP-bound and GDP-bound states (Fig. 6), this exchange is controlled by GEFs and GAPs (Pfeffer, 2007; Segev, 2001). Rabs also undergo a cycle of membrane insertion and extraction. This is partially coupled to the nucleotide cycle.

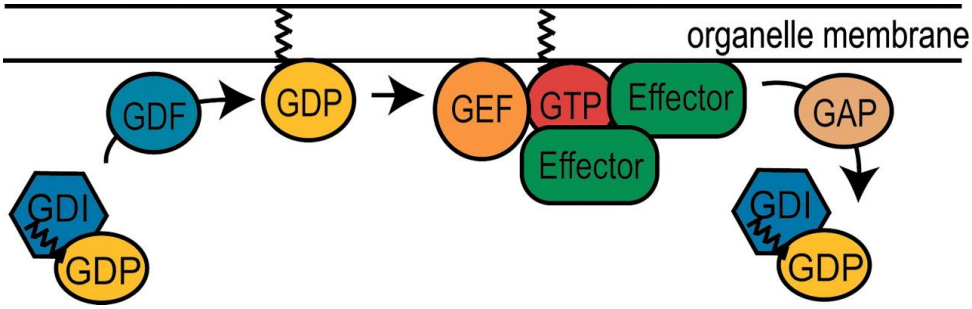

Fig. 6: ***The nucleotide and membrane attachment/detachment cycles of Rab GTPases***
(taken from Grosshans et al., 2006)
Inactive (GDP-bound) prenylated Rab GTPases are bound to GDI, which masks their isoprenyl anchor and thereby keeps the Rabs in a soluble, cytosolic form. Membrane attachment of Rabs requires the function of a GDF that dissociates the GDI-Rab complex and allows the prenyl anchor to be inserted into the membrane. Subsequently, a specific GEF exchanges the bound GDP for GTP, thereby activating the Rab GTPase. The active, membrane-bound Rab is then able to fulfil its various functions in membrane traffic by binding to specific effector proteins. Finally, a specific GAP inactivates the Rab by accelerating the hydrolysis of the bound GTP into GDP. The inactive, GDP-bound Rab can then be extracted from the membrane by GDI and recycled for another round of activation.

The modification of two C-terminal cysteins with isoprenyl lipid (geranylgeranyl) moieties is required for membrane insertion (Kinsella & Maltese, 1992). A GDP dissociation inhibitor (GDI) binds to prenylated Rabs in their GDP-bound form (Garrett *et al.*, 1994; Shapiro &

Pfeffer, 1995; Shisheva *et al.*, 1999) thereby masking their isoprenyl anchor (Rak *et al.*, 2003) and retaining them in the cytosol (Goody *et al.*, 2005). A GDI displacement factor (GDF) is therefore required for the membrane attachment of Rab (Pfeffer & Aivazian, 2004). As soon as the Rabs are dissociated from GDI they bind GTP stimulated by a GEF. Such active, membrane-bound Rabs then take part in a variety of functions in vesicular trafficking by binding to specific effectors. After specific GAPs inactivate the Rabs, they are extracted from the membrane by GDI and recycled back to the cytosol (Araki *et al.*, 1990; Ullrich *et al.*, 1993). Rab GTPases have been shown to be implicated in the regulation of almost all steps in membrane traffic. Several publications provide evidence for a role of Rabs in cargo selection, vesicle formation and the identification of maturing endosomes (Carroll *et al.*, 2001; de Hoop *et al.*, 1994; Jedd *et al.*, 1997; McLauchlan *et al.*, 1998; Morsomme & Riezman, 2002; Pagano *et al.*, 2004, Poteryaev *et al*., 2010). Furthermore, motors and motor adapters involved in vesicle and organelle transport along actin cables and microtubules have been shown to be Rab effectors (Grosshans *et al.*, 2006; Stenmark, 2009). The most prominent role of Rab proteins, however, is probably their function in vesicle tethering and fusion. Activated GTP-bound Rabs recruit elongated tethering factors to specific locations in the endomembrane system (Cai *et al.*, 2007a; Grosshans *et al.*, 2006; Stenmark, 2009). This, in turn enables long-distance contacts between the transport vesicle and the acceptor membrane. Additionally, Rab proteins also modify SNARE proteins. Several reports indicate that Rab proteins directly interact with v- and t-SNAREs to activate them for trans-SNARE complex formation (Lian *et al.*, 1994; Lupashin & Waters, 1997). Most evidence, however, indicates an indirect regulation of SNAREs through interaction of Rabs with tethering proteins (Collins *et al.*, 2005; McBride *et al.*, 1999; Subramanian *et al.*, 2004).

2.2.2.2.2 Tethering factors

Tethering factors represent a diverse group of peripheral membrane proteins. They are responsible for the initial attachment of a vesicle to its target membrane. However, since tethering factors have been shown to interact with components of the fusion machinery and with components involved in vesicle formation, it became apparent that they are more than just static bridges (Sztul & Lupashin, 2009). Tethering factors can be divided into three different functional classes (Sztul & Lupashin, 2009). One class consists of, coiled-coil tethers like p115/Uso1p, Golgins or early-endosomal autoantigen (EEA1). The second class contains multisubunit tethering complexes (MTC) that bind to SNAREs and typically act as Rab effectors. This so-called DCGE group contains the Dsl1 tethering complex, the conserved oligomeric Golgi (COG) complex, the Golgi-associated retrograde protein (GARP) complex, and the exocyst. Finally, MTCs exist that function as GEFs for Rab proteins. This group consists of the transport protein particle complexes, TRAPP I and TRAPP II, and

HOPS, which is probably both, a GEF and a Rab effector (Fig. 7). Tethering factors localize to different compartments within the secretory and endocytic pathways.

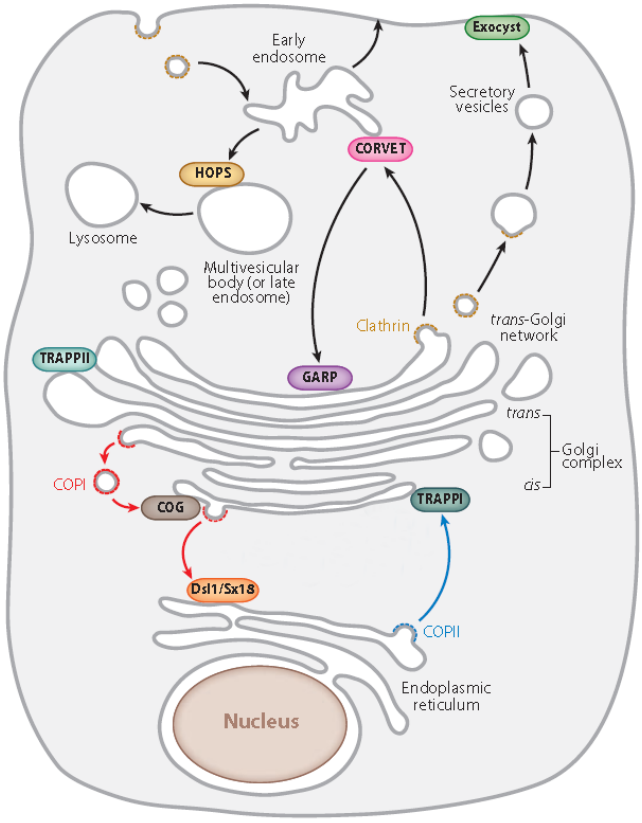

Fig. 7: ***Oligomeric tethering factors in eukaryotic cells*** *(taken from Yu & Hughson, 2010)*
The eight multisubunit tethering complexes (colored ovals) discovered so far and some of the coat proteins (COPI, COPII, clathrin) that mediate transport vesicle formation are shown. The Dsl1 complex in yeast is called the syntaxin 18 (Sx18) complex in mammals.
Abbreviations: COG, conserved oligomeric Golgi complex; CORVET, class C core vacuole/endosome tethering complex (variant of HOPS). ERGIC, endoplasmic reticulum-Golgi intermediate compartment; GARP, Golgi associated retrograde protein complex; HOPS, homotypic fusion and vacuole protein sorting (or class C vacuolar protein sorting) complex; TRAPP, transport protein particle.

As mentioned above almost all known tethering factors interact with Rab GTPases. TRAPPI, TRAPPII and HOPS act as Rab GEFs (Jones *et al.*, 2000; Sacher *et al.*, 2001; Sztul & Lupashin, 2009; Wang *et al.*, 2000). They are recruited to Rab-free membranes, where the exertion of their GEF activity leads to the recruitment of specific GTP-bound Rabs, which in turn can recruit other tethering factors.

Furthermore, many tethering factors have been shown to interact with SNAREs (Sztul & Lupashin, 2009; Yu & Hughson, 2010). On one hand, this might ensure that only the vesicles carrying the “correct SNARE equipment” get tethered and consequentially fuse (Sztul & Lupashin, 2009). On the other hand, it was shown that tethering factors play an active, regulatory role in the assembly and stability of SNARE complexes (Andag & Schmitt, 2003; Aoki *et al.*, 2009; Perez-Victoria & Bonifacino, 2009; Ren *et al.*, 2009; Shestakova *et al.*, 2007; Shorter *et al.*, 2002). Tethering factors might also influence SNARE mediated fusion by binding to and enhancing the action of SNARE regulating proteins, the so-called Sec1/Munc18 SNARE master (SM) proteins (Laufman *et al.*, 2009; Wiederkehr *et al.*, 2004). Finally, it was shown that numerous tethering factors interact with vesicle coat components. For example, Dsl1, COG, TRAPII and p115 bind to subunits of coatomer (Guo *et al.*, 2008; Sztul & Lupashin, 2009; Yamasaki *et al.*, 2009), TRAPPI and Uso1 in turn bind to the

Sec23/24p subcomplex of COPII vesicles (Behnia *et al.*, 2007; Yamasaki *et al.*, 2009). The interaction between coat proteins and tethering factors is thought to destabilize the coat (Andag *et al.*, 2001; Sztul & Lupashin, 2009; Zink *et al.*, 2009). Based on known interactions of tethering factors with various players in vesicular trafficking, a model in which tethers employ several proofreading mechanisms to identify incoming vesicles was proposed (Sztul & Lupashin, 2009). In this model, as a first step tethering factors would interact with the vesicle coat to identify which kind of vesicle is approaching. In a second step they then might trigger or facilitate uncoating, and finally, they would modify the SNARE machinery to provide a more stringent level of recognition

2.2.2.2.3 SNARE proteins

SNAREs proteins (Soluble NSF-Attachment protein Receptor proteins) (Block *et al.*, 1988; Clary *et al.*, 1990) are membrane-bound proteins that play an essential role in all vesicle and organelle fusion events in secretory and endocytic pathways (Chen & Scheller, 2001; Jahn *et al.*, 2003; Jahn & Scheller, 2006; Sudhof & Rothman, 2009). Based on studies of fusion events at the neuronal synapse (Sollner *et al.*, 1993), SNARE proteins have been divided in three subfamilies: the syntaxin-like SNAREs, the SNAP 25-like SNAREs and the synaptobrevins (Weimbs *et al.*, 1998). All SNAREs show a similar domain structure (Chen & Scheller, 2001; Jahn & Scheller, 2006; Malsam *et al.*, 2008). The variable N-terminal domains of SNAREs perform regulatory functions. Syntaxins and some synaptobrevins contain long N-terminal extensions. These can fold independently, have autoregulatory functions and can serve as platform for SNARE-regulating proteins. The middle part of SNARE proteins is occupied by a homologous α-helical domain of 60-70 amino acids composed of specialized heptad repeats, the so-called SNARE-motif (Weimbs *et al.*, 1998). Most of the SNAREs contain only one such motif, others like SNAP 25 bear two SNARE-motifs. The C-termini of SNAREs are responsible for the membrane anchoring. SNARE proteins are mainly tail-anchored proteins and insert their C-terminal transmembrane domain post-translationally in the ER membrane (Jantti *et al.*, 1994; Kutay *et al.*, 1995). Some, however, make use of hydrophobic modifications like palmitoylation or phosphoinositide-binding domains for reversible membrane localization (Cheever *et al.*, 2001; Dietrich *et al.*, 2005; McNew *et al.*, 1997). SNAREs undergo a well defined cycle during membrane fusion (Fig. 8). During the fusion process, four SNARE domains contributed by the v- and the t-SNAREs, form trans-SNARE-complexes. These progressively zipper up from the membrane-distal end and thereby pull the vesicle and the acceptor membranes in close proximity (Fiebig *et al.*, 1999). Since the unstructured SNARE domains interact and form a highly structured α-helix bundle, energy is released (Fasshauer *et al.*, 1997; Poirier *et al.*, 1998; Sutton *et al.*, 1998). This energy in turn is responsible for overcoming the repulsive forces between the two membranes.

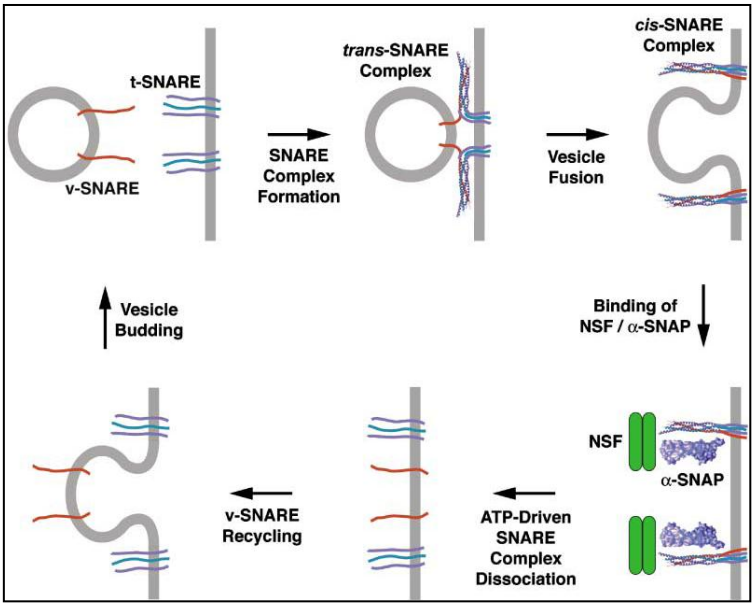

Fig. 8: ***SNARE cycle*** *(taken from Bonifacino & Glick, 2004)*
A trans-SNARE complex assembles when v-SNAREs on the vesicle binds to t-SNAREs on the target membrane, forming a stable four-helix bundle that promotes fusion. The result is a cis-SNARE complex in the fused membrane. α-SNAP binds to this complex and recruits NSF, which hydrolyses ATP to dissociate the complex. Unpaired v-SNAREs can then be packaged again into vesicles.

One member of each of the SNARE subfamilies mentioned above is required for SNARE complex formation. Furthermore, the four SNARE domains contributing to the trans-SNARE-complexes are normally provided by four different SNARE proteins (Hay, 2001). This contributes to a high degree of specificity in vesicle targeting and fusion. After the fusion event, the SNARE complexes are located in the target membrane and therefore called cis-SNARE complexes. To enable further fusion events, these cis-SNARE complexes have to be dissolved, and the v-SNAREs have to be retrieved back to the donor component. For this to happen, α-SNAP (soluble NSF attachment protein) and NSF (N-ethylmaleimide sensitive factor) (in yeast: Sec17p and Sec18p) resolve the cis-SNARE complexes, and v-SNAREs are then incorporated into vesicles travelling to their donor compartment.

2.2.2.2.4 Sec1/Munc18 SNARE master (SM) proteins

Another family of proteins involved in all intracellular fusion events are the so-called SNARE master (SM) proteins (Rizo & Sudhof, 2002). They are composed of a conserved 600 amino acid sequence that folds back into an arch-shaped “clasp” structure (Misura *et al.*, 2000; Sudhof & Rothman, 2009). They have been shown to associate with SNARE complexes (Carr *et al.*, 1999; Scott *et al.*, 2004; Wickner & Schekman, 2008). Furthermore, they interact with the “open conformation” of certain syntaxin family members, like Syntaxin4 (Dulubova *et al.*, 2002; Wickner & Schekman, 2008; Yamaguchi *et al.*, 2002), an N-terminal peptide region within the syntaxins is involved in this interaction. Furthermore, SM proteins also interact with the folded N-terminal domain (the Habc domain of the syntaxins folds back on the SNARE motif) of other syntaxins, like syntaxin 1 (Dulubova *et al.*, 1999; Wickner & Schekman, 2008). The exact role of SM proteins in regulating vesicle fusion events, however, remains elusive. Due to the discovery that Munc18-1, as mentioned above, binds to the individual synaptic t-SNARE subunit syntaxin-1, forming a complex that includes part of the SNARE motif, and

therefore disabling the formation of SNARE complexes they first were thought to be negative regulators (Sudhof & Rothman, 2009). However it has been shown that SM proteins play a positive role in all fusion reactions (Brenner, 1974; Novick *et al.*, 1980; Schoch *et al.*, 2001; Sudhof & Rothman, 2009; Verhage *et al.*, 2000). One mechanism could be that the arch-shaped body of SM proteins folds back on and clasps across the zippering up SNARE domains during trans-SNARE complex assembly. This is supported by the finding that SM proteins, as mentioned before, were found to be associated with SNARE complexes and that they bind the N-terminal peptide region of certain syntaxin family members (Sudhof & Rothman, 2009). SM proteins could therefore cooperate in trans-SNARE complex assembly and organization, spatially and temporally (Dulubova *et al.*, 2007; Shen *et al.*, 2007; Sudhof & Rothman, 2009). Since SM proteins have been shown to be involved in the regulation of Rab GTPases, another potential role for SM proteins is the regulation of tethering events.

2.2.2.2.5 Vesicle consumption in the ER-Golgi interface of yeast

As mentioned above the anterograde vesicular transport within the ER-Golgi interface is mediated by COPII-coated vesicles, whereas COPI vesicles travel back from the Golgi to the ER. The tethering of COPII vesicles to the Golgi membrane (Fig. 9) is mediated by the Rab GTPase Ypt1p, the coiled-coil tethering factor Uso1p, and the multisubunit tethering complex TRAPPI (Allan *et al.*, 2000; Cao *et al.*, 1998; Peng & Gallwitz, 2002; Sinka *et al.*, 2008; Spang, 2009). In the course of the tethering process, multiple molecules of Uso1p supposedly act as “tentacles” to capture vesicles loaded with specific Rab GTPases (Sinka *et al.*, 2008; Spang, 2009). TRAPPI however, on one hand acts as a GEF for the Rab GTPase Ypt1p (Cai *et al.*, 2008; Jones *et al.*, 2000; Wang *et al.*, 2000) and on the other hand tethers the COPII vesicles via an interaction with the Sec23/24p subcomplex (Cai *et al.*, 2007b).

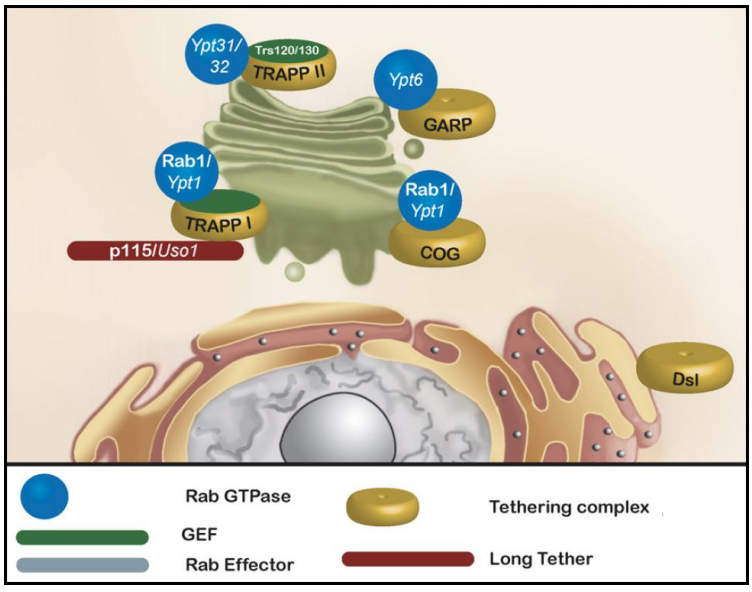

Fig. 9: ***Rab GTPases and tethering factors in the ER-Golgi interface***
(adapted from Markgraf et al., 2007)

The trans-SNARE complexes responsible for the fusion of ER-derived COPII vesicles with the cis-Golgi (Fig. 10) are formed by the t-SNARE Sed5p and the v-SNAREs Bos1p, Bet1p and Sec22p or Ykt6p, which can functionally replace each other in vivo (Cao & Barlowe, 2000; Jahn & Scheller, 2006; Liu & Barlowe, 2002; Parlati *et al.*, 2000; Spang & Schekman, 1998). The SM protein Sly1p has been shown to enhance and confer specificity to the formation of Golgi trans-SNARE complexes (Kosodo *et al.*, 2002; Peng & Gallwitz, 2002; Peng & Gallwitz, 2004).

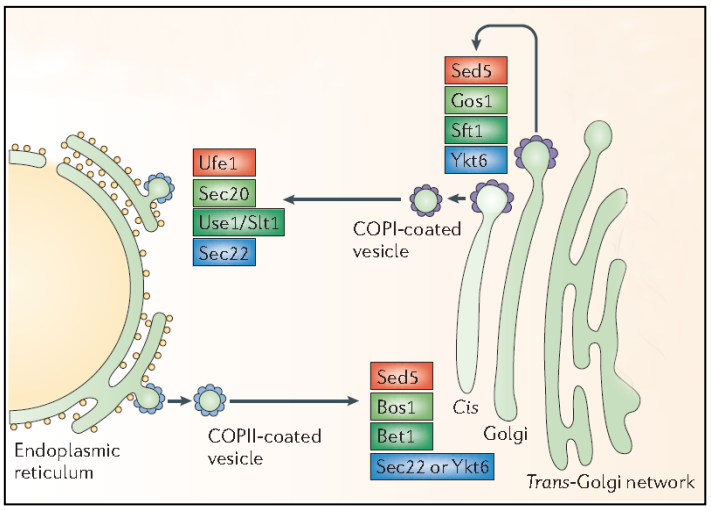

Fig. 10: ***SNARE proteins in the ER-Golgi interface*** *(adapted from Jahn & Scheller, 2006)*

The tethering factor for Golgi-derived COPI vesicles to the ER is the multisubunit tethering complex, Dsl1 (Fig. 11) (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009; Yu & Hughson, 2010; Zink *et al.*, 2009). The Dsl1 complex consists of three subunits, Dsl1p, Dsl3p and Tip20p. All of these subunits are encoded by essential genes and temperature-sensitive mutations in any of them cause a block in retrograde transport from the Golgi to the ER (Kamena & Spang, 2004; Kraynack *et al.*, 2005; Zink *et al.*, 2009). A lasso-like structure within Dsl1p interacts with subunits of coatomer, probably tethering COPI vesicles to the ER, and has also been shown to assists in the final uncoating of these vesicles (Andag *et al.*, 2001; Andag & Schmitt, 2003; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009; Yu & Hughson, 2010; Zink *et al.*, 2009). The trans-SNARE-complexes, responsible for the fusion of Golgi-derived COPI vesicles (Fig. 10) with the ER are comprised of the v-SNARE Sec22p and the three t-SNAREs Sec20p, Ufe1p and Use1p (Burri *et al.*, 2003; Dilcher *et al.*, 2003; Jahn & Scheller, 2006; Lewis *et al.*, 1997). Furthermore, it was suggested that another v-SNARE, Bet1p, also plays a role in the fusion of retrograde transport carriers (Spang & Schekman, 1998). The SM protein Sly1p was shown to be also involved retrograde transport from the Golgi back to the ER (Li *et al.*, 2005). The Dsl1 tethering complex associates with the three t-SNAREs (Sec20p, Ufe1p and Use1p) at the ER and this association is believed to be responsible for the localization of the complex at the ER (Andag *et al.*, 2001; Andag & Schmitt, 2003;

Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009). The interaction sites for Use1p and Sec20p in the Dsl3p and the Tip20p subunits, respectively, have been identified (Ren *et al.*, 2009; Tripathi *et al.*, 2009).

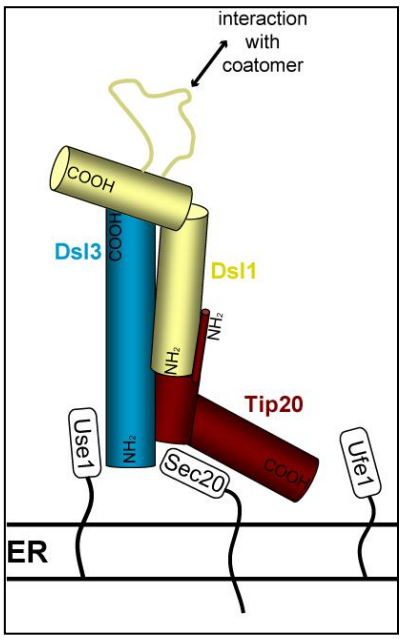

Fig. 11: ***Dsl1 tethering complex and interacting ER t-SNAREs***
(adapted from Ren et al., 2009)

In addition, it was shown recently that the Dsl1 tethering complex has a stimulatory effect on the assembly of ER trans-SNARE complexes (Ren *et al.*, 2009). A Rab GTPase that plays a role in the fusion of COPI vesicles with the ER, however, had not been identified prior to this work.

3 Aim of this study

Proteins destined for secretion from the cell or for diverse intracellular organelles like the ER, the Golgi, or the lysosomal/endosomal system are transported along the so-called secretory pathway. The ER-Golgi interface represents the first stage of this pathway. COPII-coated vesicles transport secretory cargo form the ER to the Golgi. During this process ER-resident proteins are transferred from their resident organelle. In order to maintain the identity and integrity of the ER, components are retrieved back to the compartment they originated from. This process is mediated by COPI-coated vesicles. In the last years some players involved in the fusion of these Golgi derived vesicles with the ER membrane have been identified. Nevertheless, one unanswered question concerning vesicular fusion with the ER was the involvement of Rab/Ypt proteins. One aim of this study therefore was to examine the requirement of Rab/Ypt proteins in the transport from the Golgi to the ER and potentially to identify the responsible candidate. Rab proteins and tethering factors cooperate in making the first contact between vesicles at their target membrane. The ER tethering complex Dsl1 is known to capture COPI-coated vesicles via an interaction with their coat. However, it also was shown that the Dsl1 complex is associated with ER target SNAREs (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009). Furthermore, a temperature-sensitive mutant of one of the Dsl1 complex members, the *tip20-8* mutant, shows a remarkable phenotype. It does not interfere with the generation of COPII vesicles from the ER, but allows these vesicles to fuse back to their donor compartment (Kamena & Spang, 2004), a process which is normally prohibited in the cell. To further examine this phenotype and the underlying mechanisms we analyzed mutant alleles of *TIP20* in the second part of this study.

4 Results and Discussion

At the ER-Golgi interface newly synthesized proteins travel in COPII vesicles from the ER to the Golgi, whereas COPI vesicles transport cargo, e.g. escaped ER-resident proteins, from the Golgi back to the ER. In this work, we investigated this retrograde transport, in particular the fusion of COPI vesicles with the ER.

It previously was shown that Ypt1p is the Rab GTPase responsible for the fusion of COPII vesicles with the Golgi (Allan *et al.*, 2000; Cao *et al.*, 1998; Peng & Gallwitz, 2002; Sinka *et al.*, 2008). If a Rab GTPase is also required for the fusion of COPI vesicles at the ER, however, was not known. The so-called round trip or retrieval assay (Spang & Schekman, 1998), essentially recapitulates the transport of a reporter protein from the ER to the Golgi complex and back to an acceptor ER. It allows the manipulation of transport from the Golgi to the ER without affecting the forward transport. In the first part of this study, we therefore used this assay to address the question whether fusion of COPI vesicle with the ER is dependent on Rab/Ypt proteins and, if so, which Rab/Ypt protein is responsible for this event. GDP dissociation inhibitors (GDIs) bind the GDP-bound form of Rab proteins and thereby keep them inactive (Sasaki *et al.*, 1990). Only one Rab-specific GDI, Gdi1p, which most likely acts on all Rabs/Ypts, exists in yeast. We found that addition of Gdi1p significantly reduced the amount of the reporter retrieved to the ER in wildtype cells in the round trip assay. Thus, we could establish a requirement for a Ypt protein in retrograde transport from the Golgi to the ER. Testing temperature-sensitive (ts) mutants of multiple Rab proteins, we found that a ts-mutant of *ypt1* affects the efficient retrieval of the reporter to the ER, indicating that Ypt1p is involved in retrograde transport. Secretion of the ER-resident protein Kar2p is a generally used indicator for retrograde transport defects. The observation that Kar2p was secreted by *ypt1* mutant cells therefore substantiated the assumption that Ypt1p is involved in the fusion of COPI vesicles at the ER. The round trip assay, however, does not allow to discriminate between a defect in COPI vesicle generation at the Golgi or a defect of COPI vesicle consumption at the ER. In order to pinpoint the defect observed in the *ypt1* ts-mutant, we performed budding assays to generate COPI vesicles from enriched Golgi membranes. Unexpectedly, in comparison to Golgi membranes from a wildtype strain, membranes from the *ypt1* mutant formed abnormal COPI-coated vesicles. These vesicles were of lower buoyant density, contained coatomer but lacked the ER-Golgi t-SNARE Sec22p and the cargo protein Emp47p. These results indicated that Ypt1p is required for the proper formation of COPI vesicles at the Golgi. Based on this observation, we wondered if the defect in COPI vesicle generation arose from an altered Golgi. To address this question, we analyzed Golgi membranes obtained from a wildtype strain and the *ypt1* mutant for their content of different Golgi proteins and Golgi-associated proteins. We found that the concentrations of the Golgi

enzymes Anp1p and Mnn1p, the cargo Emp47p and the v-SNARE Sec22p were all dramatically reduced, whereas the concentration of coatomer remained constant. The level of the small GTPase Arf1p was increased, and about half of the t-SNARE Sed5p and the v-SNARE Bos1p were lost from Golgi membranes. We furthermore observed a change in Golgi morphology and a loss of Golgi cisternae at the restrictive temperature in the *ypt1* mutant strain. Taken together, this showed that in the *ypt1* mutant the Golgi integrity is severely affected and led us to the assumption that Ypt1p is necessary for maintaining the Golgi morphology and its protein composition. Since we observed this dramatic effect on the Golgi in the *ypt1* mutant we wondered if it was still functional. *Ypt1* mutant cells were insensitive to osmotic stress and could still perform glycosylation efficiently. We therefore concluded that the Golgi of the *ypt1* mutant is at least partially functional. Yet, the question whether Ypt1p is involved in the fusion of COPI vesicles with the ER remained unsolved. During the fusion of COPII vesicles to the Golgi, Ypt1p binds to the t-SNARE Sed5p. Conversely, Ypt1p, if involved in the fusion of COPI vesicles with the ER, might also interact with a t-SNARE there. One of the t-SNAREs at the ER is Ufe1p. Ufe1p only displays weak homology to other SNARE proteins, its closest homolog is Sed5p. Moreover, Sed5p and Ufe1p were shown to bind the SM protein Sly1p using the same motif at the N-terminus (Yamaguchi *et al.*, 2002). We therefore decided to test for direct binding of Ypt1p to Ufe1p by *in vitro* pulldown assays and found specific binding of Ypt1p to Sed5p and Ufe1p. In addition, a genetic interaction between *UFE1* and *YPT1* was established, demonstrating that Ypt1p interacts with Ufe1p physically and genetically.

Taken together, we were able to show that retrograde transport is dependent on the action of a Rab/Ypt. Although the retrieval defect in the round trip assay observed for the *ypt1* mutant strain is probably mainly due to the defect in COPI vesicle budding, we still can propose a function of Ypt1p in the fusion of COPI vesicles with the ER. The physical and genetic interaction with Ufe1p, which acts as t-SNARE at the ER, support a direct involvement of Ypt1p in the fusion process at the ER. Interestingly Golgi membranes of a *ypt1* mutant strain could not form normal COPI vesicles. This links Ypt1p with COPI vesicle formation. It previously was shown that Ypt1p plays a role in the fusion of COPII with the Golgi (Allan *et al.*, 2000; Cao *et al.*, 1998; Peng & Gallwitz, 2002; Sinka *et al.*, 2008). Moreover, (Morsomme & Riezman, 2002) showed that Ypt1 is needed for sorting of GPI-anchored proteins therefore functions in the generation of COPII vesicles from the ER. Thus, our data suggest that Ypt1p might be required at each organelle-vesicle transition step in the ER-Golgi shuttle, (I) the formation of COPII vesicles at the ER; (II) the consumption of COPII vesicles at the Golgi; (III) the budding of COPI-coated vesicles from the Golgi; and (VI) the fusion of Golgi-derived vesicles with the ER. Moreover, Ypt1p seems to be important for Golgi maintenance *in S. cerevisiae.* Since Ypt1p is implicated in various processes, it is likely that many more

interactors and regulators than currently described exist. The identification of these would be of great use in better understanding both the function of Ypt1p as well as the mechanisms behind its recruitment to various locations. This in turn would greatly improve the understanding of the regulation of vesicular fusion at the ER-Golgi interface.

It is known that the first contact between an arriving vesicle with its acceptor membrane is mediated by the combined action of Rabs/Ypts and tethering factors. Only one tethering factor involved in the fusion of COPII vesicles, the Dsl1 tethering complex, has been identified to date. This makes the Dsl1 complex an ideal candidate for a novel interactor of Ypt1p. Three essential, peripheral membrane proteins, Dsl1p, Dsl3p and Tip20p, form the Dsl1 complex. During the tethering of COPI vesicles to the ER, the Dsl1p subunit of the complex interacts with the coat of these vesicles (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009). Previously it was shown that a specific temperature-sensitive allele of one of the Dsl1 tethering complex members, the *tip20-8* allele, does not interfere with COPII vesicle generation from the ER, but allows these vesicles to fuse back to their donor compartment (Kamena & Spang, 2004). This process normally does not occur in the cell. Furthermore, another allele of *TIP20*, the *tip20-5* allele, also displayed a temperature-sensitive growth phenotype but did not show such a backfusion phenotype.

In order to examine the phenotypes of the *tip20* ts-mutants further, and to investigate their impact on the Dsl1 complex, we analyzed the *tip20-5* and *tip20-8* alleles in the second part of this study. Sequencing revealed 9 and 6 amino acid changes in *tip20-5* and *tip20-8*, respectively. In both cases, the mutations do not cluster on the linear sequence, and mapping them in the crystal structure showed that they are quite evenly distributed over the protein. In the next step, we wanted to examine if individual point mutations could recapitulate the growth phenotype of *tip20-8*. For this reason, we analyzed yeast strains expressing variants of Tip20p that contain only one selected single point mutation identified in *tip20-8* for a potential temperature-sensitive growth phenotype. None of the single point mutations showed any growth defect. This demonstrates that the function of Tip20p is not severely altered by any of the individual point mutations. Thus an individual mutation alone may not be responsible for the *tip20-8* phenotype. Since in differential centrifugation experiments Tip20-8p still could be found in the P13 membrane fraction, which contains mainly ER, we concluded that a mislocalization of the protein is probably not the cause for the defects in *tip20-8*. In order to analyze how the mutations in Tip20-8p affect the structure of the protein, molecular dynamics simulations were performed. These showed that Tip20-8p is more flexible than wildtype Tip20p. The increased flexibility is most evident in the N-terminus and in several areas within the α-helical stalk of the protein, including the binding site for the t-SNARE Sec20p. Within the Dsl1 complex, the N-terminus of Tip20p interacts

with the N-terminus of Dsl1p. Because of the observed increased fluctuations of the N-terminus, we wondered if this part is important for the function of Tip20p. Analyzing strains expressing variants of Tip20p, which can no longer interact with Dsl1p, for a potential temperature sensitive growth phenotype and membrane association showed that the N-terminus of Tip20p is not required for growth or membrane association. The direct interaction of Dsl1p and Tip20p therefore appears not to be essential for the function of the Dsl1 complex. Thus, we analyzed if other known interactions of Tip20p were impaired in *tip20-5* and *tip20-8* mutants. For this purpose, we performed affinity purifications and found that Tip20-5p and Tip20-8p can no longer efficiently interact with their binding partners, e.g. Dsl1p and Sec20p. *In vitro* binding studies also confirmed this decreased binding of Tip20-5p and Tip20-8p to Dsl1p and Sec20p. Therefore, we wanted to analyze the effect of Tip20p mutant proteins on *in vitro* reconstituted Dsl1 complexes. We found that in presence of Tip20-8p or Tip20-5p less Dsl1 complexes are formed *in vitro*. The Dsl1 complex stably associates with the three t-SNAREs found at the ER (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009). In a recent study, it was suggested that the Dsl1 complex modestly accelerates the assembly of the SNARE complexes that are responsible for the fusion of COPI vesicles with the ER (Ren *et al.*, 2009). These complexes consist of the t-SNAREs Sec20p, Ufe1p, Use1p and the v-SNARE Sec22p and/or Bet1p. Since Tip20-5p and Tip20-8p cannot efficiently bind to Sec20p and other Dsl1 complex members, we wanted to examine how the assembly of the ER SNARE complex is affected by Tip20-8p and Tip20-5p. *In vitro* reconstitution assays showed that the Tip20p mutants cause the depletion of Sec20p, Use1p, Sec22p and Bet1p from trans-SNARE complexes at the ER. Taken together, our data indicate that Tip20p is required for proper assembly of cognate SNARE complexes at the ER. Interestingly, Ykt6p, a SNARE that can substitute for Sec22p in the fusion of COPII with the Golgi, could efficiently interact with Ufe1p, irrespective of the presence of wildtype or Tip20p mutants and even under competition conditions with Sec22p. However, this interaction did not improve the recruitment of Sec20p, Use1p or Sec22p to the complex. Moreover, non-cognate SNAREs, e.g. the v-SNARE at the Golgi, Bos1p, or the plasma membrane v-SNARE Snc1p, could not be recruited into SNARE complexes with ER t-SNAREs, demonstrating that the observed defects are specific for the cognate ER SNARE complexes. Our data therefore indicate that in the presence of Tip20p mutant proteins, ER trans-SNARE complex assembly is severely altered and that the number of these complexes is dramatically decreased in *tip20-5* and *tip20-8*. Taken together, our results provide evidence for a novel function of the Dsl1 tethering complex in the proofreading and stabilization of cognate ER trans-SNARE complexes. The association with SNARE proteins and a proofreading of trans-SNARE complex assembly has also been observed for other tethering factors. The conserved

oligomeric Golgi (COG) complex in mammalian cells e.g. interacts with the t-SNARE at the Golgi and, when knocked down, decreases the steady-state levels of intra-Golgi SNARE complexes (Shestakova *et al.*, 2007). Furthermore, the HOPS complex suppresses the formation of non-cognate trans-SNARE complexes in vacuolar fusion in yeast (Starai *et al.*, 2008). Finally, we found, that the presence of the Tip20p mutants in the Dsl1 complex disturbs its ability to bind to COPI vesicle coat. Therefore, in the *tip20-8* strain a defect in the proofreading or stabilization of cognate trans-SNARE complexes together with an inability of the Dsl1 complex to interact with COPI vesicles might allow the atypical, unspecific fusion of COPII vesicles. Interestingly, despite the fact that a backfusion phenotype could not be observed for *tip20-5* in the *in vitro* assay used to test for this phenotype, the short cut assay (Kamena & Spang, 2004), Tip20-5p behaved similar to Tip20-8p in the affinity purifications and in the *in vitro* pulldowns. The *tip20-8* strain, however, displays a growth phenotype at 30°C and higher, whereas the *tip20-5* strain only ceases to grow at 37°C. Our results therefore indicate, that *tip20-5* maybe a weaker allele than *tip20-8.* The experimental setup in the short cut assay may thus not be stringent or sensitive enough to detect backfusion of COPII vesicles in *tip20-5*. One open question that remains, is the mechanism by which the Dsl1 complex proofreads and stabilzes the SNARE complex formation. Due to the flexible hinges within its structure, the Dsl1 complex can assume an open and a closed conformation (Ren *et al.*, 2009). Our Molecular Dynamics studies revealed an increase of the flexibility of Tip20-8p. The mutations in Tip20-8p might therefore affect the SNARE complex assembly directly by changes in the binding site for Sec20p and/or indirectly by affecting the overall structure of the Dsl1 complex. The observed decrease of coatomer recruitment to Dsl1 complexes in the presence of Tip20-5p and Tip20-8p is most likely a direct consequence of the lack of binding of Dsl1p. However it cannot be excluded that an additional binding site for coatomer in Tip20p itself exists that potentially could be affected by the mutations in Tip20-8p. One further topic to be addressed is, if the Rab Ypt1p and the Dsl1 complex indeed interact as would be predicted when comparing the Dsl1 complex to other tethers. The role of such a potential interaction in the SNARE complex proofreading and/or the coatomer binding function would need to be established. Moreover, questions about the involvement of SM proteins, like Sly1p, in tethering complex-dependent SNARE complex assembly will have to be addressed in the future.

5 Publications and Manuscripts

5.1 Ypt1p is essential for retrograde Golgi-ER transport and for Golgi maintenance in *S. cerevisiae*

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The following authors have contributed to the manuscript:

Faustin Kamena performed the experiments represented in the following figures:
Fig. 1 A, B, C, E; Fig. 2 A, B, C; Fig. 3 A, B and Fig.8
He wrote parts of the manuscript and provided critical comments on the rest.

Melanie Diefenbacher performed the experiments represented in the following figures:
Fig. 1 D; Fig. 4 A, B, C; Fig. 5; Fig. 6A and Fig. 9
She wrote parts of the manuscript and provided critical comments on the rest.

Cornelia Kilchert performed the experiment represented in the following figure:
Fig. 6 B
She wrote parts of the manuscript and provided critical comments on the rest.

Heinz Schwarz performed the EM analysis represented in the following figure:
Fig. 7

Anne Spang contributed to:
Fig. 1 D and Fig. 7.
She wrote the manuscript.

Research Article 1293

Ypt1p is essential for retrograde Golgi-ER transport and for Golgi maintenance in *S. cerevisiae*

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Summary

The small GTPase Ypt1p of the Rab family is required for docking of ER-derived transport vesicles with the Golgi prior to fusion. However, the identity of the Rab protein that mediates docking of Golgi-derived COPI vesicles with the ER in retrograde transport remains elusive. Here, we show that in yeast Ypt1p is essential for retrograde transport from the Golgi to the ER. Retrieval of gpαF-HDEL (glycolylated pro-α-factor with an HDEL tag at the C-terminus) was blocked in *Δypt1/SLY1-20* membranes at the restrictive temperature in vitro. Moreover, Ypt1p and the ER-resident t-SNARE Ufe1p interact genetically and biochemically, indicating a role for Ypt1p in consumption of COPI vesicles at the ER. Ypt1p is also essential for the maintenance of the morphology and the protein composition of the Golgi. Interestingly, the concentrations of the Golgi enzymes Anp1p and Mnn1p, the cargo protein Emp47p and the v-SNARE Sec22p were all substantially reduced in Golgi from a *Δypt1/SLY1-20* strain as compared with wild-type Golgi, while the concentration of Arf1p and of coatomer were mildly affected. Finally, COPI vesicles generated from *Δypt1/SLY1-20* Golgi membranes in vitro were depleted of Emp47p and Sec22p. These data demonstrate that Ypt1p plays an essential role in retrograde transport from the Golgi to the ER.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/8/1293/DC1

Key words: ER-Golgi shuttle, YPT1, Rab, Retrograde transport, Yeast

Introduction

Proteins destined for secretion are first translocated into the lumen of the ER where they are core glycosylated. Subsequently, they are incorporated into COPII vesicles, which are en route to the Golgi complex. Upon arrival in the Golgi complex, proteins are further modified and sorted at the *trans*-Golgi network to reach their final destinations. Protein and membrane traffic at the ER-Golgi interface is bi-directional. Transport factors such as SNARE proteins, cargo receptors and ER-resident proteins that have escaped the ER retention system, are packaged into COPI vesicles and retrieved back to the ER in order to preserve organelle integrity of both the ER and the Golgi complex.

Fusion of both COPII and COPI vesicles with their respective target membranes is tightly regulated. In principle, the general fusion process can be subdivided into tethering, docking and fusion (Bonifacino and Glick, 2004). Tethering factors at the Golgi are Uso1p, TRAPPI and the COG complex, whereas at the ER membrane this function seems to be fulfilled by Dsl1p, Tip20p and probably other yet unspecified proteins (Andag et al., 2001; Kamena and Spang, 2004; Ram et al., 2002; Reilly et al., 2001). Docking of vesicles employs Rab/Ypt proteins. Ypt1p is the Rab protein required for anterograde ER-Golgi transport, whereas the Rab essential for retrograde transport remains elusive (Segev, 2001b). Finally, a recognition step mediated by the SNARE proteins leads to close opposition of the membranes of the vesicle and the target compartment and subsequently to fusion of the lipid bilayers. The precise mechanism by which fusion occurs is still a matter of debate (Peters et al., 2001; Weber et al., 1998).

Rab/Ypt proteins are small GTPases of the ras superfamily. They cycle between a GTP-bound (active) and a GDP-bound (inactive) form. The yeast genome encodes 11 *YPT*s. Ypt51/52/53p, Ypt10p and Ypt7p act in endocytosis en route to the vacuole, whereas Ypt1p, Ypt31p and Ypt32p function in exocytosis (Buvelot Frei et al., 2006; Segev, 2001a). Ypt6p is thought to be involved in both endocytosis and exocytosis (Li and Warner, 1996). Ypt11p seems to be involved in ER inheritance (Buvelot Frei et al., 2006). Ypt1p is crucial for the ER to Golgi transport and is also important for intra Golgi transport (Bacon et al., 1989; Baker et al., 1990; Jedd et al., 1995; Segev et al., 1988). Ypt31p and Ypt32p are partially functionally redundant and are both involved in protein exit from the trans-Golgi (Benli et al., 1996; Jedd et al., 1997). The switch between the GDP-bound and the GTP-bound state is mediated by specific guanine nucleotide exchange factors (GEFs) whereas GTPase activating proteins (GAPs) regulate the hydrolysis of GTP. GDI, the GDP dissociation inhibitor, is an additional regulator of Rab/Ypt proteins that sequesters the GDP-bound form of the GTPase and prevents the exchange of GDP for GTP, thereby inhibiting recruitment to the membrane (Araki et al., 1990; Garrett et al., 1994; Sasaki et al., 1990). A single GDP dissociation inhibitor, Gdi1p, has been identified in *Saccharomyces cerevisiae*. *GDI1* is essential for cell viability and can probably act on all Ypt proteins (Dirac-Svejstrup et al., 1994; Haas et al., 1995; Peter et al., 1994; Ullrich et al., 1993).

Despite the importance of Rab/Ypt proteins and although vesicle fusion at the ER-Golgi interface has been subject of intensive research, it is still not known which Rab/Ypt protein functions at

the fusion step of COPI vesicles with the ER. In this study, we identify Ypt1p as the Rab involved in the retrograde transport from the Golgi to the ER. In addition, we provide evidence for an additional function of Ypt1p in maintaining Golgi identity and integrity.

Results

Gdi1p inhibits retrieval of [^{35}S]gpαF-HDEL from the Golgi to ER in vitro

To identify the Rab protein involved in fusion of retrograde transport vesicles with the ER, we used a cell-free transport system called the round-trip assay (Spang and Schekman, 1998). The round-trip assay has been used successfully to characterize several proteins involved in retrograde transport from the Golgi to the ER (Kamena and Spang, 2004; Poon et al., 1999; Spang et al., 2001). Essentially, the round-trip assay recapitulates the transport of a reporter protein from the ER to the Golgi complex and back to an acceptor ER (supplementary material Fig. S1). As a reporter we use radioactively labeled prepro-α-factor with an HDEL tag at the C-terminus ([^{35}S]ppαF-HDEL) (Dean and Pelham, 1990). A glucan trimming reaction provides the means to determine successful retrograde transport to the acceptor ER. Trimmed and untrimmed forms can be distinguished by their differential mobilities on SDS-PAGE, and the trimmed form of [^{35}S]gpαF-HDEL is produced only upon successful return to the ER. The untrimmed band represents [^{35}S]gpαF-HDEL present in the Golgi or in vesicles that have docked but not fused and cannot be correlated to the amount of trimmed [^{35}S]gpαF-HDEL.

We first asked, whether retrograde transport actually requires the action of a Rab protein. The GDP dissociation inhibitor Gdi1p binds the GDP-bound form of Rab proteins and can inhibit the exchange of GDP for GTP (Sasaki et al., 1990). We took advantage of the presence of only one Rab-specific GDI in yeast, which most probably acts on all yeast Rabs (Ypts). We performed a round-trip assay and compared the retrieval efficiency of [^{35}S]gpαF-HDEL to the ER in the presence and in the absence of exogenously added purified Gdi1p. The amount of [^{35}S]gpαF-HDEL that reached the ER in the presence of Gdi1p was significantly reduced compared with the control (Fig. 1A, compare lanes 1 and 2). This reduction of the signal in the presence of Gdi1p was independent of its ability to inhibit COPII vesicle fusion with the Golgi because purified Gdi1p was only added to the reaction after the COPII vesicles had fused with the Golgi. Furthermore, after the fusion of the COPII vesicles with the Golgi, the membranes were washed extensively prior to the addition of cytosol, in order to avoid any residual fusion activity of COPII vesicles with the Golgi complex. Therefore, Gdi1p blocks retrieval of [^{35}S]gpαF-HDEL to the ER in vitro, and therefore establishes a requirement for a Ypt protein in retrograde transport from the Golgi to the ER.

Retrieval of [^{35}S]gpαF-HDEL is defective in *Δypt1/SLY1-20* in vitro

The result above indicates that retrograde transport depends on the action of at least one Ypt. We sought to determine which Ypt is involved in retrograde transport from the Golgi to the ER and decided to follow those Rabs involved in exocytosis, namely Ypt1p, Ypt31/32p and Ypt6p. *YPT1* is essential, but the loss of *YPT1* can be rescued by the expression of a mutation in *SLY1*, *SLY1-20*, which renders the strain temperature sensitive (ts) (Dascher et al., 1991; Ossig et al., 1991). Sly1p was isolated as suppressor of *YPT1* loss of function. Sly1p is a member of the Sec1p family and may promote target SNARE complex formation at the Golgi (Dascher et al., 1991; Kosodo et al., 2002; Peng and Gallwitz, 2002). We also used a second ts mutant, *ypt1-3*, which has been used extensively for in

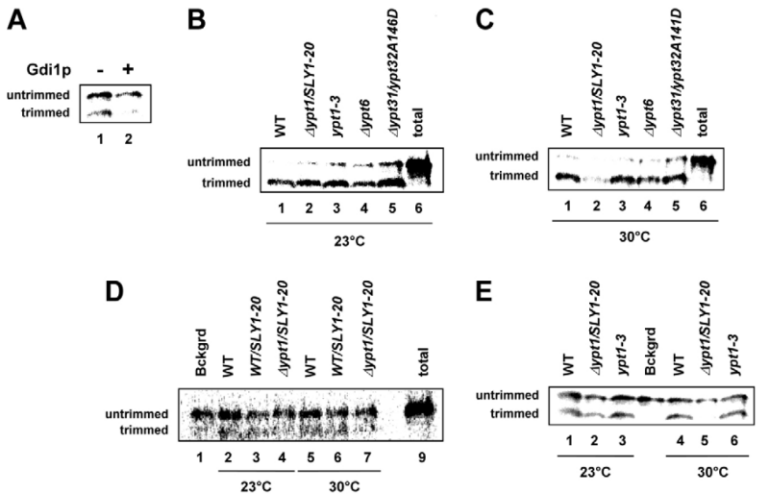


Fig. 1. Ypt1p is required for retrograde transport from the Golgi to the ER. (A) Addition of GDI reduced the retrieval efficiency of [^{35}S]gpαF-HDEL to the ER. A round-trip assay was performed with wild-type semi-intact cells. GDI was added to the round-trip assay after the fusion of COPII vesicles with the ER. The amount of trimmed [^{35}S]gpαF-HDEL in lane 2 was reduced to 63% compared with that in the buffer control (100%). In the buffer control 1.14% and after addition of GDI 0.73% of the [^{35}S]gpαF-HDEL incorporated into COPII vesicles at the donor ER reached the acceptor ER and was trimmed. (B) *YPT* mutants are not defective in retrograde transport at the permissive temperature. Semi-intact cells from different ts-mutants and wild type were used as acceptor membranes in a round-trip assay. The last step, the retrieval from the Golgi to the ER, was performed at the permissive temperature, 23°C. All semi-intact cells gave a comparable signal of trimmed [^{35}S]gpαF-HDEL. (C) Deletion of *YPT1* results in a reduction of retrograde transport from the Golgi to the ER. In parallel to the assay in B an assay was performed, in which the temperature was raised in the last step to 30°C. This temperature should represent at least a semi-restrictive temperature for most ts-mutants. The signal did not alter significantly for most mutants when compared with wild type, but the signal of retrieved and trimmed [^{35}S]gpαF-HDEL was strongly diminished in *Δypt1/SLY1-20* acceptor membranes. (D) *SLY1-20* expression does not contribute to the defect in retrograde transport. A retrieval assay was performed as described above. In *Δypt1/SLY1-20* membranes the signal in the retrograde transport assay was drastically reduced at 30°C, but the transport in WT/*SLY1-20* was as efficient as in WT. (E) *ypt1-3* is not defective in retrograde transport in vitro. A retrieval assay was performed to compare two different *YPT1* mutants side by side. *Δypt1/SLY1-20* membranes were unable to allow retrograde transport of [^{35}S]gpαF-HDEL at the restrictive temperature, *ypt1-3* membranes behaved like wild-type semi-intact cells. 'Bckgrd' is an assay using wild-type membranes, but cytosol was omitted in the last incubation step (transport from the Golgi to the ER). The retrieval efficiency was determined as percentage of trimmed [^{35}S]gpαF-HDEL of the reporter that was incorporated into COPII-coated vesicles at the ER. At 20°C: WT 2.9 %, *Δypt1/SLY1-20* 2.34 %, *ypt1-3* 3.15 %; at 30°C: WT 2.92 %, *Δypt1/SLY1-20* 1.28%, *ypt1-3* 3.05%.

vivo studies (Cao et al., 1998; Morsomme and Riezman, 2002). The *Δypt31/ypt32A141D* is a temperature-sensitive strain bearing a point mutation in *YPT32* in a *Δypt31* background (Jedd et al., 1997). *YPT6* is a non-essential gene but deletion of the gene results in growth defects at 37°C (Li and Warner, 1996). The use of semi-intact cells derived from these strains in the round-trip assay combined with a temperature shift in the last step of the assay should enable us to determine, which Ypt is required for retrograde transport from the Golgi to the ER.

When the round-trip assay was performed at the permissive temperature, the retrieval of [^{35}S]gpαF-HDEL in the *ypt* mutant membranes was indistinguishable from that in the wild-type membranes (Fig. 1B). By contrast, when we shifted the incubation temperature in the last stage (retrieval from the Golgi to the ER) of the round-trip assay to 30°C, which should be at least a semi-restrictive temperature for these mutants, the amount of retrieved [^{35}S]gpαF-HDEL to the ER in *Δypt1/SLY1-20* membranes was strongly reduced (Fig. 1C, lane 2). The presence of the *SLY1-20* plasmid itself did not exert any negative effect on the retrograde transport, because the retrieval efficiency of [^{35}S]gpαF-HDEL was not altered in a wild-type strain expressing *SLY1-20* (Fig. 1D). All other mutants, including *ypt1-3*, showed retrieval efficiencies similar to that of the wild type (Fig. 1C,E). The temperature shift to 30°C in the last step of the assay might still be permissive for *ypt1-3* in vitro. However, raising the temperature further is not possible in this in vitro system, because the membranes become leaky and make the interpretation of the assay impossible. In addition, Ballew et al. (Ballew et al., 2005) showed that Ypt6p becomes essential in *Δypt1/SLY1-20*, indicating that it can take over part of the Ypt1p functions. Therefore, it is possible that Ypt6p compensates for the transport defect in *ypt1-3* in vitro. Nonetheless, the results indicate that the defect in retrograde transport might be due to the loss of Ypt1p function in *Δypt1/SLY1-20*.

Ypt1-3 semi-intact cells are permissive for anterograde transport to the Golgi in vitro

To determine whether *ypt1-3* might be functional at 30°C in vitro, we performed an anterograde ER-Golgi transport assay (Fig. 2). The role of Ypt1p in fusion of COPII vesicles with the Golgi is well established (Cao et al., 1998). Furthermore, a constitutive defect at the permissive temperature in the fusion of COPII vesicles with the Golgi could potentially abolish the round-trip and lead to a reduction of the amount of trimmed [^{35}S]gpαF-HDEL as was seen with the *Δypt1/SLY1-20* strain. To test this possibility, we measured the amount of anti-α-1,6-linked mannose-precipitable [^{35}S]gpαF-HDEL, which is an indicator of the arrival of the reporter in the Golgi. At the permissive temperature, the amount of precipitable [^{35}S]gpαF-HDEL was similar in all membranes tested (Fig. 2A). Additionally, as expected, at the restrictive temperature, the *Δypt1/SLY1-20* mutant strain displayed a drastic reduction in the transport of [^{35}S]gpαF-HDEL to the Golgi (Fig. 2B). This result confirms that there is no constitutive defect in anterograde transport in *Δypt1/SLY1-20* under the conditions of our assay. However, the *ypt1-3* semi-intact cells were permissive for anterograde transport from the ER to the Golgi *in vitro* even at the restrictive temperature (Fig. 2B). We conclude, therefore, that the decrease in function of the *ypt1-3* mutant is insufficient to be detectable in our assay. Taken together, these results suggest that Ypt1p is essential for retrograde transport form the Golgi to the ER.

To substantiate our finding that Ypt1p is involved in retrograde transport, we scored the secretion of Kar2p. The chaperone Kar2p is an ER-resident protein, which can escape to the Golgi and is retrieved back to the ER by the HDEL-receptor Erd2p (Semenza et al., 1990). However, if retrograde transport is defective, Kar2p is secreted. We assessed secretion of Kar2p by colony blot after incubation at 30°C (Fig. 2C). We chose 30°C as semi-permissive temperature for the secretion assay, because all strains still grew at about the same rate at this temperature, so that unspecific effects caused by differences in cell number and growth behavior could be excluded. Although no Kar2p was secreted in the wild-type strain, both *ypt1-3* and *ypt1D136A* secreted Kar2p efficiently, similar to that of *tip20-5* and *dsl1-22*, two retrograde transport mutants with established Kar2p secretion phenotypes (Andag et al., 2001; Cosson et al., 1997). Hence *ypt1-3* and *ypt1D136A* are defective in retrograde transport in vivo. This phenotype can probably be compensated for in our in vitro assays. The *Δypt1/SLY1-20* strain also secreted Kar2p, though to a lesser degree. This result was not unexpected, since *Δypt1/SLY1-20* is a much stronger mutant than the other *ypt1* alleles in the assay and has defects at lower temperatures. Anterograde transport is likely to be more strongly affected in *Δypt1/SLY1-20* than in *ypt1-3* and *ypt1D136A*. The *ypt6* and *ypt31/32* mutants also showed some Kar2p secretion, which is most likely due to an interference with Golgi function. However, a more direct effect is also possible at least for Ypt6 (Ballew et al., 2005). The Kar2p signal was not due to cell lysis, because the cytoplasmic protein Pgk1p was never detected for any of the strains grown at 30°C. Taken together, we find that multiple mutants in *YPT1* secrete Kar2p, supporting a role for Ypt1p in retrograde transport from the Golgi to the ER.

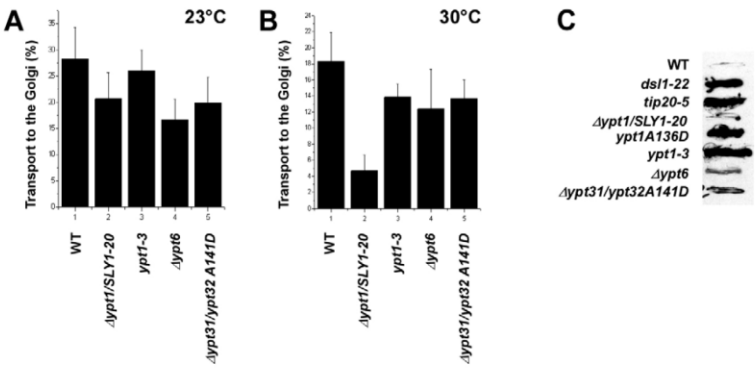


Fig. 2. Behavior of *YPT* mutants in anterograde transport and in a Kar2p secretion assay. (A,B) An ER-Golgi transport assay. The arrival of [^{35}S]gpαF-HDEL in the Golgi was monitored by precipitation with α-1,6-mannose antibodies and protein A-Sepharose. The amount of gpαF-HDEL precipitated by α-1,6-mannose antibodies was normalized to the amount of budded gpαF-HDEL from the ER. The average and standard deviation from at least three independent experiments are shown. The ER-Golgi transport assay was performed at (A) 23°C and (B) 30°C. (C) Mutants in *YPT1* secrete Kar2p. Colony blot of different *YPT* mutants grown overnight at 30°C. Secreted Kar2p was detected by immunoblotting.

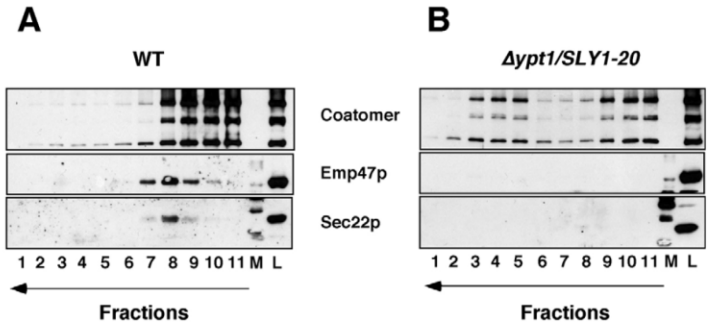


Fig. 3. COPI-coated vesicles from *Δypt1/SLY1-20* Golgi are depleted from cargo and SNAREs. Golgi membranes enriched from either wild-type (A) or *Δypt1/SLY1-20* (B) cells were incubated with Arf1p, coatomer and GTP-γ-S, to generate COPI vesicles. The vesicles were separated by velocity sedimentation centrifugation. The vesicle peak was collected and floated on a buoyant density gradient. Fractions were collected from the top, precipitated, and analyzed by immunoblotting. Sec22p is a v-SNARE in the ER-Golgi shuttle and Emp47p is a vesicle cargo. The arrows indicate the movement of the lipid particles in the gradient.

Enriched *Δypt1/SLY1-20* Golgi membranes are unable to bud functional COPI vesicles in vitro

The best-characterized function of Ypt1p is in the docking of ER-derived vesicles to the Golgi membrane (Cao et al., 1998). Yet, recent investigations have shown that Ypt1p is also required for the sorting of GPI-anchored cargo molecules into ER-derived vesicles (Morsomme and Riezman, 2002). Furthermore, another recent study reports a role for Rab1, the mammalian orthologue of Ypt1p, in cargo uptake into COPI vesicles from the Golgi (Alvarez et al., 2003). If Ypt1p plays a similar role in yeast, then the defect observed in our round-trip assay might arise either from a failure in COPI vesicle generation at the Golgi or, alternatively, from defective consumption of COPI vesicles at the ER. Therefore, we investigated a possible role for Ypt1p in COPI vesicle formation in an in vitro Golgi budding assay (Spang and Schekman, 1998). Enriched Golgi membranes from wild-type or *Δypt1/SLY1-20* cells were incubated with coatomer, Arf1p and guanine nucleotide. The resulting COPI vesicles were purified, first over a sedimentation gradient and then by buoyant density centrifugation. As expected, the wild-type membranes formed normal COPI-coated vesicles as judged by the presence of the cargo Emp47p and the v-SNARE Sec22p in the vesicle fraction (Fig. 3A). By contrast, Golgi membranes from the *Δypt1/SLY1-20* mutant formed abnormal COPI-coated vesicles (Fig. 3B). Although, a coatomer signal was obtained in higher migrating fractions, which indicates a lower buoyant density, these fractions contained much less Emp47p and Sec22p (Fig. 3B). Thus, these vesicles might represent at least partially defective COPI vesicles because they did not contain normal amounts of Sec22p (Spang and Schekman, 1998). Together, these data support a requirement of Ypt1p in the formation of COPI-coated vesicles at the Golgi.

The Golgi is altered in *Δypt1/SLY1-20* mutant cells

Does the defect in COPI vesicle generation arise form a partially dysfunctional Golgi complex? To investigate this possibility, we first compared, by immunoblotting, the content of different Golgi proteins and Golgi-associated proteins in wild-type and *Δypt1/SLY1-20* Golgi membranes. As for the experiments above, the Golgi used for this analysis was enriched from cells that had been grown at the permissive temperature for *Δypt1/SLY1-20*, 23°C. Surprisingly, the concentrations of the Golgi enzymes Anp1p and Mnn1p, the cargo Emp47p and the v-SNARE Sec22p were all dramatically

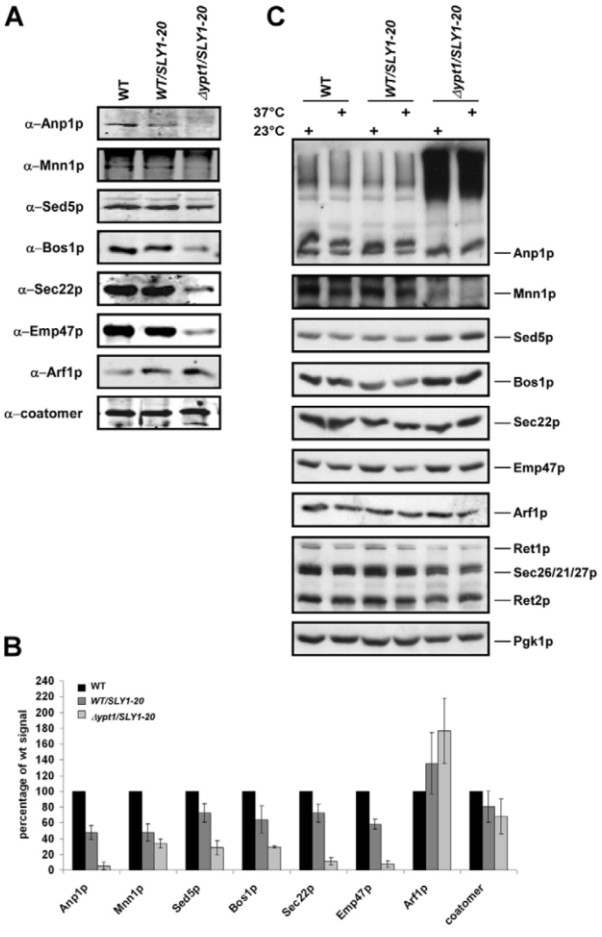


Fig. 4. Wild-type and *Δypt1/SLY1-20* Golgi differ in their protein content. Immunoblots of equal amounts of Golgi membranes enriched from wild-type, wild-type expressing *SLY1-20* and *Δypt1/SLY1-20* cells that were grown at 23°C. The blots were developed with antibodies directed against the Golgi enzymes Anp1p and Mnn1p, the SNAREs Sed5p, Bos1p and Sec22p, the cargo Emp47p, and the COPI components Arf1p and coatomer. (A) A representative collection of immunoblots. (B) The immunoblots were quantified using a Licor Odyssey system. The average and standard deviation from at least three independent experiments are shown. (C) Proteins that are lost from *Δypt1/SLY1-20* Golgi are not degraded. Total cell lysates were prepared from cells that were grown at 23°C or shifted to 37°C. Equal amounts of protein were loaded for SDS-PAGE and analyzed by immunoblotting with antibodies as described above and with anti-Pgk1 as a loading control.

reduced, whereas the concentration of coatomer remained constant and the level of Arf1p was increased (Fig. 4A,B). About half of the t-SNARE Sed5p and the v-SNARE Bos1p were lost in the *Δypt1/SLY1-20* Golgi. Expression of *SLY1-20* in the wild-type background also caused a reduction of Anp1p, cargo and SNAREs, although this reduction was less dramatic. This loss of proteins from the *SLY1-20* Golgi did not cause any obvious growth phenotype (data not shown) nor did it interfere with COPI vesicle formation in vitro.

Sed5p-GFP and Emp47p-myc accumulate in the ER and Anp1p-GFP is dispersed in *Δypt1/SLY1-20* mutants

Next, we wanted to determine the fate of the proteins lost from *Δypt1/SLY1-20* Golgi membranes. They could either be degraded or accumulate in other organelles or structures in the cell. To distinguish between these two possibilities, we compared the protein levels in total yeast lysates derived from strains grown at 23°C or shifted for 1 hour to 37°C, which allows the induction of a comparable acute response in the different strains. Surprisingly, no big changes in the protein level of Golgi or Golgi-associated proteins were detected irrespective of the strain background or the temperature (Fig. 4C). Therefore, we next determined the subcellular localization of the Sed5p-GFP, Emp47p-myc, Anp1p-GFP and Sec7p-dsRed in wild-type, WT/*SLY1-20* and *Δypt1/SLY1-20* at 23°C or after shifting to 37°C (Fig. 5). Consistent with the immunoblot data, all proteins were detected also at 37°C. Although no significant difference in protein localization was observed upon shift to 37°C in wild-type and WT/*SLY1-20* cells, Sed5pGFP and Emp47p-myc accumulated in the ER and the Anp1p-GFP and the Sec7p-dsRed signal became diffuse in *Δypt1/SLY1-20*. Moreover, a partial defect had been observed already for Emp47p, Anp1p and Sec7p at 23°C, indicating that this mutant has defects even at 23°C and these defects gradually increase with a rise in temperature. The effects observed could be due to a strong decrease in anterograde traffic, a defect in retrograde transport or a combination of both, because Ypt1p is required for the fusion of vesicles at the Golgi and the ER. Taken together our data suggest that Ypt1p plays a role in the maintenance of the Golgi complex.

The *Δypt1/SLY1-20* Golgi is at least partially functional at 23°C

Since, the localization of Anp1p and Sec7p was already disturbed at 23°C in *Δypt1/SLY1-20* cells, we wondered whether efficient glycosylation could still take place. Surprisingly, the extent of glycosylation observed in the *Δypt1/SLY1-20* mutant strain was comparable to or even greater than that in the wild type, independent of the incubation temperature (Fig. 6A). This result confirms that, despite the loss of Anp1p and other proteins, the Golgi was still functional. A similar result was observed when we analyzed a mutant in the ARF-GEF *SEC7*, which possesses an abnormal Golgi morphology at the restrictive temperature (Achstetter et al., 1988). In addition, *Δypt1/SLY1-20* cells were not osmo-sensitive, which indicates that the extracellular matrix was functional. Taken together, our results suggest that despite the lack of at least one glycosylation enzyme, glycosylation in the Golgi occurred very efficiently.

The result above was somewhat unexpected, because Ypt1p is essential for the fusion of COPII vesicles with the Golgi and hence a reduction of extensively glycosylated proteins could have been expected. One explanation for the observed glycosylation pattern might be that, most of the glycosylated proteins were not turned over during the 1-hour shift to 37°C. To further investigate this possibility, we performed a pulse-chase experiment and followed the maturation of the vacuolar carboxypeptidase C (CPY). Upon arrival in the ER, proCPY is core-glycosylated (p1). Further glycosylation occurs in the Golgi complex (p2), and in the vacuole the mature form (m) is present. In wild-type and WT/*SLY1-20* cells all CPY was converted into the mature form after 30 minutes of chase at 23°C and at 37°C (Fig. 6B). By contrast, maturation of CPY was strongly delayed in *Δypt1/SLY1-20* at 23°C. However, the processing of CPY seemed to occur normally, indicating that

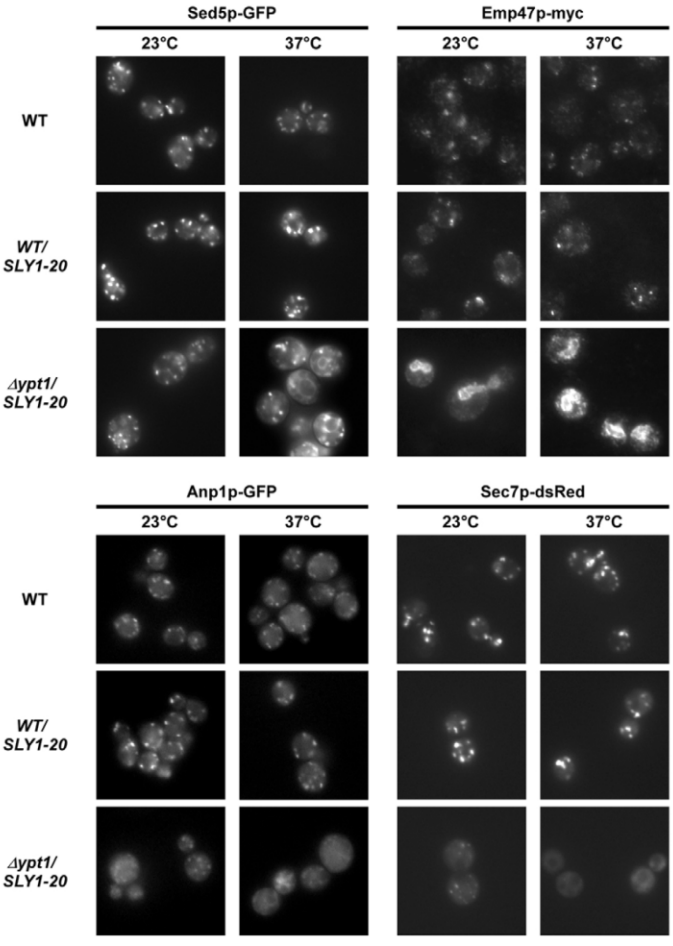


Fig. 5. The localization of various Golgi proteins is altered in *Δypt1/SLY1-20* cells. Cells were grown to early log phase at 23°C. One half of the culture was shifted to 37°C for 1 hour. Cells were either examined directly (Anp1p-GFP, Sed5p-GFP, Sec7p-dsRed) or treated for immunofluorescence (Emp47p-myc) with mouse anti-myc and goat-anti mouse IgGs coupled to CY3.

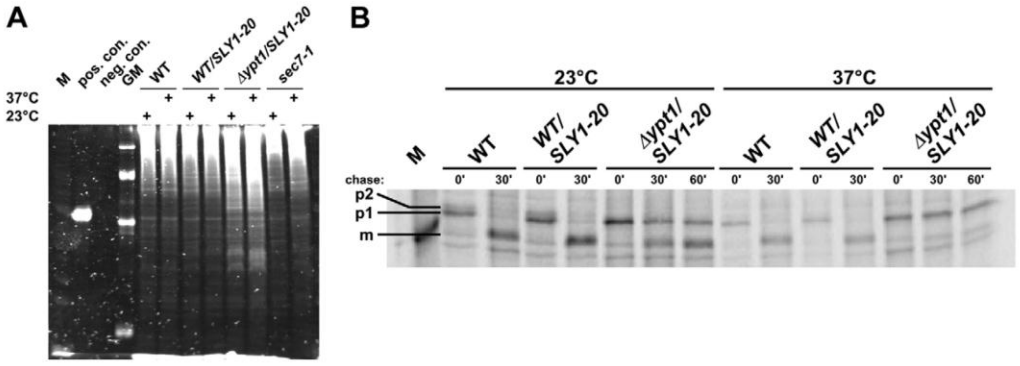
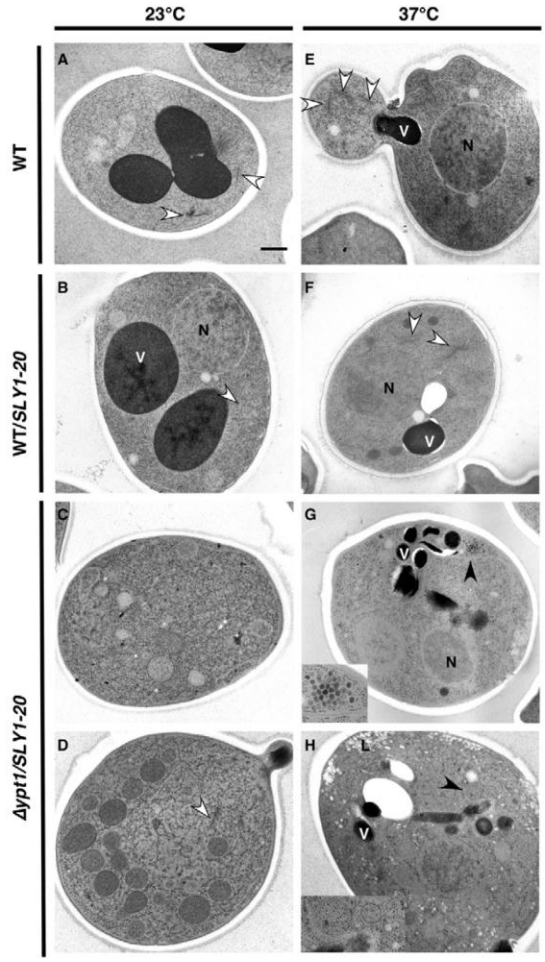


Fig. 6. Golgi function is impaired in *Δypt1/SLY1-20* cells. (A) Glycosylation is enhanced in *Δypt1/SLY1-20* cells. Strains were grown overnight at 23°C to early to mid log phase and shifted for 1 hour to 37°C where indicated. Total yeast lysates were prepared and equal amounts of protein were separated by SDS-PAGE. Glycosylated proteins were visualized with a Pro-Q Emerald 300 Kit. More glycoproteins were detected in *Δypt1/SLY1-20* cells irrespective of the temperature. M, marker; pos. con., positive control (HRP); neg. con., negative control, Sec24p; GM, CandyCane glycoprotein molecular mass standards: (from top to bottom) 180 kDa; 82 kDa; 42 kDa; 18 kDa. (B) CPY outer chain glycosylation still occurs at 23°C and is abolished 37°C in *Δypt1/SLY1-20* cells. A pulse-chase experiment was performed at either 23°C or 37°C. Transport of CPY to the vacuole was delayed, yet the glycosylation occurred normally at 23°C in *Δypt1/SLY1-20* cells. At 37°C transport between the ER and the Golgi is blocked. Hence no CYP maturation could occur. Note that a wild-type strain expressing *SLY1-20* behaved like the wild type in the assay.



the Golgi must be at least partially functional. As expected, the p1 of CPY accumulated in the *Δypt1/SLY1-20* cells at 37°C as the transport to the Golgi was blocked.

Therefore, we decided to investigate the Golgi morphology further by electron microscopy (Fig. 7). Although expression of *SLY1-20* in wild-type yeast had no effect on the morphology of the cells and its organelles, Golgi cisternae were less frequently observed in *Δypt1/SLY1-20* cells even at 23°C. In addition the vacuole seemed fragmented (compare Fig. 7A,B with C,D). Upon shift of *Δypt1/SLY1-20* cells for 1 hour to 37°C, no normal Golgi cisternae could be identified, the ER was dilated and vesicle accumulations were frequently observed, consistent with a block in the ER-Golgi transport shuttle (Fig. 7G). Furthermore, lipid-rich structures were present (Fig. 7G,H). Therefore, *Δypt1/SLY1-20* cells have a vesicle transport and a Golgi morphology defect.

Ypt1p interact physically and genetically with Ufe1p

Our results suggest a role for Ypt1p in Golgi maintenance and in the generation of functional COPI vesicles. Is Ypt1p also required for COPI vesicle consumption? If so, we should be able to establish a direct interaction between Ypt1p and an ER t-SNARE. Ypt1p binds to the t-SNARE Sed5p at the Golgi (Lupashin and Waters, 1997). Similarly, Ypt1p should bind the t-SNARE on the ER membrane protein Ufe1p. We performed GST-pull down assays using GST-SNARE fusion proteins and purified His_6-Ypt1p. GST-Ufe1p as well as GST-Sed5p could specifically recruit His_6-Ypt1p (Fig. 8, lanes 2 and 6). By contrast, the GST fusion protein of Snc1p, which

Fig. 7. Ultrastructural analysis of *Δypt1/SLY1-20* cells. Wild-type, wild-type expressing *SLY1-20* and *Δypt1/SLY1-20* strains were grown to early to mid-log phase and half of the culture was shifted to 37°C for 1 hour. The cultures were processed for ultrastructural analysis. Thin sections were stained with lead citrate and uranyl acetate. (A-D) Cells grown at 23°C; (E-H) cells shifted for 1 hour to 37°C. (A,E) wild-type; (B,F) wild-type expressing *SLY1-20*; (C,D,G,H) *Δypt1/SLY1-20*. White arrowheads point to individual Golgi cisternae. The black arrowhead in G points to an accumulation of vesicles [an enlargement (×2) is shown in the inset]. The black arrowhead in H is directed towards strange membraneous profiles. An enlargement (×2) is shown in the inset. N, nucleus; V, vacuole. Bar (in A), 500 nm.

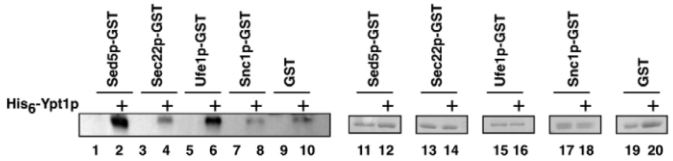


Fig. 8. Ypt1p interacts directly with Ufe1p-GST. The transmembrane domain of the SNAREs was replaced by GST. Equal amounts of GST-tagged SNAREs were immobilized on GSH-agarose and incubated with purified His_6-Ypt1p. His_6-Ypt1p bound to the SNAREs was detected by immunoblotting (lanes 1-10). The amount of immobilized SNARE proteins is shown in lanes 11-20.

functions in post Golgi transport, bound only background amounts of His_6-Ypt1p (Fig. 8, compare lanes 8 and 10). GST-Sec22p binding was slightly over the background (Fig. 8, lane 4). Binding of His_6-Ypt1p to GST-Ufe1p was specific because when we used His_6-Ypt7p instead of His_6-Ypt1p no association with GST-Ufe1p was detected (data not shown). Ypt7p is the Rab protein required for all fusion steps with the vacuole. These results demonstrate a direct physical interaction between the Rab Ypt1p and the ER t-SNARE Ufe1p. To corroborate these findings, we aimed to establish a genetic interaction between *YPT1* and *UFE1*. For that we crossed the temperature-sensitive *ypt1-3* strain with a strain in which *UFE1* was chromosomally deleted and the temperature-sensitive *ufe1-1* mutation was present on a CEN plasmid (Fig. 9A). Sixty-eight tetrads were dissected, 21 of which had only two viable spores at 23°C and 37°C, indicating that they carried the wild-type alleles of *YPT1* and *UFE1*. Because *ufe1-1* was only present on a plasmid, we determined the transmission rate during sporulation (Fig. 9B). The plasmid was transmitted in the wild-type spores with 36% efficiency. Given this transmission rate, we should have been able to recover tetrads with three or four spores growing at 23°C and only two spores growing at 37°C, reflecting the temperature-sensitive phenotype of p*ufe1-1* *Δufe1* and *ypt1-3* (Fig. 9A). However, this combination was never obtained, demonstrating that *UFE1* and *YPT1* interact genetically. By contrast crossing *ypt1-3* with *tip20-8,* a mutant that was recently shown to allow back fusion of COPII vesicles with the ER (Kamena and Spang, 2004), we could isolate viable double mutant haploids, indicating that the genetic interaction between *YPT1* and *UFE1* is indeed specific. These results demonstrate that Ypt1p interacts with Ufe1p both physically and genetically, consistent with an involvement of Ypt1p in the fusion process of COPI vesicles with the ER.

Discussion

Ypt/Rab proteins are essential for the fusion of membranes in the cell by displacing inhibitory factors from the SNAREs. Here, we demonstrate that Ypt1p is the Rab required for retrograde transport of COPI vesicles from the Golgi to the ER. Remarkably, Ypt1p might be required at each organelle-vesicle transition step in the ER-Golgi shuttle, namely (1) budding of COPII vesicles from the ER; (2) fusion of COPII vesicles with the Golgi; (3) generation of COPI-coated vesicles at the Golgi; and (4) consumption of Golgi-derived vesicles at the ER.

Ypt1p is required for sorting of GPI-anchored proteins into COPII vesicles at the ER (Morsomme and Riezman, 2002) and the role of Ypt1p in fusion of COPII vesicles with the Golgi is well established (Cao et al., 1998; Cao and Barlowe, 2000). However, no requirement of Ypt1p in retrograde transport from the Golgi to the ER had been demonstrated. Using an in vitro assay that recapitulates retrograde transport from the Golgi to the ER, we were able to show that Ypt1p is involved in this pathway. Consistent with data by Morsomme and Riezman (Morsomme and Riezman, 2002) on the role of Ypt1p in cargo inclusion we found that, in the absence of Ypt1p, COPI vesicles were still formed at the Golgi, yet these vesicles contained less of the cargo Emp47p and of the v-SNARE Sec22p than did

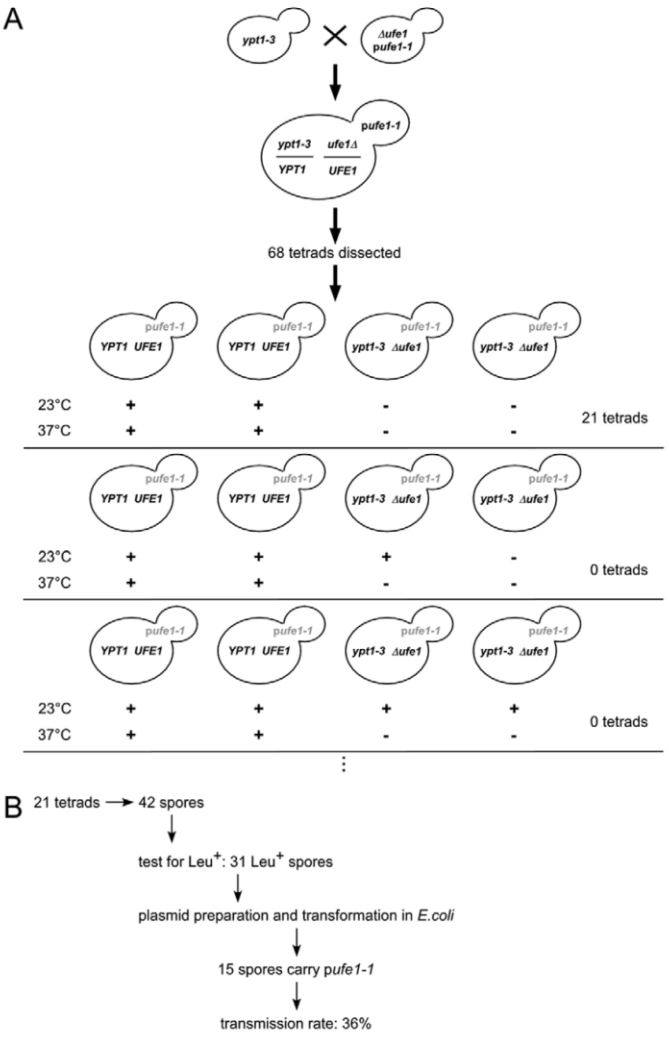


Fig. 9. *UFE1* and *YPT1* interact genetically. (A) Schematic outline of the cross between *ypt1-3* and *Δufe1* p*ufe1-1*. A subset of the meiotic outcomes is shown. (B) Determination of the transmission rate of p*ufe1-1* in wild-type yeast spores.

vesicles from wild-type cells. These COPI vesicles might only contain minor amounts of cargo, because the protein to lipid ratio was significantly decreased compared with COPI vesicles derived from wild-type Golgi membranes. In mammalian cells, the overexpression of a dominant-negative form of Rab1 causes displacement of coatomer from Golgi membranes and a BFA phenotype, indicating that Rab1 is required in a Arf1-dependent recruitment step of coatomer at the Golgi (Alvarez et al., 2003). In yeast, we could not detect a loss of coatomer from Golgi membranes in the *Δypt1/SLY1-20* mutant. However, the expression of *SLY1-20* might prevent the dissociation of coatomer from the Golgi. Yet, the protein composition of the mutant Golgi was altered. Surprisingly, the cells still performed glycosylation efficiently, and the cells were still inert against osmotic stress. In addition, we observed a change in morphology of the Golgi or a loss of Golgi cisternae by electron microscopy, which indicates that neither the absolute concentration of some Golgi proteins or peripheral Golgi proteins nor the Golgi morphology are important for survival in yeast.

Finally, to complete the cycle, we propose that Ypt1p is required for fusion of COPI vesicles with the ER. This hypothesis is based on our demonstration of a direct interaction between Ypt1p and the ER t-SNARE as well as on genetic analysis. Collectively, these data suggest that Ypt1p is not only involved in vesicle consumption but also vesicle generation at each step in the ER-Golgi shuttle. This rather surprising mechanism might be re-used at different places in the cell by different Rabs/Ypts. Recently, Vonderheit and Helenius (Vonderheit and Helenius, 2005) suggested a role for Rab7 in cargo sorting at the early endosome and in the formation of late-endosome-targeted transport vesicles. Rab7 has been implicated previously in fusion of transport intermediates with the late endosome and the lysosome (Bucci et al., 2000; Meresse et al., 1995) and the conversion from early to late endosomes (Poteryaev et al., 2007; Rink et al., 2005). Whether Rab7 is necessary for the generation of vesicles from the late endosomes that might be targeted to the TGN to recycle SNAREs and other transport factors remains to be determined. Yet, a common picture seems to emerge where the role of Ypts/Rabs is not limited to docking and fusion of vesicles and organelles, but also extends to cargo recruitment and transport carrier formation. However, this also implies that the regulation of the Ypts/Rabs must be more complicated than thus far anticipated. A focus of research in the near future must be to identify new upstream regulators of Rab proteins, in order to understand their regulation, which can no longer be limited to cycles of activation and inactivation.

Materials and Methods

Yeast methods, strains and antibodies

Standard yeast genetic techniques and media were used throughout (Sherman, 1991). Yeast strains used in this study are listed in Table 1.

Polyclonal rabbit antibodies directed against coatomer, Arf1p, Sec22p, Bos1p, Sed5p, Emp47p, Kar2p, α-1,6-linked mannose residues, Anp1p, Mnn1p, and Ypt1p and mouse monoclonal anti-myc antibodies were used in this study. His_6-Ypt7p was detected using the Super Signal West HisProbe Kit (Pierce Biotechnology Inc., Bonn, Germany)

Preparation of perforated yeast spheroplasts and cytosol

Perforated yeast spheroplasts (semi-intact cells) were prepared as described previously (Spang and Schekman, 1998).

To obtain cytosol, yeast cells were grown to early- to mid-log phase in YPD medium at either 23°C or 30°C. Cells were harvested by centrifugation and washed twice with water. The cell pellet was resuspended in a minimal volume of buffer B88 (20 mM Hepes pH 6.8, 250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate) and pipetted into liquid nitrogen. The cell beads were ground up under liquid nitrogen in a blender (Worthington Biochemical, Lakewood, NJ) for large-scale preparations or in a mortar for small-scale preparations. The cell powder was thawed in an ice-water bath, and was then diluted 1:1 with B88, and 1 mM DTT (dithiothreitol) and protease inhibitors were added. The lysate was centrifuged (5 minutes at 3000 ***g***, 15 minutes at 20,000 ***g***, 1 hour at 100,000 ***g***). The 100,000 ***g*** supernatant was collected, carefully avoiding the pellet and the lipids that floated to the top.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
RSY1169	*MATa leu2-3,112 ura2-53 pep4::URA3 gls1-1*	R. Schekman
SEY6210	*MATa ura3 leu2 his4 trp1 lys2 suc2-Δ9*	P. Cosson
tip20-8	*MATα ura3 leu2 his4 trp1 lys2 suc2-Δ9 tip20-8*	P. Cosson
CBY900	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63*	C. Barlowe
CBY901	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20*	C. Barlowe
CBY903	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3*	C. Barlowe
Δypt6	*MATα ade2-101oc his3-200 leu2-1 lys2-801am trp1-63 ura3-52 ypt6::kan*	A. Spang
Ypt31/32	*MATa ypt32 A141 D-his ypt31::kan*	D. Gallwitz
PC137	*MATa ura3-1 leu2–1 his4-619 trp1–9 lys2-801am suc2–9 tip20-5*	P. Cosson
YUA1-9c	*MATα ade2 ura3 leu2 his3 lys2 dsl1-22*	H. D. Schmitt
RSY1163	*MATa ura3-52 leu2,3,-112 ade2-101 kar2-133*	R. Schekman
RSY976	*MATa ura3-52 ypt1-3*	R. Schekman
YAS134	*MATα his4 ura3 YPT1::ypt1-A136 D-LEU*	N. Segev
YAS959	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 pRS316*	This study
YAS976	*MATa ura3 leu2 his4 lys2 bar1 sec7-1 pRS316*	This study
YAS2058	*MATα ura3-52 ufe1::TRP1 pRS315-ufe1-1*	This study
YAS1751	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 pRS315-SED5-GFP*	This study
YAS1752	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 pRS315-SED5-GFP*	This study
YAS1753	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3 pRS315-SED5-GFP*	This study
YAS1742	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 LEU2::EMP47-myc*	This study
YAS1743	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 LEU2::EMP47-myc*	This study
YAS1744	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3 Yiplac128*$^{+}$	This study
YAS1745	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 ANP1::ANP1-xyEGFP-kanMX4*	This study
YAS1746	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ANP1::ANP1-xyEGFP-kanMX4*	This study
YAS1747	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3 ANP1::ANP1-xyEGFP-kanMX4*	This study
YAS1748	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 pTPQ128**	This study
YAS1749	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 pTPQ128**	This study
YAS1750	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3 pTPQ128**	This study

*pTPQ128: pRS415-ADH-SEC7-dsRed.

Purification of coatomer, Sec23/24p, Sec13/31p, Sar1p, Lma1p, Uso1p, Sec18p, Ypt1p, Ypt7p and *N*-myristoylated Arf1p

The purifications of Sar1p, Sec23/24p, Sec13/31p, coatomer, myc-tagged Uso1p, Sec18-His_6, *N-myr*-yArf1p and the Lma1p complex were performed as described previously (Barlowe, 1997; Barlowe et al., 1994; Hosobuchi et al., 1992; Kahn et al., 1995; Salama et al., 1993; Xu et al., 1997). His_6-Ypt1p and His_6-Ypt7p were prepared following the ΔN17-Arf1p-His_6 purification protocol (Rein et al., 2002).

In vitro round-trip assay

Stage I

Translocation. The translocation reaction using [^{35}S]ppαF-HDEL and *gls1-1* as donor membranes was performed as described previously (Spang and Schekman, 1998).

Stage II

Budding. To the membranes of the stage I reaction, we added 25 μg/ml Sar1p, 25 μg/ml Sec23/24p complex, 75 μg/ml Sec13/31p complex, 50 μM GTP and an ATP regeneration system (Baker et al., 1988). The reaction mixture was incubated for 30 minutes at 20°C, chilled for 5 minutes on ice, and subjected to a medium speed centrifugation (12,000 ***g***, 30 seconds), which retained COPII vesicles in the supernatant fraction (MSS). An aliquot of the MSS was saved to determine the efficiency of the retrograde transport.

Stage III

Fusion. The MSS from stage II was supplemented with an ATP regeneration system, 50 μM GTP, 1 μM Lma1p, 1 μg/ml Sec18p, 1.5 μg/ml Uso1p and 600 μg/ml perforated spheroplast membranes from the *GLS1* strain. Fusion was allowed to take place for 20 minutes at 20°C.

Stage IV

Retrieval. Cytosol was added to a final concentration of 2 mg/ml or the same volume of B88 was added to the non-cytosol control. Reactions were incubated for 30 minutes at either 23°C (permissive temperature for ts strains) or 30°C (semi-permissive temperature for ts strains). The reaction mixture was chilled on ice for 5 minutes and the acceptor ER sedimented by centrifugation at 12,000 ***g*** for 30 seconds. The pellet was washed once with 2.5 M urea in B88 for 10 minutes on ice and twice with B88. Fusion with the acceptor ER was measured by precipitation of protease-protected [^{35}S]gpαF-HDEL with concanavalin A-Sepharose followed by separation of untrimmed [^{35}S]gpαF-HDEL from trimmed [^{35}S]gpαF-HDEL by SDS-PAGE. The read out takes advantage of the different glycosylation patterns of the reporter in the donor ER and the acceptor ER. The donor ER is defective in glucosidase I, the enzyme, which together with glucosidase II, is responsible for trimming the *N*-glycans of glucosylated proteins prior to their ER exit. The acceptor membranes always contained a functional glucosidase I, so that successful retrieval of the reporter to the acceptor ER is monitored by trimming of the *N*-glycans. Trimmed and untrimmed forms of [^{35}S]gpαF-HDEL have different electrophoretic mobilities and can be discriminated by SDS-PAGE. In the experiments using Gdi1p, the membranes were washed twice with B88 after stage III and retrieval was performed either in presence of purified Gdi1p (1.72 μg/ml) or complemented with the same volume of B88.

In vitro forward-transport assay

Stage I

Translocation. The translocation reaction using either [^{35}S]ppαF-HDEL or [^{35}S]ppαF was performed as described above.

Stage II

Transport. An aliquot (10 μl) of the stage I membranes was incubated with an ATP regeneration system, 50 μM GTP, and 2.5 mg/ml cytosol for 1 hour at 30°C. Each reaction was performed in quadruplicate, and the final volume of each reaction was 50 μl. After the incubation, the samples were chilled for 5 minutes at 4°C and centrifuged for 30 seconds at 12,000 ***g***. A 30 μl aliquot of the supernatant collected from the meniscus was treated with trypsin followed by trypsin inhibitor as described previously (Rexach and Schekman, 1991). The membranes were solubilized with 1% SDS and heated for 5 minutes at 95°C. For each set of experiments, two reactions were precipitated with concanavalin A-Sepharose or antibodies directed against α-1,6-linked mannose modifications and protein A-Sepharose. Washed immunoprecipitates were quantified in a liquid scintillation counter (Beckman Instruments, Krefeld, Germany). The budding efficiency was determined by comparing the amount of protease-protected [^{35}S]gpαF-HDEL in the supernatant to the total amount of [^{35}S]gpαF-HDEL translocated into the ER in the stage I reaction precipitated with concanavalin A-Sepharose. The efficiency of the fusion of COPII vesicles with the Golgi is given as the ratio of anti-α-1,6-linked mannose precipitated counts over concanavalin A-Sepharose precipitated counts.

Golgi-budding assay

The in vitro Golgi-budding assay was performed as described by Spang and Schekman (Spang and Schekman, 1998) with modifications. Enriched Golgi membranes were incubated with 0.1 mM GTP, coatomer (100 μg/ml) and Arf1p (80 μg/ml) for 30 minutes at 30°C in a volume of 200 μl. After chilling on ice, samples were loaded on top of a Ficoll-sucrose gradient consisting of 0.4 ml 60% (wt/wt) sucrose, 0.8 ml 7.5% (wt/wt) Ficoll, 1 ml 5%, 4%, 3% and 0.8 ml 2% (wt/wt) Ficoll in 15% sucrose, 20 mM Hepes pH 6.8, and 5 mM magnesium acetate. Vesicles were separated from the Golgi by centrifugation for 2 hours at 35,000 rpm (SW55 rotor; Beckman Instruments). Fractions (400 μl) were collected from the top of the gradient. Fractions 5-7 were pooled, mixed with an equal volume of 80% Nycodenz in 20 mM Hepes pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate (B150), and overlaid with 600 μl 30%, 25%, 20% and 15%, and 400 μl 10% Nycodenz in B150. The gradient was centrifuged for 16 hours at 40,000 rpm (SW55 rotor). Fractions (300 μl) were collected from the top, TCA-precipitated, and analyzed by immunoblotting.

CPY pulse-chase analysis

Cells were grown to an OD_{600} of 1, and the cell equivalent of an OD_{600} of 2.5 was resuspended in 200 μl HC-MET-D. Cells were preincubated for 15 minutes, incubated at 23°C or 37°C for 30 minutes and then labeled for 10 minutes with 100 μCi/ml [^{35}S]methionine (GE Healthcare, Freiburg, Germany). The radioactive product was chased with an excess of methionine and cysteine for the indicated time points. Cells were lysed and carboxypeptidase C (CPY) was immunoprecipitated using anti-CPY antibodies and protein A-Sepharose (GE Healthcare). The immunoprecipitates were resolved by SDS-PAGE, and the bands were visualized with a PhosphoImager (GE Healthcare).

GST pull-down assay

SNARE-GST fusion proteins (5 μg) were immobilized onto 25 μl 50% glutathione-agarose slurry (GE Healthcare) for 30 minutes at 4°C. Unbound proteins were removed by three washes with BBP (25 mM Hepes, pH 6.8, 1 mM DTT, 0.5 mM $MgCl_2$, 300 mM potassium acetate, 0.2% Triton X-100). Beads were incubated with His_6-Ypt1p (100 pmol) or His_6-Ypt7p (100 pmol) in BBP for 1 hour at 4°C. The total reaction volume was 500 μl. After binding, beads were washed three times with BBP, transferred to a fresh tube, washed once with 20 mM Hepes, pH 6.8, and then heated to 65°C for 10 minutes in sample buffer. Eluted proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining or immunoblot.

Kar2p secretion assay and glycostain

Secretion of the ER-resident Kar2p was assessed as described by Andag et al. (Andag et al., 2001). The detection was performed with antibodies directed against Kar2p or Pgk1p.

For the detection of glycosylated proteins, equal amounts of total yeast lysates were separated by SDS-PAGE and visualized using a Pro-Q Emerald 300 Kit from Molecular Probes (Mobitec, Göttingen, Germany) according to the manufacturer's recommendations. The yeast lysates were prepared according to Spang et al. (Spang et al., 2001).

Fluorescence microscopy and electron microscopy

Yeast cells were grown to early log phase and where indicated shifted for 1 hour to 37°C. The cells were immobilized on ConA-coated slides, mounted with Citifluor and GFP fluorescence was detected using an Axioplan microscope equipped with an Axiocam (Zeiss, Oberkochen, Germany). The detection of Emp47-myc by immunofluorescence was performed as described previously (Spang et al., 2001). For electron microscopy analysis, the cells were frozen under high pressure and treated as described in Sandmann et al. (Sandmann et al., 2003).

Genetic interaction analysis between *YPT1* and *UFE1*

To test for genetic interaction between *YPT1* and *UFE1*, *ypt1-3* was crossed to a deletion of *UFE1*, which is kept alive by the ts-sensitive mutation *ufe1-1* on a CEN plasmid. For sporulations, diploids were grown overnight at 37°C as a patch on GNA pre-sporulation plates (5% glucose, 1% yeast extract, 3% nutrient broth, 2% agar). Cells from the patch were incubated for 5 days at 30°C in liquid sporulation medium (1% potassium acetate, 0.005% zinc acetate) on a roller wheel. Tetrads were dissected on YPD plates and incubated at 23°C. Tetrads with two living and two dead spores were analyzed further. The two living spores always carried the wild-type copy of both *YPT6* and *UFE1*. Deletion of *UFE1* is lethal by itself. Therefore, we had to determine the inheritance rate of the *ufe1-1* ts plasmid in the surviving wild-type spores. Plasmid DNA was isolated and retransformed into *E. coli*. Thirty-six percent of the wild-type spores contained the *ufe1-1* plasmid. However, we never recovered a spore with the *Δypt6 Δufe1* p*ufe1-1* genotype in our tetrad analysis.

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5.2 The Dsl1 tethering complex actively participates in SNARE complex assembly at the endoplasmic reticulum in *S. cerevisiae*

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Holmfridur Thorsteinsdottir performed the experiments represented in the following figures:
Fig. 3 A, B, C, D
She contributed to Fig. 1 A and B, wrote parts of the manuscript and provided critical comments on the rest.

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The Dsl1 tethering complex actively participates in SNARE complex assembly at the endoplasmic reticulum in *S. cerevisiae*

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Abstract

The first contact of an incoming vesicle with its target membrane is mediated by a tethering factor. The tethering factor responsible for the docking of Golgi-derived vesicles to the ER is the Dsl1 tethering complex, which is comprised of the essential proteins Dsl1p, Dsl3p and Tip20p. We probed for the role of Tip20p in tethering by analyzing two mutants, *tip20-5* and *tip20-8*. Both mutants contain multiple mutations that were scattered throughout the *TIP20* sequence. Individual mutations could not reproduce the ts-phenotype of *tip20-5* and *tip20-8*, indicating that the overall structure was probably changed in the mutant proteins. Using molecular dynamics simulations, we determined that at least Tip20-8p, especially in the N-terminus and in some regions within its stalk is more flexible than the wildtype protein. *Tip20-5* and *tip20-8* mutants are not only defective in Dsl1 complex assembly but also in the formation of SNARE complexes at the ER. Thus, we provide evidence for a direct role of the Dsl1 complex in the formation and stabilization of ER SNARE complexes.

Introduction

The correct targeting and delivery of proteins and lipids to various organelles, including the cell membrane, is one of the most essential processes in eukaryotic cells. Vectorial transport ensures directionality and provides the order in which proteins travel through organelles along the secretory pathway. This process is fundamental to any exocytic and endocytic pathway. The traffic between different membrane-bound compartments is mediated mostly by a variety of transport vesicles. In yeast, anterograde transport of cargo from the endoplasmic reticulum (ER) to the Golgi apparatus is accomplished by COPII-coated vesicles, whereas retrograde trafficking is mediated by COPI-coated vesicles. The basic principles for the generation and the consumption of COPII and COPI vesicles are very similar. In either case, a small GTPase (Sar1p for COPII and Arf1p for COPI) is recruited to the donor membrane and activated. The GTPase in turn then recruits cargo, SNAREs -which are important for the subsequent fusion event- and additional coat proteins (Bonifacino & Glick, 2004; Lee *et al.*, 2004). Next to the GTPases, the COPII vesicle coat is composed of the Sec23/34 and Sec13/31 subcomplexes, whereas COPI vesicles are coated by the heptameric coatomer complex (Barlowe *et al.*, 1994; Letourneur *et al.*, 1994). The polymerization of coat proteins on the membrane surface leads to membrane deformation and, ultimately, the release of transport vesicles. Through the activation of the small GTPase, the vesicles are believed to be partially uncoated on their way to the acceptor compartment (Antonny *et al.*, 2001; Spang, 2009). The first contact of the arriving vesicle with the acceptor membrane is mediated by the concerted action of a Rab/Ypt-protein and a tethering factor. When vesicles are in vicinity of the membrane, a vesicle SNARE (v-SNARE) and the SNAREs on the target membrane (t-SNAREs) zipper up to form a four-helix bundle (trans-SNARE complex) bringing the opposing membranes in close contact and promoting fusion of the lipid bilayers. (Fasshauer *et al.*, 1997; Fiebig *et al.*, 1999; Poirier *et al.*, 1998; Sutton *et al.*, 1998). Some SNAREs can functionally replace each other in vivo (Borisovska *et al.*, 2005; Gotte & Gallwitz, 1997; Liu & Barlowe, 2002). These SNAREs are usually present on several membranes along the secretory pathway (Pelham, 2001; Tsui & Banfield, 2000), and they participate in multiple reactions (Fasshauer *et al.*, 1999). Therefore, additional factors are needed to provide specificity in the fusion process. Rab/Ypt GTPases, tethering factors as well as Sec1/Munc18 (SM) proteins have been shown to orchestrate, stabilize and proofread the assembly of cognate v-t-SNARE complexes (Cai *et al.*, 2007; Carr & Rizo, 2010; Jahn & Scheller, 2006; Markgraf *et al.*, 2007; Perez-Victoria & Bonifacino, 2009; Ren *et al.*, 2009; Starai *et al.*, 2008; Ungermann & Langosch, 2005).

In yeast the trans-SNARE-complexes that are responsible for the fusion of Golgi-derived COPI vesicles with the ER are comprised of the v-SNARE Sec22p and the three t-SNAREs

Sec20p, Ufe1p and Use1p (Burri *et al.*, 2003; Dilcher *et al.*, 2003; Jahn & Scheller, 2006; Lewis *et al.*, 1997). In addition, another v-SNARE, Bet1p, could also participate in the fusion of retrograde transport carriers with the ER (Spang & Schekman, 1998). In contrast, the trans-SNARE complexes formed during the fusion of ER-derived COPII vesicles at the Golgi contain the t-SNARE Sed5p and the v-SNAREs Bos1p, Bet1p and Sec22p or Ykt6p, which seems to be functionally redundant in this process *in vivo* (Jahn & Scheller, 2006; Liu & Barlowe, 2002). In this case, the v-SNAREs seem to provide three helices and the t-SNARE only one during the formation of the trans-SNARE complex.

The Dsl1 tethering complex is essential for the fusion of retrograde COPI vesicles from the Golgi with the ER (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009; Zink *et al.*, 2009). This tethering complex consists of the three peripheral membrane proteins Dsl1p, Dsl3p and Tip20p. The Dsl1 complex is localized to the ER through the interaction of Dsl3p and Tip20p with Use1p and Sec20p, respectively, whereas Dsl1p interacts with the coatomer complex of the incoming vesicles (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009). Recent evidence suggests that the complex also accelerates the formation of ER trans-SNARE complexes (Ren *et al.*, 2009). Thus, the Dsl1 complex appears to have two functions: one is tethering COPI vesicles through Dsl1p and the second is increasing the efficiency of the fusion process through acceleration of SNARE assembly.

Previously, we showed that a temperature-sensitive allele of *TIP20*, *tip20-8*, caused the backfusion of COPII vesicles to the ER (Kamena & Spang, 2004), a process that does normally not occur in the cell. In contrast, another allele, *tip20-5*, or *dsl1-1* did not show this backfusion phenotype. In this study, we aimed to understand the molecular basis of the *tip20-8* phenotype. We found that *tip20-8* contained multiple mutations that were not clustered to a specific part of the protein. Single point mutant analysis revealed that none of the individual mutations might be responsible for the loss-of-function in *tip20-8*. Molecular dynamics simulations showed that Tip20-8p is generally more flexible than the wild type protein. In particular the N-terminal hinge region, which is in immediate vicinity of the Dsl1p interactions site and several residues within the long α-helical stalk region that also includes the binding site for Sec20p, showed increased fluctuations. Over all, these mutations led to the decreased presence of assembled Dsl1 complex, an effect also observed for *tip20-5*, which shows a similar distribution of mutations. As a consequence, SNARE complex assembly was strongly reduced. We provide evidence that the Dsl1 complex does not only accelerate SNARE complex assembly *in vitro*, but may play a more active role in the formation of trans-SNARE complexes at the ER.

Materials and Methods

Alignment and evolutionary conserved residues

The alignment and evaluation of evolutionarily conserved residues were performed using the ConSurf database (Goldenberg *et al.*, 2009). For the alignment, the algorithm used PSI-Blast to extract in total 56 related sequences from the UNIPROT database and aligned these using standard methods. The evolutionary conservation of each amino acid position is calculated using the Rate4Site algorithm (Pupko *et al.*, 2002). The conservation scores are normalized and translated to 9 colour codes which represent the grade of conservation, 1 is maximum variability and 9 is maximum conservation.

Mapping of mutations in crystal structure

The x-ray structure for Tip20p was downloaded from the Protein Data Bank (PDB ID: 3FHN). This structure has a number of missing loops which were rebuilt using the ModLoop server (Fiser & Sali, 2003) for automated modelling of loops in protein structures. The *tip20-5* and the *tip20-8* mutations were incorporated into the structure using the mutation tool in the Swiss-pdb Viewer (Guex & Peitsch, 1997). The side-chain conformations of the mutated residues were regenerated from the backbone structure of 3FHN using the program SCWRL (Canutescu *et al.*, 2003).

Strain construction

Standard techniques for DNA manipulation (Sambrook *et al.*, 1989) and standard yeast genetic techniques and media (Sherman, 1991) were used throughout. Yeast strains used in this study are listed in Suppl. Table 1, sequences of the primers are listed in Suppl. Table 2, and constructs are listed in Suppl. Table 3.

Yeast strains that express variants of Tip20p containing only one of the mutations identified in *tip20-8* or *tip20(∆1-81)*, *tip20(I10D,L28E)* or *tip20(V17E)*, were constructed as follows. Expression plasmids (kindly provided by F. M. Hughson) of the according constructs or wildtype *TIP20* were subcloned into a *LEU2*-plasmid carrying a fusion construct of the 5'- and 3'-UTR of *TIP20*. These plasmids then were transformed into a yeast strain in which *TIP20* was chromosomally deleted. *TIP20* is essential, therefore a *URA3*-plasmid with a wildtype copy of *TIP20* was present in the Δ*tip20* strain to keep it viable. After transformation of the *LEU2*-plasmids, the *URA3*-plasmid was shuffled out of the strains using 5-FOA, leaving a *tip20* variant as the sole source of Tip20p.

Antibodies

Polyclonal rabbit antibodies directed against Tip20p, Arf1p (Spang & Schekman, 1998), Sec61 (generous gift from M. Spiess), Dsl1p, Dsl3p (both generous gifts from H. D. Schmitt), Ykt6p (generous gift from C. Ungermann), Bos1p and coatomer (Rexach *et al.*, 1994), mouse monoclonal anti-HA (Sigma), anti-His (AbD Serotec and GE Healthcare), anti-Pgk1p

(Invitrogen) antibodies and HRP-conjugated anti-His antibody (Sigma) were used in this study.

Growth Assays

For growth assays, cells of the indicated strains were grown to the logarithmic phase in YPD-medium, diluted to a cell density of OD_{600} 0.1, followed by 4 serial dilutions of 10-fold each. Drops were spotted on YPD plates and incubated at indicated temperatures for appropriate times.

Preparation of yeast total cell extract

Of each of the indicated strains, cells from logarithmically growing cultures were harvested (8 OD_{600}), washed once with H_2O_{dd} and resuspended in 200 µl buffer B88 (20 mM HEPES pH 6.8, 150 mM KAc, 5 mM $Mg(Ac)_2$, 250 mM sorbitol) supplemented with 1 mM dithiothreitol (DTT), aprotinin, leupeptin, pepstatin A. About 160 µl glass beads were added. After vigorous vortexing for 15 min at 4°C, cell debris and glass beads were sedimented (5 min, 300 g, 4°C), and the supernatant (= total cell extract) was transferred to a fresh reaction tube. For subsequent analysis by SDS-PAGE and immunoblot, 30 ng of the total cell extracts were used.

Subcellular fractionation

Overnight cultures were diluted to 0.1 OD_{600} and grown at the permissive temperature (23°C) to OD_{600} 0.4-0.6. Cells equivalent of 13-26 OD_{600} were harvested by centrifugation at 1800 x g for 5 min, washed once with water, resuspended to 5 OD_{600}/ml in Buffer A (100 mM Tris-Cl pH 9.4, 10 mM DTT) and incubated for 5 min at RT. Afterwards, the cells were harvested by centrifugation at 1800 x g for 5 min and converted into spheroblasts by incubation at 5 OD_{600}/ml in Buffer B (0.75 × YP, 0.7 M sorbitol, 0.5 % glucose, 50 mM Tris-Cl pH 7.5) containing 25 µl Zymolase-T20 (25 mg/ml)/ml for 30 min at 23°C. In a subsequent step, the spheroblasts were collected by centrifugation at 200 x g for 3 min, resuspended in 170-340 µl B88* buffer (20 mM HEPES pH 6.8, 250 mM sorbitol, 150 mM NaAc, 5 mM $Mg(Ac)_2$) supplemented with 1 mM DTT, aprotinin, leupeptin, pepstatin A., transferred to a microfuge tube and disrupted with a Dounce homogenizer on ice. Unlysed spheroplasts and cell debris were removed by centrifugation at 2000 × g for 2 min at 4°C. The supernatant was transferred to a fresh microfuge tube and centrifuged at 13000 × g for 10 min at 4°C. Afterwards the supernatant was centrifuged at 100000 × g for 60 min at 2°C. Pellets were solubilized in the starting volume of modified B88* buffer. Samples were analyzed by immunoblot.

Molecular dynamics simulations

The molecular dynamics simulations for the native and mutant protein were carried out using the software package GROMACS (Van Der Spoel *et al.*, 2005). The protein structures were immersed into a water box of dimension 112.40 Å x 106.23 Å x 164.92 Å with periodic

boundary conditions. A steepest-descent minimization was performed to minimize the energy of each system and to relax the water molecules. Then the systems were equilibrated to 300 K and a production simulation for 6 ns was performed. The program Gromacs was used for the subsequent analysis of RMSD and RMSF with the modules g_rms and g_rmsf. For the principal component analysis the g_covar module was used to calculate and diagonalize the covariance matrix. The corresponding eigenvectors were analysed with the g_anaeig module.

Protein Purification

The C-terminal GST-tagged cytoplasmic region of Ufe1p was purified from cell lysates via glutathione (GSH)-agarose (Sigma-Aldrich). STE-buffer (25 % [w/v] sucrose, 50 mM Tris-Cl, pH 8.0, and 40 mM EDTA) served as lysis buffer. For washes PBS, 15 % glycerol was used and the elution was performed with 150 mM Tris-Cl pH 8.0, 120 mM NaCl, 50 mM glutathione, 5 mM DTT, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The N-terminal GST-tagged cytoplasmic tail of Sec20p and the N-terminal GST-tagged Dsl1p were purified from cell lysates via GSH-agarose. B88 buffer (20 mM HEPES pH 6.8, 150 mM KAc, 5 mM $Mg(Ac)_2$, 250 mM sorbitol) supplemented with 0.5 % Triton X-100 was used for lysis and washes. For elution 20 mM HEPES, pH 6.8, 150 mM KAc, 5 mM $Mg(Ac)_2$, 250 mM sorbitol, 50 mM glutathione, 0.5 % Triton X-100 was used. The N-terminal His_6-tagged Dsl1p, the N-terminal His_6-tagged Tip20p, the N-terminal His_6-tagged cytoplasmic region of Use1p co-expressed with Dsl3p and the N-terminal His_6-tagged cytoplasmic region of Sec20p were purified from cell lysates via Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen). Lysis and washes were done in 50 mM Tris-Cl, pH 8.0, 200 mM NaCl, 20 mM imidazole, 2 mM ß-mercaptoethanol. The elution was performed with 50 mM HEPES pH 7.5, 150 mM KCl, 270 mM imidazol, 10 % Glycerol, 2 mM ß-mercaptoethanol. N-terminal His_6-tagged Tip20-5p and Tip20-8p were purified from cell lysates via Ni-NTA-agarose (Qiagen). The lysis and washes were performed in 50 mM Tris-Cl, pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 % Triton X-100, 10 % glycerol, 1 mM TCEP. Protein was eluted with 50 mM Tris-Cl pH 7.5, 500 mM NaCl, 300 mM imidazole, 1 % Triton X-100, 10 % glycerol, 1 mM TCEP. The C-terminal His_6-tagged cytoplasmic regions of Sec22p, Ykt6p, Bet1p, Bos1p and Snc1p were purified from cell lysates via Ni-NTA-agarose (Qiagen) according to manufacturer's instructions. Coatomer was purified as described previously (Hosobuchi *et al.*, 1992).

GST pull-down assay

GST fusion proteins (2.5 µg) were immobilized onto 25 µl 50 % glutathione-agarose slurry (GE Healthcare) for 60 minutes at 4°C. Unbound proteins were removed by three washes with Buffer C (25 mM Tris-Cl, pH 7.5, 150 mM KCl, 10 % Glycerol, 1 % Triton X-100, 2 mM ß-mercaptoethanol). The beads were incubated with recombinant His_6-tagged proteins (5 µg or 100 µg) in Buffer C at 4°C on a rotating wheel. The total reaction volume was 200-500 µl.

After binding, beads were washed three times with Buffer C, transferred to a fresh tube, washed once with 25 mM Tris-Cl pH 7.5 and heated to 65°C for 10 minutes in sample buffer. Eluted proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining or immunoblotting.

Results

Mutations in *tip20-8* and *tip20-5* are distributed all over *TIP20*.

To better understand the phenotype of the *tip20-5* and *tip20-8* temperature-sensitive mutants we sequenced both alleles. The *tip20* mutant alleles were generated by error-prone PCR (Cosson *et al.*, 1997), suggesting that more than a single point mutation should be found in the mutants. Indeed, *tip20-5* and *tip20-8* contain 9 and 6 amino acid changes, respectively (Fig. 1A and Suppl. Fig. 1). All mutations, except for L435S in *tip20-8* and E370G, S475P and K588E in *tip20-5* also occur naturally in sequences of *TIP20* homologues in other species (Fig. 1A and Suppl. Fig. 1). Therefore, the conserved changes may not be the major cause of to the *tip20-8* phenotype. The L435S mutation in *tip20-8* is in a semi-conserved position of the sequence and represents the change from an aliphatic non-polar residue to a small polar residue. In contrast, in *tip20-5*, three mutations do not occur in other *TIP20* homologues (E370G, S475P and K588E). Since the mutations found in *tip20-5* and *tip20-8* did not seem to cluster on the linear sequence, we determined whether they could cluster detect clustering in the 3D space, by introducing the mutations in the model of the crystal structure (Fig. 1B). Still, the mutations in Tip20-8p and Tip20-5p are more or less evenly distributed throughout the protein, with some enrichment along the long helical axis. Taken together, by comparing the distribution of the mutations within the two alleles, we could not identify a specific region in Tip20p that would be responsible for the growth phenotype of *tip20-5* and *tip20-8* cells.

Individual point mutations do not recapitulate the growth phenotype of *tip20-8*

Next, we wanted to test whether an individual mutation in *tip20-8* could be responsible for the growth phenotype. In order to determine such a potential key residue, we constructed yeast strains that express variants of Tip20p containing only one of the mutations identified in *tip20-8* (Fig. 2A). For this purpose, we used existing expression plasmids of the single point mutations occurring in *tip20-8* or wildtype *TIP20* and subcloned these into a *LEU2*-plasmid carrying a fusion construct of the 5'-and 3'-UTR of *TIP20*. These plasmids were then transformed into a yeast strain in which *TIP20* was chromosomally deleted. Since *TIP20* is essential, a *URA3*-plasmid with a wildtype copy of *TIP20* kept the cells alive. After transformation of the point mutation containing *LEU2*-plasmid, the *URA3*-plasmid was shuffled out of the strains using 5-FOA, leaving the single point mutation-constructs as the sole source of Tip20p.

First, we had to ensure that all the single point mutation constructs were expressed. To this end, we prepared native lysates of the different yeast strains and checked for Tip20p expression by immunoblot (Fig. 2B). All single point mutation-construct strains expressed Tip20p to similar extent than the wild-type constructs, indicating that none of the single point

mutations caused severe protein instability. Next, we assayed the *tip20* mutant strains for temperature-sensitive growth. The *tip20-8* strain does not grow at 30°C and above, while *tip20-5*, only ceased to grow at 37°C (Cosson *et al.*, 1997; Kamena & Spang, 2004; Kraynack *et al.*, 2005). In contrast none of the single point mutations showed any growth defect at any tested temperature (Fig. 2C). The data suggest that the individual point mutations do not severely interfere with Tip20p function in the cell, and hence more global changes should be responsible for the phenotypes in *tip20* mutants.

The membrane association of Tip20p is not altered in the *tip20-8*-strain or in any of the strains carrying single point mutations.

Tip20p acts as part of the Dsl1 complex at the ER membrane (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009; Zink *et al.*, 2009), and a more subtle effect, which may not lead to a growth defect, could be the less efficient recruitment of Tip20p variants to the ER. Possible changes in the distribution of the protein could lead to the perturbed protein function in the *tip20-8* strain. Therefore, we analyzed the membrane association of Tip20p variants by immunoblot. For this purpose, we performed differential centrifugation experiments (Fig. 2D) and found that in comparison to the wildtype strain neither the *tip20-8* strain nor any of the single point mutation strains showed any changes in the membrane association of Tip20p. In all the cases, most of the protein could be found in the pellet fraction after a 13,000 x g spin (P13), which contains most of the ER as shown by the presence of the ER-resident (Sec61p). A smaller portion of Tip20p was found in the S100-cytoplasmic pool. This finding is in agreement with Tip20p being a peripheral membrane protein and a member of the ER-localized Dsl1 complex (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009; Zink *et al.*, 2009). Our data indicate that all *tip20* mutants still localize correctly. Moreover, the data suggest that the *tip20-8* phenotype is not due to mislocalization of the protein.

The mutations occurring in Tip20-8p lead to an increased flexibility of the N-terminal hinge region

Since none of the point mutations in *tip20-8* gave a noticeable phenotype. We analyzed to which extend the overall structure of Tip20p is affected by mutations occurring in Tip20-8p. For this purpose, we performed molecular dynamics simulations using the software package Gromacs. The Tip20-8p mutations were modelled onto the Tip20p structure (Tripathi *et al.*, 2009). Then both structures were subjected to identical conditions, and their molecular dynamics trajectories were calculated for 6 ns each. To estimate the quality and convergence of the MD trajectory, the backbone root mean square deviation (RMSD) values of each protein structure relative to their starting structures were calculated (Fig. 3A). We found that while the Tip20p is quite stable during the simulation, the Tip20-8p simulation

showed dramatic changes in the RMSD as an effect of the mutations. To further probe the source of these differences, the root mean square fluctuation (RMSF), which measures the movement of each residue in the system with respect to the average position of that residue, was calculated for both structures (Fig. 3B). These calculations showed a striking difference between the Tip20p and Tip20-8p for the first 25-30 residues and indicated that the N-terminus in Tip20-8p is very flexible. In addition, further differences in the regions of residues 250-260, 330-350 and at the C-terminus (residues 650-701) could be observed. In order to determine the exact range and location of the motions and the difference between Tip20p and Tip20-8p, we performed a Principle Component Analysis (PCA) on both of the structures. This technique is used to discriminate the background atomic fluctuations from larger more relevant movements of the protein. The PCA confirmed the findings from the RMSF calculation. Analysis of the first two largest components showed very large movements in the first 30 N-terminal amino acids of Tip20-8p (Fig 3C) (component 2), and some further smaller movement, particularly in regions of residues 250-255, 330-350 and 650-701 (component 1) (Fig 3D). Taken together the molecular dynamics simulations showed that Tip20-8p is in general more flexible compared to wildtype. This flexibility is most apparent in the N-terminal hinge region which is in close vicinity of the Dsl1p binding site and in 3 parts within the long α-helical stalk region. Interestingly, the affected residues in the stalk region include the binding area (aa 82- 356) of the ER t-SNARE Sec20p (Ren *et al.*, 2009; Tripathi *et al.*, 2009).

The N-terminus of Tip20p is not required for growth or ER localization

The increased flexibility of the hinge region connecting the N-terminal finger to the downstream α-helices in *tip20-8* suggested that the N-terminus might be critical for Tip20p function. The N-terminus of Tip20p (aa 1-81) appears to be necessary and sufficient for interaction with Dsl1p and thus for the correct assembly of the Dsl1 complex (Ren *et al.*, 2009; Tripathi *et al.*, 2009). We used the same strategy as described above to construct yeast strains that contain either a version of Tip20p that is lacking the amino acids 1-81 (∆1-81), or contains two point mutations (I10D L28E) or one point mutation (V17E), respectively, within the N-terminal region of the protein (Fig. 4A). These point mutant constructs were shown to abolish the interaction with Dsl1p similarly to the ∆1-81 construct (Tripathi *et al.*, 2009). Extracts from the different *tip20* variant strains revealed that the expression levels of the point mutations was comparable to those of wildtype Tip20p (Fig. 4B). In contrast, the signal for the ∆1-81 construct was very low and appeared to co-migrate with another band, which might also represent a degradation product of Tip20p. To ascertain that the lower molecular weight band in Fig. 4B corresponded to Tip20Δ1-81p, we constructed yeast strains in which a 3HA-tag was added to the C-terminus of wild type and the N-terminal deletion construct. The HA-antibody recognized a band for wild type Tip20p-HA and Tip20∆1-81p-HA

at the same height as the Tip20p-antibody (Fig. 4B). Moreover, the band intensities for both constructs were comparable, indicating, that Tip20Δ1-81p is not less stable than wild type Tip20p, and that the main epitope, which is recognized by our polyclonal antibody resides in the N-terminal part of Tip20p.

Next we checked whether the N-terminal mutation constructs are essential for growth at various temperatures (Fig. 4C). None of the N-terminal mutations showed a growth phenotype, indicating that the direct interaction between Dsl1p and Tip20p is not essential for the function of the Dsl1 complex. One explanation for this observation could be that the two proteins do not need to interact for their proper localization. To test this possibility, we performed differential centrifugation experiments (Fig. 4D). All Tip20p constructs were membrane associated to the same extent as wild type, suggesting that Tip20p localizes to membranes, most likely the ER, independent of its interaction with Dsl1p. This finding is supported by data from (Ren *et al.*, 2009), that show that the Dsl3p and the Tip20p subunits of the Dsl1 complex bind independently to the ER t-SNAREs Use1p and Sec20p, respectively.

Tip20-5p and Tip20-8p can no longer interact efficiently with their binding partners

Since the interaction between Dsl1p and Tip20p does not seem to be essential for Dsl1 complex function, we wanted to determine whether interactions of Tip20p with other proteins were impaired in *tip20-5* and *tip20-8* mutants. For this purpose, we chromosomally tagged the *TIP20*, *tip20-5* and *tip20-8* with a Strep-tag and performed affinity purifications under native conditions followed by LC/MS-analysis and immunoblotting in parallel. We identified, as previously reported (Kraynack *et al.*, 2005), Sec22p and Ufe1p as interactors of Tip20p, and found that these interactions were strongly reduced in the mutants (data not shown). However, we could not detect the previously reported interaction with the ER t-SNARE Sec20p (Kraynack *et al.*, 2005; Sweet & Pelham, 1993; Tripathi *et al.*, 2009). To overcome this shortcoming, we performed purifications under denaturing conditions and included a crosslinking step. For this purpose, we chromosomally tagged *SEC20* with a Histidin-Biotin-Histidin (HBH) tag in our *TIP20* strains. This approach allowed us to show that less Tip20-5p and Tip20-8p was associated with Sec20p, when compared to wildtype (data not shown). Consistent with the wide distribution of the mutations over the entire sequence of *TIP20* in the mutants and an increase of flexibility in various parts of at least Tip20-8p, we found that the interaction with all known Tip20p binding proteins was strongly reduced in both Tip20-5p and Tip20-8p.

Tip20-8p and Tip20-5p interaction with known interactors, Dsl1p and Sec20p, is decreased *in vitro*

To further extend our studies and to confirm the above results, we performed in vitro pull down assays using Dsl1p-GST and His_6-tagged Tip20p variants. The affinity of Tip20-8p and

Tip20-5p for Dsl1p was significantly decreased compared to wild-type Tip20p in vitro (Fig. 5A). Since Tip20p interacts directly with Sec20p, we tested next the binding of Tip20p variants to Sec20p-GST. Similarly to the Dsl1p-GST experiment, the binding ability of the mutants was strongly reduced (Fig. 5A). The increased flexibility ofthe N-terminal hinge close to the Dsl1p interaction site and in residues that are important for the interaction with Sec20p (Ren *et al.*, 2009; Tripathi *et al.*, 2009) might, at least for Tip20-8p, provide an explanation for the observed loss of these interactions. Taken together, these experiments confirmed the results of the affinity purification experiments and are consistent with previously published data (Kraynack *et al.*, 2005).

Tip20-8p and Tip20-5p destabilize the Dsl1 tethering complex *in vitro*

Since both, Dsl1p and Tip20p, are members of the ER-associated Dsl1 complex (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009) we wanted to analyze the effect of Tip20p mutant proteins on *in vitro* reconstituted Dsl1 complexes. Although it had been shown previously that Dsl1p interacts directly with Dsl3p (Ren *et al.*, 2009), our attempts to pull down His_6-Dsl3p with immobilized GST-Dsl1p failed, probably due to non-functional Dsl3p. Dsl3p directly interacts with the ER t-SNARE Use1p (Kraynack *et al.*, 2005; Ren *et al.*, 2009) and Use1p could be only efficiently purified when co-expressed with Dsl3p (Ren *et al.*, 2009). Thus, we used as a source of Dsl3p (or Use1p) the co-purified complex His_6-Use1p-Dsl3p. Given this slight complication, we decided to build up the Dsl1 complex from the SNARE site. To this, end we used a GST fusion to the third ER t-SNARE, Ufe1p, (Lewis *et al.*, 1997), which has been shown to interact with both Use1p and the Dsl1 complex. (Burri *et al.*, 2003; Dilcher *et al.*, 2003; Kraynack *et al.*, 2005). To *in vitro* reconstitute the Dsl1 complex, we performed pulldowns using GST-Ufe1p as bait and Dsl1 complex members as prey (Fig. 5B). Interestingly, Tip20-5p and Tip20-8p bound to Ufe1p with similar efficiencies than wild-type, indicating that the mutations in those proteins may not affect the binding site for Ufe1p. Consistent with the results above, Dsl1p was only present at background levels in these pulldowns. Since Dsl3p is expressed in a complex with Use1p, and Use1p can bind directly to Ufe1p, we refrained from drawing any conclusions about Dsl3p. The strong interaction of the Tip20p mutant proteins with Ufe1p may explain why Tip20p mutants still show a strong membrane association, despite the loss of interaction with Sec20p. Moreover, these data provide strong evidence that the Dsl1 complex is destabilized in *tip20-5* and *tip20-8* mutants, and probably also *in vivo*.

Tip20p mutants inhibit trans-SNARE complex assembly *in vitro*

It is assumed that formation of an SNARE-complex consisting of the ER-localized SNAREs Sec20p, Ufe1p and Use1p with the v-SNAREs Sec22p and/or Bet1p is necessary for the fusion of COPI vesicles with the ER is necessary (Burri *et al.*, 2003; Dilcher *et al.*, 2003; Jahn

& Scheller, 2006; Lewis *et al.*, 1997). Moreover, a recent study suggest that the Dsl1 complex accelerates SNARE complex assembly at the ER (Ren *et al.*, 2009). Since Tip20-5p and Tip20-8p failed to bind to Sec20p and to other Dsl1 complex members efficiently, we asked how Tip20-8p and Tip20-5p would influence the assembly of the ER-SNARE-complex. To this end, GST-Ufe1p was immobilized on glutathione beads and incubated with the remaining ER t-SNAREs, the v-SNARE Sec22p and Dsl1 complex members (Fig. 5C). As expected SNARE complex assembly occurred in the presence of wildtype Tip20p. In contrast, mutant Tip20p proteins did not only inefficiently recruit Sec20p and Use1p, but also the v-SNARE Sec22p was less efficiently incorporated into SNARE complexes. Tip20p must have a regulatory function during SNARE complex assembly at the ER because SNARE complexes containing Sec20p, Use1 and Sec22p were formed properly, when first SNAREs complexes were formed and then Dsl1 complex members were added in a second incubation step (Fig. 5C). Dsl1p was recruited efficiently to these SNARE complexes. However, this binding was most likely dependent on the Dsl3p-Use1p complex, which was present during the SNARE complex assembly step. These data are consistent with the observation that ER SNARE complex assembly is accelerated *in vitro* by the presence of the Dsl1 complex (Ren *et al.*, 2009). Taken together, our data indicate that Tip20p is required for proper SNARE complex assembly.

Tip20p and not Dsl1p is required for proper SNARE complex assembly

So far, the defect in SNARE complex assembly could be related to either the reduced interaction of Tip20-5p and Tip20-8p with Dsl1p or the phenotype could be completely independent of Dsl1p. To distinguish between those possibilities, we repeated the above experiment in the absence of Dsl1p (Fig. 5D). Again, SNARE complexes assembled in the absence of Dsl1p when wildtype Tip20p was present, and this assembly was reduced when mutant Tip20p was added to the incubation mixture. Interestingly, the reduction in SNARE complex assembly was independent of the presence of Dsl1p in the assay. Therefore, we conclude that Tip20p may have a more prominent role in SNARE complex assembly than Dsl1p. Moreover, the failure to form proper ER SNARE complexes might be direct effect of *tip20* mutants, and their most prominent phenotype could be due to malfunctioning during the assembly of ER-SNARE-complex.

The ER SNARE complex assembly is not rescued by alternative v-SNAREs in *tip20* mutants

Sec22p is not the only v-SNARE that could potentially engage in a trans-SNARE complex at the ER. Bet1p and Ykt6p have been shown to be substitutes for Sec22p (Liu & Barlowe, 2002; Spang & Schekman, 1998). In addition, it has been shown that SNARE–SNARE interactions under some circumstances are promiscuous, and that the formations of non-physiological SNARE-complexes could take place (Fasshauer *et al.*, 1999; Tsui & Banfield,

2000; Wendler & Tooze, 2001; Yang *et al.*, 1999). Therefore, we tested whether in the presence of Tip20-5p and Tip20-8p ER SNARE complexes would become more promiscuous. First, we decided to look at Ykt6p, which seems to be able to substitute for v-SNAREs in more than one type of SNARE complexes (Fischer von Mollard & Stevens, 1999; Jahn & Scheller, 2006; Liu & Barlowe, 2002) Ykt6p interacted with Ufe1p equally well, independent of the presence of wild-type or a mutant form of Tip20p in the assay (Fig. 6A). However, this interaction did not improve the recruitment of Use1p or Sec20p to the complex. Moreover, adding Ykt6p and Sec22p together did not improve the SNARE complex assembly at the ER. Since neither Sec20p nor Use1p were efficiently recruited to Ufe1p in the presence of Ykt6p, Ykt6p binding might not be productive in SNARE complex formation at the ER.

Next, we tested if Bet1p was efficiently recruited into SNARE complexes. Bet1p behaved similar to Sec22p and was only found to be incorporated into SNARE complexes in the presence of wildtype Tip20p (Fig. 6B). Tip20p mutants caused a strong reduction, and less ER SNARE complexes were formed. The effects for the ER v-SNARE were specific because non-cognate SNAREs as the v-SNARE at the Golgi, Bos1p or the plasma membrane v-SNARE Snc1p could not be recruited at all to engage into SNARE complex formation (Fig. 6B). Our data indicate that in the presence of Tip20p mutant proteins, SNARE complex assembly at ER is severely altered but proper recognition of the v-SNAREs is maintained as non-cognate v-SNAREs cannot be recruited into ER SNARE complexes under any conditions tested in this assay.

Loss of affinity of the Dsl1 tethering-complex for coatomer in the presence of Tip20-8 or Tip20-5

So far, we have shown that the *tip20* mutants disturb the formation of ER-SNARE-complexes. Another well established function of the Dsl1 complex is the tethering of incoming COPI-vesicles to the ER-membrane (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009). During this process Dsl1p directly interacts with subunits of the COPI coat (Zink *et al.*, 2009). We wanted to check whether the *tip20* mutants also affect this second function of the Dsl1 complex. To this end, GST-Ufe1p was immobilized on glutathione beads and incubated with the members of the Dsl1 complex and coatomer. We furthermore preassembled Dsl1 complexes and added coatomer only afterwards. In both cases the Dsl1 complexes displayed a decreased interaction with coatomer if Tip20-8p or Tip20-5p were present (Fig. 6C). These results suggest that the efficiency of the COPI-vesicle recognition at the ER is affected by the *tip20* mutants.

Discussion

In this paper we investigated the molecular basis of the phenotypes of *tip20* mutants and found that these mutants interfered with the proper assembly of trans-SNARE complexes at the ER. Trans-SNARE complexes consisting of the t-SNAREs Sec20p, Ufe1p and Use1p and the v-SNARE Sec22p and/or Bet1p promote fusion of Golgi-derived COPI-coated vesicles with the ER (Burri *et al.*, 2003; Dilcher *et al.*, 2003; Jahn & Scheller, 2006; Lewis *et al.*, 1997). Ufe1p, Sec20p, Use1p, Sec22p and Bet1p could not be efficiently assembled into ER SNARE complexes in the presence of mutant Tip20p *in vitro*. This defect was not compensated for by the third vesicle SNARE, Bos1p, which was not found in the ER SNARE complex under any conditions tested. While Sec22p and Bet1p can participate in SNARE complex formation at both the ER and the Golgi (Cao & Barlowe, 2000; Liu & Barlowe, 2002; Spang & Schekman, 1998), Bos1p appears only to act in the fusion process of COPII vesicles at the Golgi (Cao & Barlowe, 2000; Jahn & Scheller, 2006; Liu & Barlowe, 2002; Parlati *et al.*, 2000; Spang & Schekman, 1998). The non-cognate v-SNARE Snc1p, which acts at the plasma membrane, could also not be engaged in SNARE complex formation. This supports that the observed defects of the Tip20p mutants in SNARE assembly are specific for the cognate trans-SNARE complexes at the ER and indicates that the amount of ER trans-SNARE complexes is dramatically decreased in *tip20-5* and *tip20-8*. Interestingly, we found that Ykt6p, another v-SNARE, which can functionally replace Sec22p in the fusion of ER-derived COPII vesicles with the Golgi (Liu & Barlowe, 2002) binds efficiently to Ufe1p, irrespective of the presence of wildtype or Tip20 mutants, and even when added together with Sec22p. Yet, the presence of Ykt6p did not improve the incorporation of Sec20p, Use1p or Sec22p *in vitro*. However, we cannot exclude that *in vivo,* when e.g. other factors like the SM-protein Sly1p are present, Ykt6p can assist in the incorporation of cognate SNAREs in the ER SNARE complex.

The finding that defective Dsl1 complexes interfere with a proper assembly of cognate ER trans-SNARE complexes and the observation that the Dsl1 complex accelerates SNARE complex formation (Ren *et al.*, 2009), provide evidence for a novel function of the Dsl1 complex, namely a role in proofreading and stabilizing ER trans-SNARE complexes. Such a function has been suggested before for other tethering complexes. Uso1p, an essential tethering factor at the Golgi in yeast, is required for the assembly of the v-SNARE/t-SNARE complexes (Sapperstein *et al.*, 1996). In mammalian cells, it was shown that defects in the function of the intra-Golgi tethering complex, the COG-complex, lead to a significant decrease in Golgi SNARE mobility, an accumulation of uncomplexed Syntaxin5, and a decrease in the steady-state level of intra-Golgi SNARE complexes (Shestakova *et al.*, 2007). Furthermore, the trans-Golgi located mammalian tethering complex GARP was found

to specifically and directly interact with SNAREs that participate in the endosome-to-TGN retrograde route. Further functional analyses placed the GARP complex upstream of the SNAREs, regulating their localization and assembly into SNARE complexes (Perez-Victoria & Bonifacino, 2009). Moreover, in yeast, the tethering complex present at the vacuole, the HOPS-complex, proofreads SNARE domain and N-terminal domain structures of vacuolar SNAREs and regulates the fusion capacity of trans-SNARE complexes, only allowing full function for wild-type SNARE configurations (Starai *et al.*, 2008).

To define a possible molecular mechanism for the observed decrease in formation of cognate SNARE complexes we performed molecular dynamics analyses of Tip20-8p. These showed that Tip20-8p in general is more flexible than Tip20p. Movements of the residues 250-255, 330-350 and 650-701 could be identified as one source of significant flexibility within the mutant protein. Since the residues 82-356 of Tip20p are necessary for binding to Sec20p (Ren *et al.*, 2009), the above mentioned fluctuations in residues 250-255 and 330-350 could potentially be responsible for the dramatic decrease in the affinity of Tip20-8p for this SNARE. They in turn might also influence the incorporation of Sec22p and Bet1p in ER SNARE complexes. Thus the interaction of Tip20p with Sec20p might be critical for the trans-SNARE complex assembly at the ER.

Apart from being associated with ER t-SNAREs the Dsl1 complex is known to interact with coatomer subunits (Andag *et al.*, 2001; Andag & Schmitt, 2003; Kraynack *et al.*, 2005; Reilly *et al.*, 2001; Ren *et al.*, 2009; Tripathi *et al.*, 2009; Zink *et al.*, 2009). It tethers the incoming COPI vesicles to the ER membrane via an unstructured loop in the Dsl1p subunit promoting the final uncoating of the vesicles. In addition, truncations of *TIP20* displayed synthetic lethality with coatomer mutants (Frigerio, 1998). Consistent with those results, we found that Tip20p mutant proteins decreased the affinity of the tethering complex for coatomer. The decrease in coatomer binding most likely just reflects our observation that Dsl1 complexes are destabilized in the presence of the Tip20p mutants and therefore the number of functional complexes is reduced. Our molecular dynamics simulations showed a dramatic increase in the fluctuation of the N-terminus in Tip20-8p. This fluctuation is due to an increased flexibility of the N-terminal hinge linking the N-terminal finger to the remaining protein. This N-terminal hinge, together with another hinge in the Dsl1p subunit, represents flexible points in the Dsl1 tethering complex (Ren *et al.*, 2009; Tripathi *et al.*, 2009). Therefore the increased flexibility of the N-terminal hinge of Tip20-8p could also indirectly prevent the loop region within Dsl1p from performing its function in capturing Golgi-derived COPI vesicles. However, we cannot exclude that coatomer can bind directly to Tip20p and that this interaction is affected in the mutant situation. In support of this possibility (Schmitt, 2010) reported potential COPI interaction sites in the N-terminus of Tip20p homologs from *Schizosaccharomyces* species. The increased movements of the N-terminus in Tip20-8p

therefore could potentially result in decreased coatomer binding to Tip20-8p. Whether these potential sites are present and functional in *S. cerevisiae* remains unclear.

Taken together our results indicate that the efficiency of the recognition of Golgi- derived COPI vesicles is impaired in *tip20-8* and *tip20-5*. Moreover, we provide evidence for a novel function of the Dsl1 complex in proofreading and stabilization of ER trans-SNARE complexes. In the *tip20-8* strain the defect in the assembly of cognate trans-SNARE complexes in combination with the decreased tethering of coatomer-coated vesicles to the ER might lead to a loss of specificity in vesicle fusion at the ER membrane and therefore cause the backfusion of COPII vesicles to their donor membrane. However, in our *in vitro* SNARE assembly and coatomer interaction assays, Tip20-5p behaved similar to Tip20-8p, although *tip20-5* did not show a backfusion phenotype (Kamena & Spang, 2004). An explanation for this discrepancy could be that *tip20-5* is weaker allele than *tip20-8* and that the assay used to detect the backfusion phenotype is not sensitive enough to show a phenotype for *tip20-5*.

How *tip20-8* and *tip20-5* interfere with SNARE complex assembly remains still unclear, but the decreased interaction with Sec20p might be key in this process. Interestingly, the binding of Tip20p to Ufe1p appears to be less important for SNARE complex formation. A plausible scenario could be that the arrival of a vesicle is signalled via the Tip20p-Sec20p interaction, leading to an efficient recruitment of cognate SNAREs. The role of other players, like SM-proteins, known to have a role in orchestrating and stabilizing fusion events at the ER, needs to be further examined in this context.

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Figure legends

Figure 1:

Mutations in *tip20-8* and *tip20-5* do not cluster in the linear sequence or the 3D space. **(A)** Sequencing of the *tip20-8* and the *tip20-5* alleles revealed 9 and 6 amino acid changes, respectively. An alignment and evaluation of evolutionarily conserved residues for Tip20p was performed using the ConSurf database (Goldenberg *et al.*, 2009). The conservation scores were normalized and translated to 9 color codes which represent the grade of conservation, 1 is maximum variability and 9 is maximum conservation. The mutations occurring in Tip20-8p (top) and in Tip20-5p (bottom) were mapped onto the linear sequence. Stars indicate mutations that do not occur naturally in sequences of *TIP20* homologues in other species. Neither the mutations found in *tip20-8* nor the ones identified in *tip20-5* cluster on the linear sequence. **(B)** The mutations in Tip20-8p and Tip20-5p are relatively evenly distributed throughout the protein, with some enrichment along the α-helical stalk region of the protein. Mutations occurring in Tip20-8p (left) and in Tip20-5p (right) were incorporated into the X-Ray crystal structure of Tip20p (3FHN) using the mutation tool in the Swiss-pdb Viewer (Guex & Peitsch, 1997). The side-chain conformations of the mutated residues were regenerated from the backbone structure using the program SCWRL (Canutescu *et al.*, 2003)).

Figure 2:

Individual point mutations behave like wildtype *TIP20* and are, as Tip20-8p, still membrane associated. (A) Schematic drawing of yeast strains expressing variants of Tip20p that contain only one of the mutations identified in *tip20-8*. **(B)** All single point mutation constructs express Tip20p to a similar extent than the wildtype constructs. Immunoblots of protein extracts from the single point mutation were performed. Detection of Arf1/2p was used as loading control. **(C)** None of the single point mutations showed any growth defect at any tested temperature. Growth assays were performed at the indicated temperatures to test the *tip20* mutant strains. The *tip20-8* strain displays a growth defect at 30°C and above, while the *tip20-5* strain only ceases to grow at 37°C. **(D)** In all strains most of Tip20p was found in the P13 fraction, which contains mostly ER membranes. A smaller portion of Tip20p was found in the S100-fraction. Subcellular fractionations of the indicated strains were performed and analyzed by immunoblots. Pgk1p was used as a marker for cytosolic proteins, whereas Sec61p served as a maker for ER-membranes.

Figure 3:

The mutations occurring in Tip20-8p lead to an increased flexibility of the N-terminal hinge region and of three regions within the α-helical stalk of the protein. (A) While Tip20p (blue) behaves rather stably during the molecular dynamics simulation, Tip20-8p (red) as an effect of the mutations shows dramatic changes in the RMSD. The backbone root mean square deviation (RMSD) values of each protein structure relative to their starting structures were calculated to estimate the quality and convergence of the MD trajectory. **(B)** A striking difference between the Tip20p (blue) and Tip20-8p (red) for the first 25-30 residues (indicated by an arrow) and further differences in the regions of residues 250-260, 330-350 and the C-terminus (residues 650-701) (indicated by dashed arrows) could be detected. The sources of the observed differences in RMSD were determined by computation of the root mean square fluctuation (RMSF). Thereby the movement of each residue in the system with respect to the average position of that residue was calculated for both structures. **(C)** Component 2 of the principal component analysis (PCA) reflects the very large movements in the first 30 N-terminal amino acids. The maximal range as well as intermediate states of the movements for the wildtype Tip20p (left, blue) and the Tip20-8p (middle, red) is shown. On the right side a superposition (Tip20p in blue, Tip20-8p in red) is displayed. **(D)** Component 1 of the PCA mirrors the observed fluctuations in the regions of the long α-helical stalk. A superposition of the maximal range as well as intermediate states of the movements for wildtype Tip20p (blue) and Tip20-8p (red) is shown. The boxes represent an enlargement of the regions (aa 250-255, aa 3330-350, aa 650-701) that displayed the biggest amplitude in movement.

Figure 4:

The N-terminus of Tip20p is not required for growth or membrane localization. (A) Schematic drawing of yeast strains expressing variants of Tip20p that contain either a version of Tip20p that is lacking the amino acids 1-81 (Δ1-81), or containing two (I10D,L28E) or one point mutation (V17E), respectively. **(B)** All constructs of N-terminal *tip20* variants express Tip20p to a similar extent than the wildtype constructs. Immunoblots of protein extracts from the N-terminal *tip20* variants were performed. Detection of Arf1/2p was used as loading control. **(C)** None of the N-terminal *tip20* variants showed a growth phenotype. Growth assays were performed at the indicated temperatures using the indicated *tip20* variant strains. **(D)** None of the N-terminal *tip20* variants showed an aberrant localization of Tip20p. Subcellular fractionations of the indicated strains were performed and analyzed by immunoblots.

Figure 5:

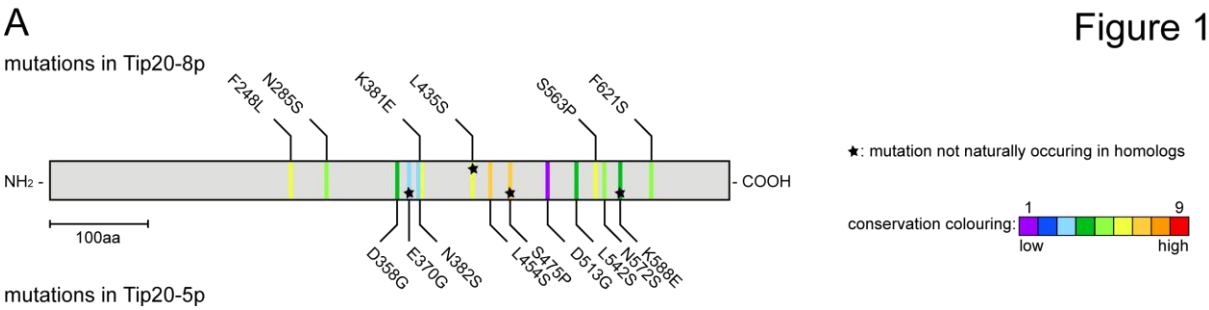
***In vitro* assembly of Dsl1 tethering complexes and ER trans-SNARE complexes is affected by Tip20p mutants. (A)** In comparison to wildtype Tip20p the binding of Tip20-8p and of Tip20-5p to GST-Dsl1p (left) and GST-Sec20p (right) is strongly decreased. To examine the interaction of Tip20p and Tip20p mutants with Dsl1p and Sec20p *in vitro* pulldown assays were performed and analyzed by SDS PAGE and Coomassie Blue staining. On the right side of each panel all the proteins used in the assay are displayed in a graphical representation, the asterisk indicates that either wildtype Tip20p, Tip20-5p or Tip20-8p (marked on top of the panel) were used. **(B)** Dsl1p binding to Tip20-5p or Tip20-8p is drastically decreased. To reconstitute the Dsl1 tethering complex *in vitro*, pulldown assays were performed as indicated in the graphical representation and analyzed by immunoblotting. Note that Dsl3p and Use1p were added as a complex. Dsl1p and Dsl3p were detected with protein specific antibodies, while an anti-His antibody was used to detect Tip20p. **(C)** In the presence of Tip20-8p or Tip20-5p the amount of Sec20p, Sec22p and Use1p that is incorporated in the ER SNARE complexes is strongly decreased (left). To reconstitute the assembly of the ER trans-SNARE complex, *in vitro* pulldown assays were performed as indicated in the graphical representation and analyzed by immunoblotting. ER trans-SNARE complexes were pre-assembled and the members of the Dsl1 complex added separately (right). Under these conditions the incorporation efficiency of Sec20p, Sec22p and Use1p was not affected. Two different anti-His antibodies were used to detect Tip20p, Sec20p, Use1p and Sec22p. **(D)** The presence of Dsl1 did not influence SNARE complex assembly. In vitro pulldown assay were performed according to (C), but Dsl1p was omitted. No difference to the experiment shown in panel (C) was observed.

Figure 6:

The assembly of ER SNARE complexes in *tip20* mutants is not rescued by alternative v-SNAREs and coatomer binding is affected in the presence of Tip20-5p or Tip20-8p. (A) Ykt6p binds to GST-Ufe1p but does not promote SNARE complex assembly. Pulldown assays were performed as indicated in the graphical representation. **(B)** Bet1p, Bos1p, and Snc1p do not improve SNARE complex assembly in the presence of the Tip20p mutants. Pulldown assays were performed as indicated in the graphical representation. For simplicity reasons only Use1p, the alternative SNAREs, as well as Tip20p and its mutants are displayed here, but all indicated proteins were present in the pulldowns. **(C)** Coatomer binding to mutant Dsl1 complexes is reduced *in vitro*. To assess if the coatomer binding function of the Dsl1 complex is affected by the Tip20p mutants, Dsl1 complexes were assembled and coatomer added either at the same time or only after Dsl1 complex pre-assembly.

Figures

Figure 1



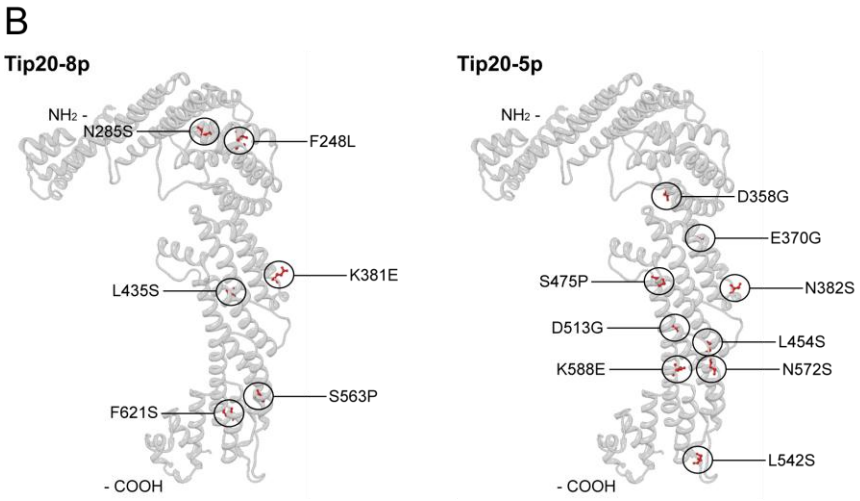
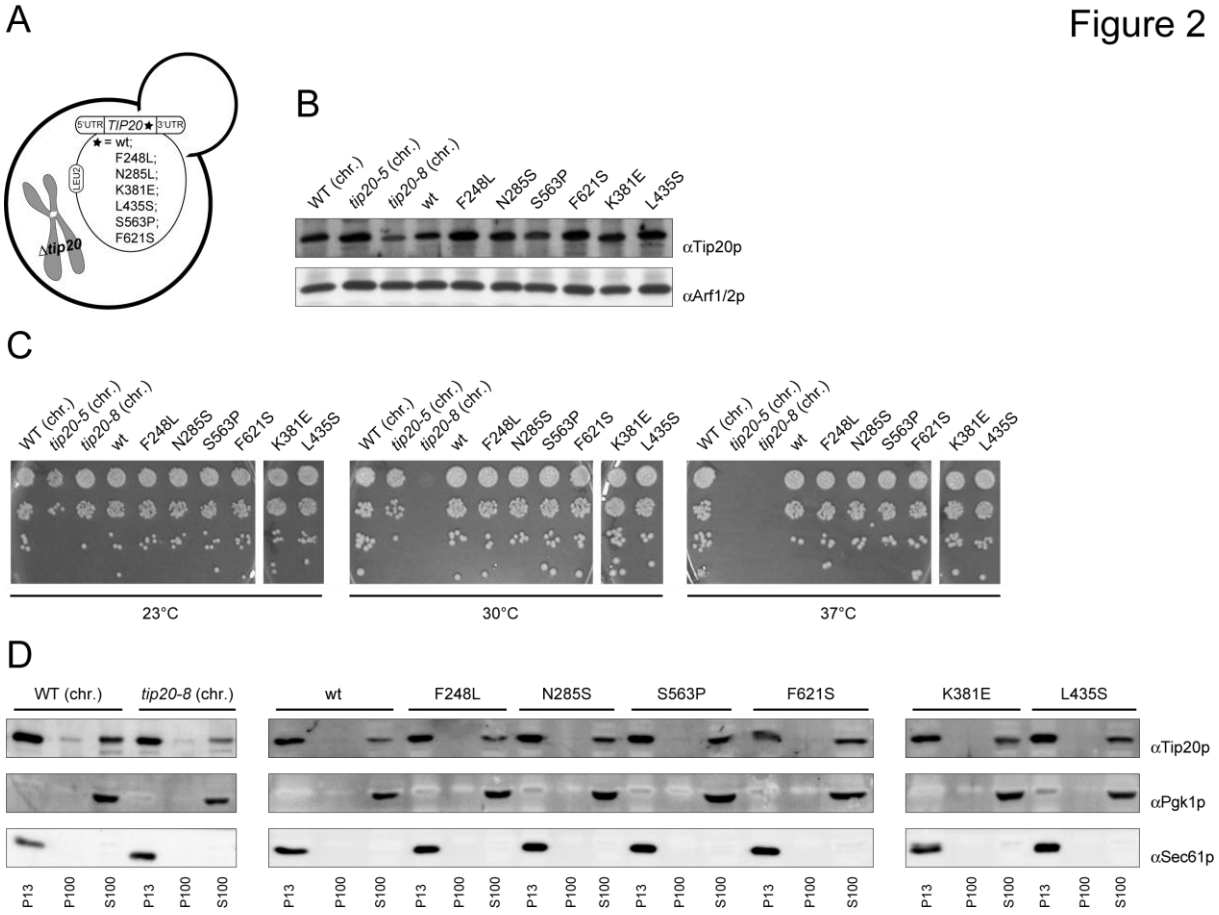


Figure 2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3'UTR
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LEU2
Δtip20
B
WT (chr.)
tip20-5 (chr.)
tip20-8 (chr.)
wt
F248L
N285S
S563P
F621S
K381E
L435S
αTip20p
αArf1/2p
C
23°C
30°C
37°C
D
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tip20-8 (chr.)
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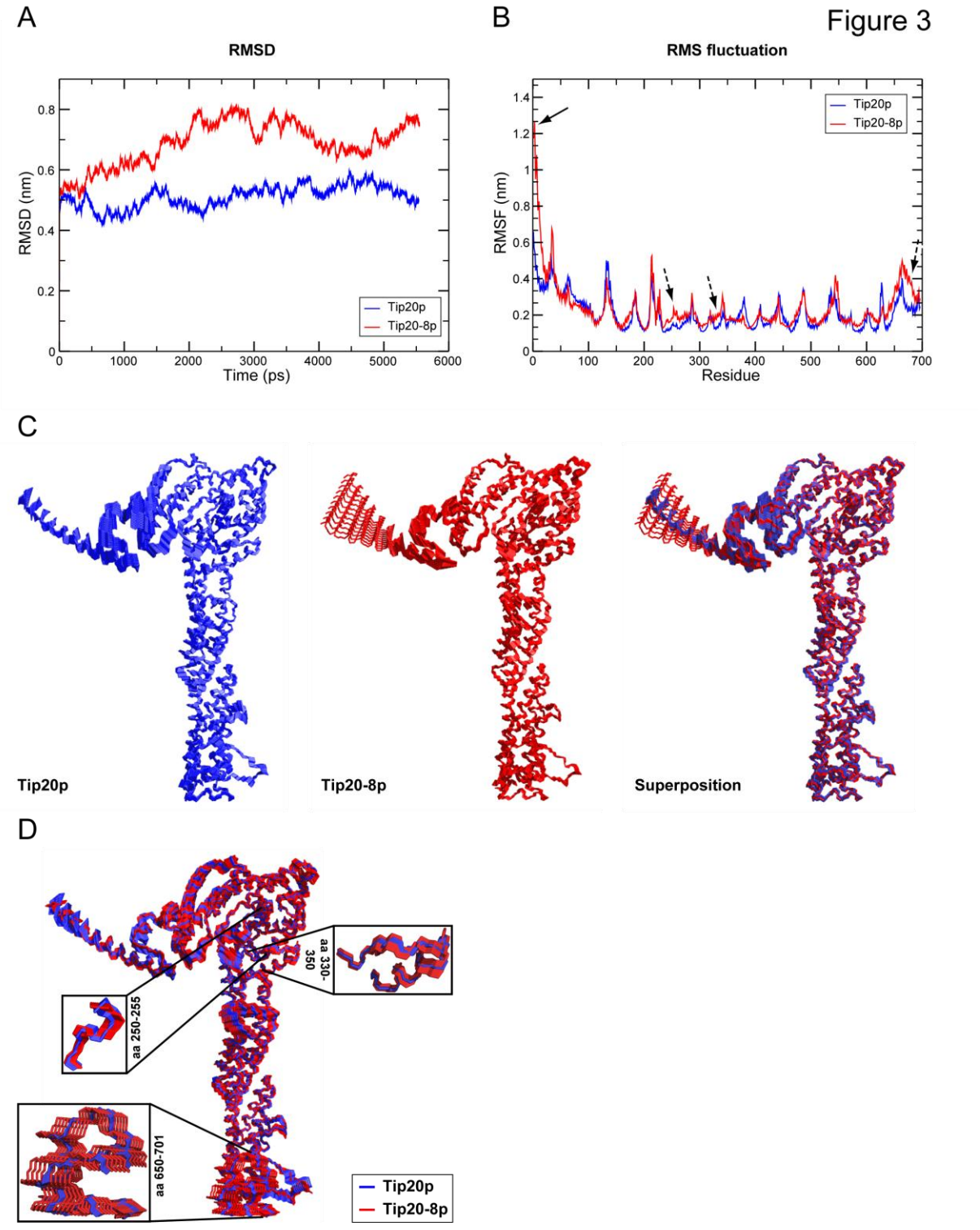
αPgk1p
αSec61p
P13
P100
S100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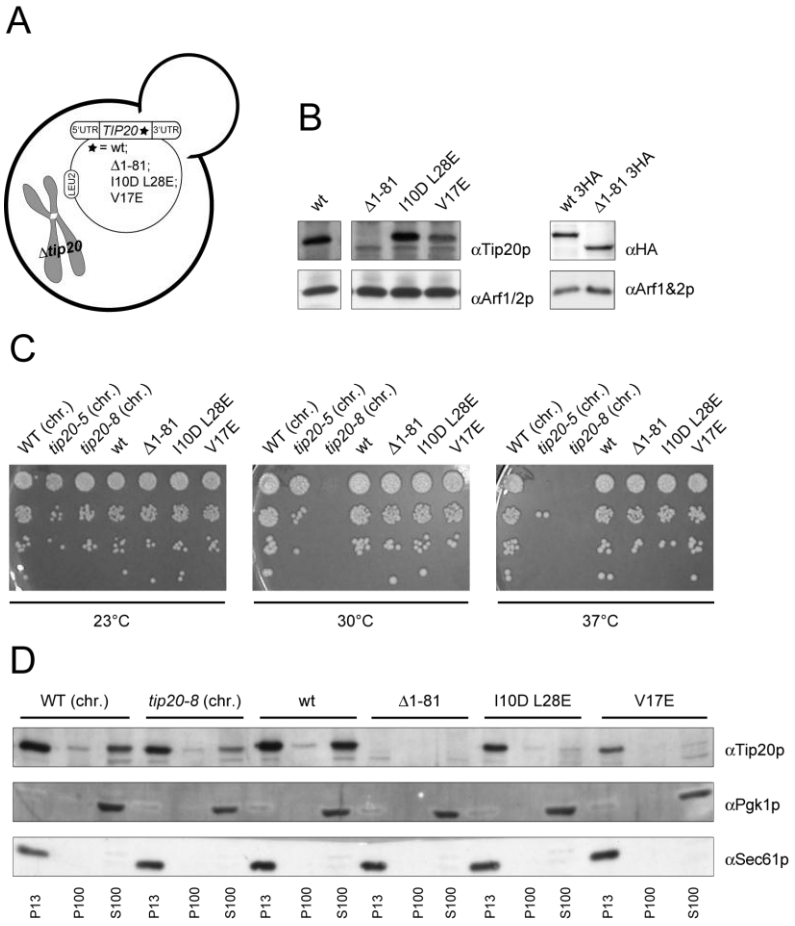
RMSD
RMSD (nm)
Time (ps)
Tip20p
Tip20-8p
B
RMS fluctuation
RMSF (nm)
Residue
Tip20p
Tip20-8p
C
Tip20p
Tip20-8p
Superposition
D
aa 330-350
aa 250-255
aa 650-701
Tip20p
Tip20-8p

Figure 3

Figure 4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LEU2
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B
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Δ1-81
I10D L28E
V17E
wt 3HA
Δ1-81 3HA
αTip20p
αHA
αArf1/2p
αArf1&2p
C
WT (chr.)
tip20-5 (chr.)
tip20-8 (chr.)
wt
Δ1-81
I10D L28E
V17E
23°C
30°C
37°C
D
WT (chr.)
tip20-8 (chr.)
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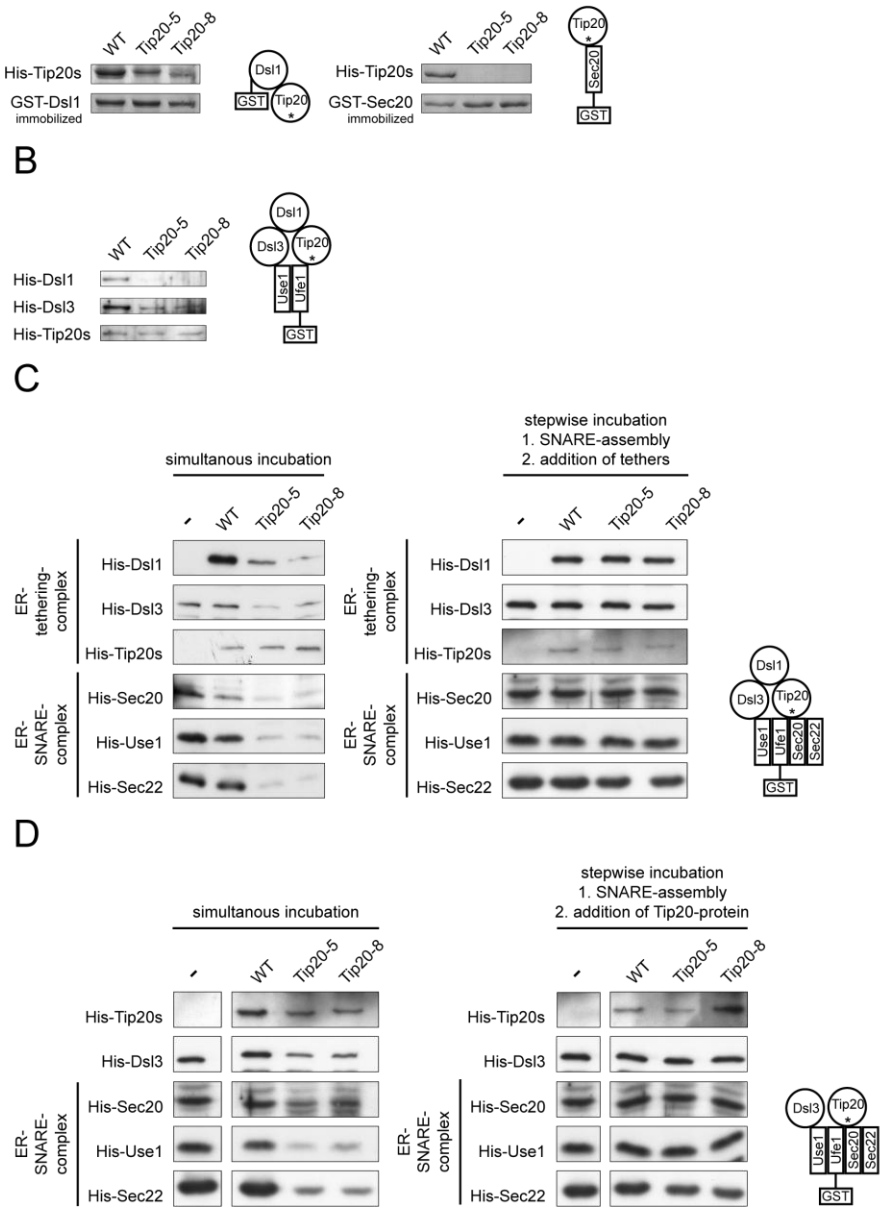
P13
P100
S100

Figure 5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WT
Tip20-5
Tip20-8
His-Tip20s
GST-Dsl1
immobilized
Dsl1
GST
Tip20
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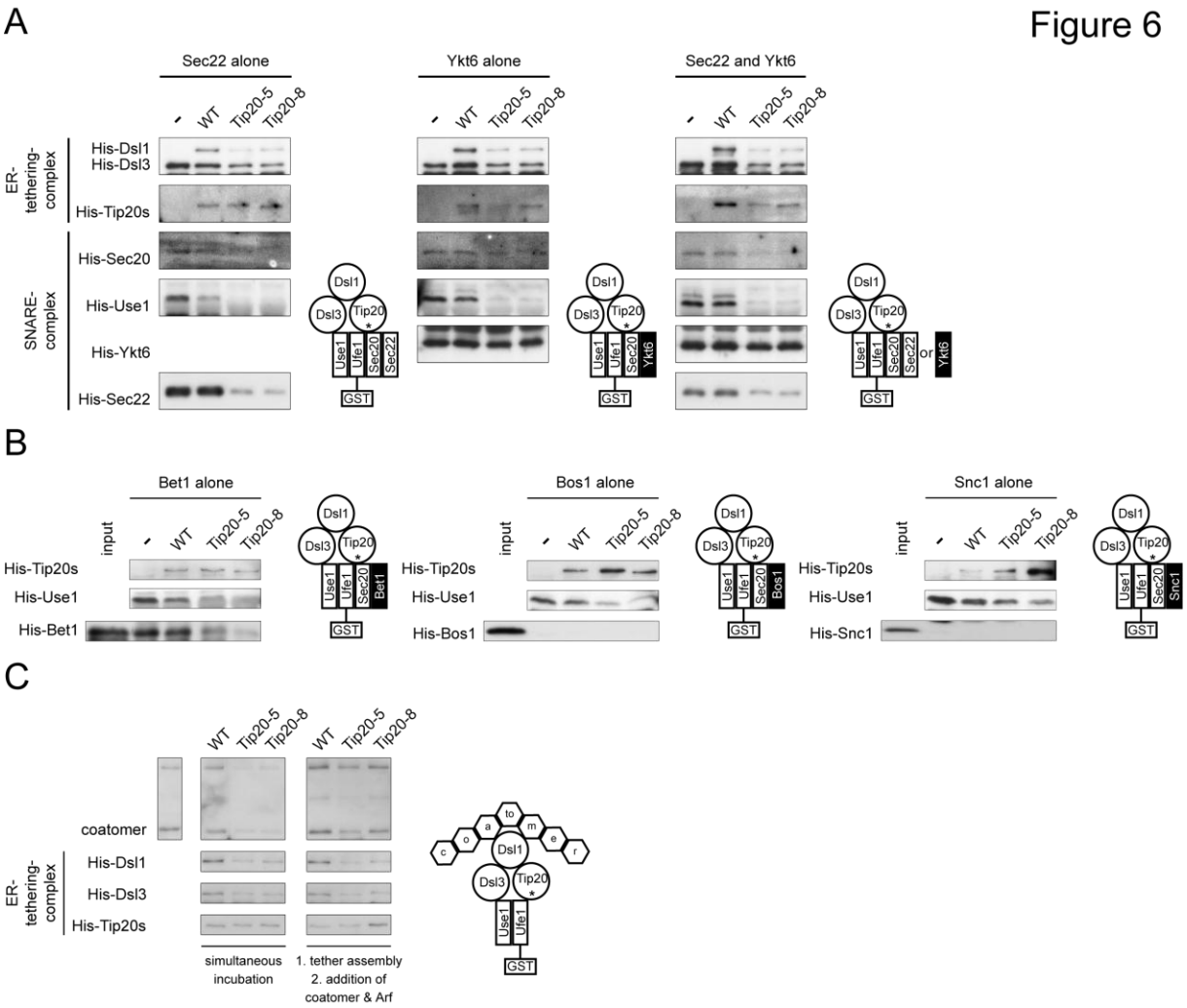
GST-Sec20
immobilized
Sec20
B
His-Dsl1
His-Dsl3
His-Tip20s
Dsl3
Use1
Ufe1
C
simultanous incubation
stepwise incubation
1. SNARE-assembly
2. addition of tethers
-
ER-tethering-complex
ER-SNARE-complex
His-Sec20
His-Use1
His-Sec22
Sec22
D
simultanous incubation
stepwise incubation
1. SNARE-assembly
2. addition of Tip20-protein

Figure 6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Sec22 alone
Ykt6 alone
Sec22 and Ykt6
-
WT
Tip20-5
Tip20-8
ER-tethering-complex
His-Dsl1
His-Dsl3
His-Tip20s
SNARE-complex
His-Sec20
His-Use1
His-Ykt6
His-Sec22
Dsl1
Dsl3
Tip20
Use1
Ufe1
Sec20
Sec22
Ykt6
GST
or
B
Bet1 alone
Bos1 alone
Snc1 alone
input
His-Tip20s
His-Use1
His-Bet1
His-Bos1
His-Snc1
Bet1
Bos1
Snc1
C
coatomer
His-Dsl1
His-Dsl3
His-Tip20s
simultaneous incubation
1. tether assembly
2. addition of coatomer & Arf
Dsl1
Dsl3
Tip20
Use1
Ufe1
GST

Supplementary material

Supplementary Table 1: Yeast strains used in this study

Strain	Genotype	Source
SEY6210	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9*	P. Cosson
PC137	*MATa ura3-1 leu2-Δ1 his4-619 trp1-Δ9 lys2-801am suc2-Δ9 tip20-5*	P. Cosson
tip20-8	*MATα ura3 leu2 his4 trp1 lys2 suc2-Δ9 tip20-8*	P. Cosson
YAS2793	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS316-TIP20-URA3*	this study
YAS2794	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-TIP20-LEU2*	this study
YAS2795	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20F248L-LEU2*	this study
YAS2796	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20N285S-LEU2*	this study
YAS2797	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20S563P-LEU2*	this study
YAS2798	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20F621S-LEU2*	this study
YAS2799	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20K381E-LEU2*	this study
YAS2800	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20L435S-LEU2*	this study
YAS2801	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20Δ1-81-LEU2*	this study
YAS2802	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20I10DL28E-LEU2*	this study
YAS2803	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20V17E-LEU2*	this study
YAS2804	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-TIP20-3HA-LEU2-TRP1*	this study
YAS2805	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::kanMX4 pRS315-tip20Δ1-81-3HA-LEU2-TRP1*	this study
YAS2806	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 TIP20::Tip20-STREP-kanMX6*	this study
YAS2807	*MATa ura3-1 leu2-Δ1 his4-619 trp1-Δ9 lys2-801am suc2-Δ9 tip20-5::tip20-5-STREP-kanMX6*	this study
YAS2808	*MATα ura3 leu2 his4 trp1 lys2 suc2-Δ9 tip20-8::tip20-8-STREP-kanMX6*	this study
YAS2809	*MATα ura3 leu2 his3 trp1 lys2 suc2-Δ9 SEC20::Sec20-HBH-TRP1*	this study
YAS2810	*MATa ura3-1 leu2-Δ1 his4-619 trp1-Δ9 lys2-801am suc2-Δ9 tip20-5 SEC20::Sec20-HBH-TRP1*	this study
YAS2811	*MATα ura3 leu2 his4 trp1 lys2 suc2-Δ9 tip20-8 SEC20::Sec20-HBH-TRP1*	this study

Supplementary Table 2: Primers used in this study	
Name	Sequence
MDSeq1 (tip20Cterm raus)	TTG AGC TTA CCG TCA CAT GC
MDSeq2 (tip20Nterm raus)	TGC AGT TTT GAT GCC AGT TC
MDSeq3 (tip20part1 left)	AAA ATT GTA GGC AGA AGT AGA TAA GAA
MDSeq4 (tip20part1 right)	TTG AGG AAC TGT AGC GAA GAC A
MDSeq5 (tip20part2 left)	TTT GAA CGT CAG TTA GAT GAG TTA GC
MDSeq6 (tip20part2 right)	CTG GCT TCG TCA ACC CAT T
MDSeq7 (tip20part3 left)	AGG TTT ACG TAC CAT TTT CAC G
MDSeq8 (tip20part3 right)	GCT CTT TAG TTC TTG TTT CAT TCA A
MDSeq9 (tip20part4 left)	GAT TAT TTG GAA CCG TTC TAC GA
MDSeq10 (tip20part4 right)	TAA CAT TTA ACA ATT CGT TCT TAA CC
MDSeq11 (tip20part5 left)	CAA GCG CTG AGT TGG TCA AT
MDSeq12 (tip20part5 right)	TGG TCT GGA GTT ACA TTT GGA
MDTIP1 (Tip20-BamHIfwd)	CGC GGA TCC GCG AGC AGC AAC GAG CGT TTA AT
MDTIP2 (Tip20-BamHIrev)	CGC GGA TCC GCG GCG CCA CAA AAG TTT CCT AC
MDTIP3 (Tip20Dfwd)	TTG TAT ATT AAG TTA TTG TTT ATA AGC ATA GTC ACA AGT GCA TAA CAG CTG AAG CTT CGT ACG C
MDTIP4 (Tip20DeletionS2)	TAA TAC TCG TCT TGT TGA TTT TTT TCC TTC TCT TTT TTT TAC GAG GCA TAG GCC ACT AGT GGA TCT G
MDTIP7 (T20-5UTR_fwd)	CGC TCT AGA TGC CTG CAT TAA ACA CGG TA
MDTIP8 (T20-5UTR_rev)	CCC GGG TAT ATA GGA TCC AGT TTT TAT GCA CTT GTG ACT ATG C
MDTIP9 (T20-3UTR_fwd)	GGA TCC TAT ATA CCC GGG CTC GTA AAA AAA AGA GAA GGA AAA AAA TC
MDTIP10 (T20-3UTR_rev)	CGC CTG CAG TGC AGG AAA GAA AGA ACG TG
MDTIP11 (ARATIforward(BamH1))	CGC GGA TCC ATG AAC GGC ATT GAT GAT CTC
MDTIP12 (ARATIreverse(Kpn1))	CGG GGT ACC TTA TAA TAT GTT ACC ATA TAT AAT CCT ATA G

Supplementary Table 3: Constructs used in this study		
construct	plasmid	source
C-terminal GST-tagged cytoplasmic region of Ufe1p (1-327)	pETGEXCT (Sharrocks, 1994)	Randy Schekmann (University of California, Department of Molecular and Cell Biology, Berkeley, USA)
N-terminal His_6-tagged cytoplasmic region of Use1p (1-217) co-expressed with Dsl3p	pQLink vector system (Scheich *et al.*, 2007)	F. M. Hughson (Princeton University, Department of Molecular Biology, Princeton, USA)
N-terminal GST-tagged cytoplasmic region of Sec20p (1-275)	pGEXTT (pGEX-2T with modified polylinker region, GE Healthcare)	R.-W. Peng (ETH Zürich, Department of Biosystems Science and Engineering, Basel, Switzerland).
N-terminal His_6-tagged cytoplasmic region of Sec20p (1 -275)	pQE30 (Qiagen)	H. D. Schmitt (Max-Planck-Institute for Biophysical Chemistry, Department of Neurobiology, Göttingen, Germany)
C-terminal His_6-tagged cytoplasmic region of Sec22p (1-180)	pET24b (Merck)	D. K. Banfield (Hong Kong University of Science and Technology, Department of Biology, Hong Kong SAR, People's Republic of China).
C-terminal His_6-tagged cytoplasmic region of Bet1p (1-123)	pET24(+)	H. D. Schmitt
C-terminal His_6-tagged cytoplasmic region of Bos1p (1-216)	pET24b (Merck)	D. K. Banfield
N-terminal His_6-tagged cytoplasmic region of Snc1p (1-93)	pTrcHisC (Invitrogen)	J. E. Gerst (Weizmann Institute of Science, Department of Molecular Genetics, Israel)
C-terminal His_6-tagged cytoplasmic region of Ykt6p (2-190)	pET24b (Merck)	D. K. Banfield
N-terminal GST-tagged Dsl1p	pGEXTT	H. D. Schmitt
N-terminal His_6-tagged Dsl1p	pProExHTb (Invitrogen)	F. M. Hughson
N-terminal His_6-tagged Tip20p	pProExHTb (Invitrogen)	F. M. Hughson
N-terminal His_6-tagged Tip20-5p	pProExHTb (Invitrogen)	this study
N-terminal His_6-tagged Tip20-8p		

Supplementary Figure legend

Figure S1

Evolutionary conservation profile for Tip20p as calculated by the ConSurf Server (Ashkenazy *et al.*, 2010). **(A)** Secondary structure representation of Tip20p. The amino acids are colored by their conservation grade according to the gradient shown, with blue (1) to red (9) indicating variable to conserved. The red triangles indicate the mutations found in Tip20-8p and the green triangles the mutations found in Tip20-5p. The amino acids that occur in the mutations are indicated under the respective triangles. **(B)** Amino acid conservation scores for the residues found to be mutated in Tip20-8p and Tip20-5p. 3LATOM: The ATOM derived sequence in three letter code, including the amino acid positions as they appear in the PDB file and the chain identifier. SCORE: The normalized conservation scores. COLOR: The color scale representing the conservation scores (9: conserved, 1: variable). CONFIDENCE INTERVAL: A confidence interval is assigned to each of the inferred evolutionary conservation scores. CONFIDENCE INTERVAL COLORS: The color scale representing the lower and upper bounds of the confidence interval. MSA DATA: The number of aligned sequences having an amino acid (non-gapped) from the overall number of sequences at each position. RESIDUE VARIETY: The residue variety at each position of the multiple sequence alignment.

A

Supplementary figure 1

Sec. struc.:  Helices labeled H1, H2 ... and strands by their sheets A, B ...

Helix Strand

Motifs: β beta turn, γ gamma turn

Conservation colouring: Low 1 2 3 4 5 6 7 8 9 High

B

SEQ		3LATOM	SCORE	COLOR	CONFIDENCE INTERVAL	CONFIDENCE INTERVAL COLORS	MSA DATA	RESIDUE VARIETY
Tip20-8p								
	F	PHE248:A	-0.519	6	-0.820,-0.332	7,6	55/56	S,F,W,I,L,Y,V
	N	ASN285:A	0.183	5	-0.140, 0.679	5,3	55/56	S,T,N,K,Y,E,V,H,Q,D,L
	K	LYS381:A	0.979	3	0.343, 1.170	4,2	55/56	A,S,T,N,K,E,H,Q,M,D,I,G
	L	LEU435:A	-0.565	6	-0.820,-0.332	7,6	46/56	Q,D,N,K,R,E,L
	S	SER563:A	-0.259	6	-0.506, 0.080	6,5	55/56	F,A,S,T,P,V,M,C,I,L
	F	PHE621:A	-0.135	5	-0.506, 0.080	6,5	54/56	A,S,F,T,Y,V,M,I,L
Tip20-5p								
	D	ASP358:A	0.307	4	-0.140, 0.679	5,3	50/56	S,A,T,N,K,P,E,H,Q,D,G,L
	E	GLU370:A	0.667	3	0.343, 1.170	4,2	56/56	A,Q,N,D,K,R,E
	N	ASN382:A	-0.465	6	-0.820,-0.140	7,5	54/56	A,S,T,N,P,E,D,G,L
	L	LEU454:A	-0.802	7	-1.113,-0.506	8,6	48/56	S,A,F,Q,C,I,E,L,V
	S	SER475:A	-1.011	7	-1.263,-0.820	8,7	56/56	A,S,T,N,E,Q,C,D,R,G
	D	ASP513:A	1.624	1	1.170, 2.360	2,1	55/56	S,T,N,K,E,V,H,Q,M,D,R,I,G,L
	L	LEU542:A	0.531	4	0.080, 0.679	5,3	55/56	S,T,N,P,K,E,V,Q,M,D,I,G,L
	N	ASN572:A	0.144	5	-0.140, 0.343	5,4	55/56	S,A,T,N,K,E,H,Q,D,C,I,G,L
	K	LYS588:A	0.355	4	-0.140, 0.679	5,3	55/56	A,S,F,T,W,N,K,Y,V,C,I,L

6 References

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