Analysis of SNAREs, Arf1p and Regulators in Intracellular Transport

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1 Zusammenfassung

Für die Kommunikation zwischen den einzelnen Organellen, die das Überleben einer eukaryotischen Zelle sichert, ist der Vesikeltransport unbedingt notwendig. Bei vielen Schritten innerhalb des sekretorischen Weges ist die kleine GTPase Arf1p ein wichtiger Regulator; z.B. bei der Bildung von Protein-umhüllten Vesikeln von einem Organell innerhalb des sekretorischen Weges. Damit ein Vesikel mit einer Akzeptororganelle verschmelzen und seine Fracht abliefern kann, muß sich die Proteinhülle vom Vesikel lösen. Dieser Prozess wird durch die Hydrolyse von GTP durch Arf1p bewerkstelligt. Hierfür benötigt Arf1p die Hilfe von ArfGAPs, die die GTPase Aktivität von Arf1p erhöhen (GTPase activating proteins). SNARE Proteine (soluble NSF attachment protein receptors) auf der Vesikel- (v-SNAREs) und der Akzeptormembran (t-SNAREs) bilden *trans*-Komplexe, die beide Membranen zusammenführen können. Schließlich können Vesikel- und Akzeptormembran verschmelzen.

Frühere Ergebnisse aus unserem Labor haben gezeigt, dass ArfGAPs katalytisch eine Konformationsänderung in v-SNARE Proteinen hervorrufen können. In der aktivierten Konformation kann das v-SNARE mit Arf1p wechselwirken, was dazu führt, dass die SNAREs effizient in neu entstehende Transportvesikel integriert werden.

Daten aus der vorliegenden Arbeit belegen, dass ArfGAPs diese aktivierte SNARE Konformation auch in t-SNAREs hervorrufen kann. Da die aktivierte Konformation die SNARE-Komplexbildung begünstigt, spielt sie nicht nur für den Einbau von SNAREs in neu entstehende Vesikel, sondern auch während des Verschmelzens des Vesikels mit der Akzeptormembran eine große Rolle. Basierend auf diesen Beobachtungen, vermuten wir, dass ArfGAPs auf SNAREs ähnlich wie Chaperone wirken könnten, indem sie die Faltung von SNAREs positiv beeinflussen. SNAREs in der aktivierten Konformation sind außerdem in der Lage mit Sec17p und der AAA-ATPase Sec18p, den in der Hefe homologen Proteinen von α-SNAP (soluble NSF attachment protein) und NSF (N-ethylmaleimide-sensitive factor), zu interagieren. Sec17p und Sec18p spielen bei der Membranfusion eine Schlüsselrolle, da sie *cis*-SNARE-Komplexe, die Überreste von vorhergehenden Membranfusionen in der Akzeptormembran darstellen, auflösen können. Hierfür ist ATP-Hydrolyse durch die ATPase Sec18p essentiell. Für ein anfängliches Erkennen von Vesikel- und Akzeptormembran sind Überreste der Vesikelhülle eventuell noch notwendig, dennoch würden diese Reste den eigentlichen

Membranfusionsprozess behindern. Hier können wir eine zweite Funktion von Sec18p zeigen, die unabhängig von der ATP-Hydrolyse und dem Co-Faktor Sec17p ist: Sec18p kann verbliebenes Arf1p von den SNARE Proteinen verdrängen. Aufgrund dieser Funktion kann der Fusionsprozess stattfinden.

V-SNARE Proteine werden nach einer erfolgreichen Vesikelfusion zum Ausgangskompartiment zurücktransportiert. Ergebnisse aus dieser Arbeit zeigen, dass das ArfGAP Gcs1p mit den SNARE Proteinen Snc1p und Snc2p, die an der Exo- und Endozytose in Hefe beteiligt sind, genetisch und physikalisch wechselwirken kann. Diese Interaktion ist für das Recycling, das über das *trans*-Golgi-Netzwerk (TGN) und die Endosomen abläuft, unbedingt notwendig. Transportvesikel, die von einer COPI Proteinhülle umgeben sind, spielen ihre Hauptrolle im Pendelverkehr zwischen dem endoplasmatischen Retikulum (ER) und dem Golgi Apparat sowie im retrograden Transport innerhalb des Golgi Apparats. Wir konnten jedoch zeigen, dass COPI-umhüllte Vesikel auch für das Recycling der Snc v-SNAREs im post-Golgi Transport eine wichtige Rolle spielen.

Das *trans*-Golgi-Netzwerk ist die wichtigste Verteilstation für Proteine, die vorwärts transportiert werden. Die kleine GTPase Arf1p ist auch an Vesikeltransportvorgängen beteiligt, die vom TGN ausgehen. Im Bestreben neue Interaktoren von Arf1p zu finden, konnten wir eine bislang unerforschte pilz-spezifische Proteinfamilie identifizieren: die ChAPs (Chs5p and Arf1p-interacting proteins). Diese Proteinfamilie hat vier Mitglieder: Bch1p, Bch2p, Chs6p and Bud7p. Alle vier Proteine sind zumindest teilweise am TGN lokalisiert; hierfür ist die Interaktion mit Chs5p, einem weiteren Faktor, notwendig. Schon früher konnte gezeigt werden, dass Chs5p für den Transport des Enzyms Chitin Synthase III (Chs3p) and den Knospungshals in Hefe essentiell ist; ein Prozess in den auch Arf1p involviert ist. Im Rahmen der vorliegenden Arbeit konnte gezeigt werden, dass Arf1p, Chs5p und einzelne Mitglieder der ChAP Familie genetisch und physikalisch interagieren und am Transport von Chs3p zur Plasmamembran beteiligt sind. Arf1p, Chs5p und die einzelnen ChAP Proteine befinden sich höhermolekularen Komplexen, die außerdem Chs3p, als mögliche Fracht von spezialisierten Transportvesikeln enthalten. Bei der Bildung dieser Transportvesikel am TGN könnten die ChAPs als Frachtrezeptoren, als Adapter für eine Proteinhülle oder sogar selbst als Proteinhülle für Transportvesikel fungieren.

Zusammengefaßt stellt diese Arbeit neue Interaktoren für die kleine GTPase Arf1p, sowie neue Funktionen für die regulatorischen Proteine von Arf1p, den ArfGAPs, vor.

2 Summary

Vesicular transport is an essential process allowing communication between different organelles in an eukaryotic cell. The small GTPase Arf1p regulates the generation of coated vesicles from donor organelles at many different levels of the secretory pathway. Arf1p cooperates with GTPase-activating proteins (GAPs) to hydrolyze the bound GTP, which subsequently induces the shedding of the proteinacious vesicle coat. Only then the vesicle is capable to undergo SNARE-mediated (soluble NSF attachment protein receptor) fusion with a target organelle to deliver its content. Previously, our lab showed that ArfGAPs can catalytically induce an altered conformation in vesicle SNARE proteins (v-SNAREs). The SNARE in the altered conformation is able to interact with Arf1p. Thus, the uptake of v-SNAREs in budding vesicles is facilitated.

The current study extends the previous results to target membrane SNAREs (t-SNAREs) and shows that the ArfGAP-induced conformation enhances the formation of SNARE complexes. SNARE complex formation is an essential step during membrane fusion. Thus, the altered SNARE conformation is not only important during vesicle generation, but also for the consumption of transport vesicles. This let us speculate that ArfGAP proteins might act in a chaperone-like function on SNARE proteins. We were also able to show that SNARE proteins in the ArfGAP-induced SNARE conformation can interact with Sec17p and Sec18p, the yeast homologs of α-SNAP (soluble NSF attachment protein) and AAA-ATPase NSF (N-ethylmaleimide-sensitive factor). Both factors play a key role in membrane fusion by resolving *cis*-SNARE complexes. Data from this study indicate that the ATPase Sec18p has a second function during vesicle fusion as it can displace Arf1p from SNAREs. This process requires neither ATP-hydrolysis nor Sec18p´s co-factor Sec17p. Residual coat on the vesicle could still be required during an initial contact between the two fusing membranes. However, it would likely obstruct the final fusion event. Thus, Sec18p might be able to remove residual coat from a vesicle and allow final fusion to take place.

After one round of vesicle fusion, v-SNAREs are recycled back to the donor compartment. Data presented in this study show that Snc1p and Snc2p, which are v-SNAREs involved in yeast exo- and endocytosis, interact physically and genetically with the ArfGAP Gcs1p, and that this interaction is essential for recycling of SNAREs via the *trans*-Golgi-Network (TGN) and endosomes. Furthermore, we were able to show that the

COPI vesicle coat, which has been implicated in retrograde trafficking from the Golgi apparatus to the endoplasmic reticulum (ER) as well as in retrograde traffic within the Golgi apparatus, plays also an important role in the recycling of the post-Golgi SNAREs Snc1p and Snc2p.

Besides Arf1p´s role in the formation of retrograde directed vesicles, Arf1p also participates in anterograde trafficking from the TGN, which is thought to be the main sorting station for anterograde cargo in an eukaryotic cell. In an attempt to identify new interactors of the small GTPase Arf1p, we found a novel fungi-specific protein family: the ChAPs (for Chs5p and Arf1p-interacting proteins). The ChAP family of proteins consists out of four members: Bch1p, Bch2p, Chs6p and Bud7p. These factors at least partially localize to the TGN, dependent on their interaction with Chs5p. Chs5p has been previously reported to be essential for the delivery of chitin-synthase III (Chs3p) to the yeast bud neck-region; a process Arf1p is also implicated in. We are able to show that Arf1p, Chs5p and individual members of the ChAP family interact genetically and physically. Arf1p, Chs5p and the ChAP proteins form high molecular complexes that contain the potential cargo Chs3p. Based on our findings, we suggest that the ChAP proteins are required for the transport of certain cargo in specialized transport vesicles. The ChAPs might function as cargo receptors, coat adaptors or even as novel coat.

Altogether, this work has highlighted new interactors for the small GTPase Arf1p and new modes of function for its regulatory GAP proteins.

3 Introduction

3.1 Intracellular transport, secretion and endocytosis

Compartmentalization of the intracellular space into discrete membrane-bound organelles is a hallmark of eukaryotic cells. This feature has many advantages as it allows a cell to have suitable environments for a great variety of biochemical reactions ranging from enzyme content to pH and redox potential. However, compartmentalisation also requires a sophisticated machinery to achieve the communication between organelles. This machinery not only allows exchange of material between the organelles, but must also ensure that an organelle, in order to function properly, retains its identity respective to lipid and protein content. A single eukaryotic cell expresses several thousand proteins, and each one needs to be localized correctly. Proteins in general follow either the cytoplasmic or the secretory pathway to reach their final destination (Palade, 1975; Lodish *et al*., 2003). For proteins that function in the cytoplasm, the nucleus, the peroxisomes or the mitochondria, protein translation is initiated on cytosolic ribosomes. After synthesis, they are released into the cytoplasm, from which they can, if necessary, be imported into organelles dependent on a specific localization sequence. Proteins, which travel along the secretory pathway, have special signal sequences. Upon recognition of these, the ribosomes relocate from the cytoplasm to the membrane of the endoplasmic reticulum (ER). The newly synthesized proteins are either released into the lumen of the ER or co-translationally inserted into the ER membrane. After folding and initial modification in the ER, proteins can be transported to the Golgi apparatus. From there they can reach the endosomal system, the lysosome or the plasma membrane (Figure 1) (reviewed by Bonifacino and Glick, 2004). Although all these proteins are termed secretory proteins, only a fraction is secreted to the extracellular space; a large portion is retained within a certain organelle along the secretory pathway to function there as a resident protein. Proteins do not only leave the cell, the cell takes up macromolecules and particulate substances from the outside or recycles proteins from the plasma membrane in a process which is called endocytosis. Furthermore, proteins that escaped their compartment of residence, need to be retrieved from later compartments. Transport along the secretory and endocytic pathway is mostly achieved by small vesicles. These are membrane-bound carriers that are initially equipped with a

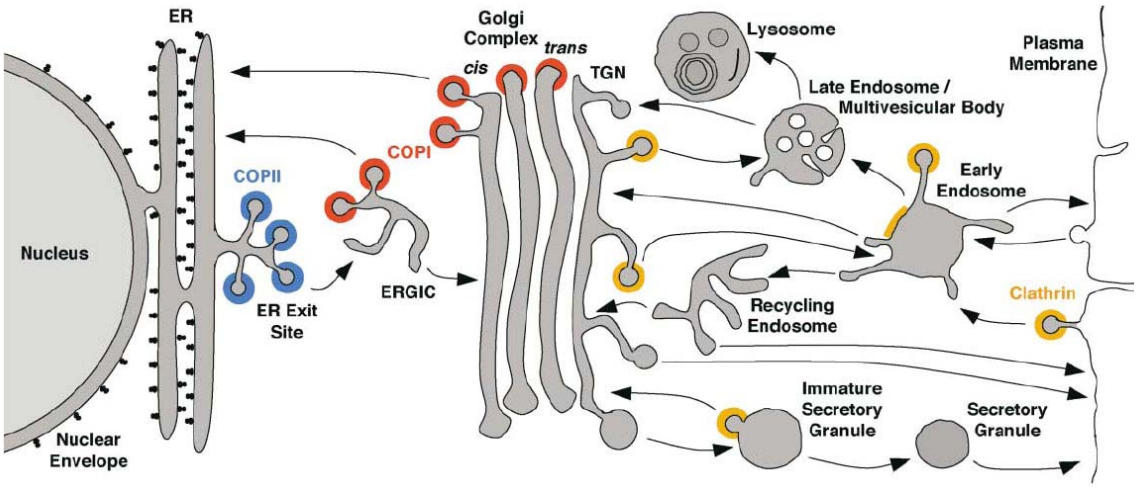

Figure 1: Secretory and endocytic pathways. Overview of important vesicular transport routes in a mammalian cell. Transport steps are indicated as arrows. Colors indicate the known or presumed locations of COPII (blue), COPI (red) and clathrin (orange). In *S. cerevisiae* the ER-Golgi-intermediate compartment (ERGIG) does not exist and the vacuole has the function of the lysosome (taken from Bonifacino and Glick, 2004).

cytoplasmic, proteinacious coat. To deliver cargo proteins as well as lipids, vesicles bud from a donor compartment and fuse with an acceptor membrane in
a series of tightly regulated, sequential steps. The mechanisms and factors involved in generation and consumption of transport vesicles are conserved from yeast to man.

3.2 Molecular mechanism of vesicle budding

Although most steps in the secretory and endocytic pathway employ different types of vesicles, the basic principles of vesicle formation and fusion are almost invariant (Figure 2). Vesicle formation is initiated when a small GTPase of the Arf-family is recruited to a donor membrane. Dependent on the interaction with the small GTPase, coat components are able to bind to the membrane. Although COPI and COPII vesicles can be formed *in vitro* using synthetic liposomes, nucleotides, Arf-family GTPases and coat components (Matsuoka *et al.*, 1998; Spang and Schekman, 1998), *in vivo* cargo proteins in the donor membrane are actively involved in coat recruitment and stabilization (Aoe *et al.*, 1998; Forster *et al.*, 2006; Pepperkok *et al.*, 2000). Initially, complexes consisting of only a few cargo molecules and coat components are generated (Springer *et al.*, 1999). A coated lattice is formed on the membrane as these complexes interact laterally and more coat components and cargo proteins are added. Already at this stage membrane-anchored fusion factors, required for the consumption of the vesicles at the target membrane, are

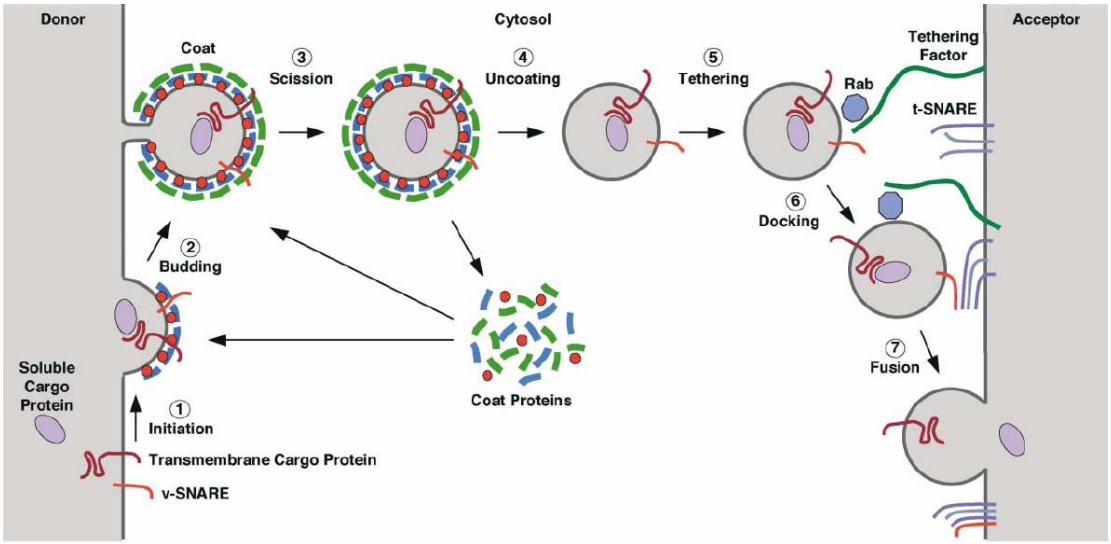

Figure 2: Steps of vesicle budding and fusion. Small GTPases are recruited to the donor membrane by cargo molecules and can attract other coat components to form an initiation complex (1). These complexes can interact laterally and more coat components and cargo proteins are included into the forming vesicle bud (2). The neck structure between the coated vesicle and the donor membrane is severed (3). The coat is shed due to GTP-hydrolysis by the small GTPase and coat components are recycled to generate new vesicles (4). The vesicle moves to the target compartment and is tethered by the action of RabGTPases and tethering complexes (5). During docking (6) SNAREs on the vesicle (v-SNAREs) and on the target membrane (t-SNAREs) form a *trans*-SNARE complex, which promotes membrane fusion and content mixing (7) (taken from Bonifacino and Glick, 2004).

included in the nascent vesicle. Coat polymerization induces membrane bending and yields a characteristic bud (Bi *et al.*, 2002). Finally, scission releases a coated vesicle into the cytoplasm. Before a vesicle can fuse with

an acceptor compartment, it has to shed its coat. The Arf-family GTPase, which was incorporated into the vesicle in its GTP-bound form (Barlowe *et al.*, 1994; Donaldson *et al.*, 1992), hydrolyzes GTP which serves as a trigger for uncoating (Tanigawa *et al.*, 1993; Yoshihisa *et al.*, 1993).

3.2.1 Small GTPases of the Arf-family

The Arf-family of small GTPases is part of the Ras superfamily of small GTPases. In mammalian cells six different Arf proteins ARF1-ARF6 are known; budding yeast possesses three Arf proteins. In the following sections `Arf´ refers to Arf-family members in yeast and higher eukaryotes, whereas Arf1p is used only for yeast Arf1 and ARF1 is used for mammalian Arf1. Yeast Arf1p and Arf2p are 96% homologous and act redundantly. Yet, 90% of Arf protein present in a yeast cell is Arf1p (Stearns *et al.*, 1990). Arf1p and Arf2p are mainly involved in the generation of vesicles that bud from the *cis*-Golgi to retrieve proteins to the ER as well as in the generation of retrograde

vesicles from medial and *trans*-Golgi compartments. Furthermore, Arf1p and Arf2p take part in the formation of vesicles that travel from the *trans*-Golgi network (TGN) in the anterograde direction. Arf3p is involved in trafficking in the endosomal system. Another member of the Arf-family is Sar1p, which is exclusively involved in formation of COPII-coated vesicles at the ER (Kuge *et al.*, 1994; Nakano and Muramatsu, 1989). Besides a role of Arf proteins in vesicular trafficking, members of the Arf-family help to regulate the membrane phospholipid composition, the actin cytoskeleton and organelle morphology. Furthermore, they function in mitotic growth and entry in the cell cycle (reviewed in Nie *et al.*, 2003; Randazzo *et al.*, 2000; Spang, 2002). Like all small GTPases, Arf-family members exist in two nucleotide states. Therefore, small GTPases can be considered as binary molecular switches (for review see Takai *et al.*, 2001).

There is continuous cycling between the two nucleotide states with the help of regulator proteins (Figure 3). The GDP-bound form is inactive and localizes to the cytoplasm. ArfGTPases require guanine-nucleotide-exchange factors (GEFs) for nucleotide exchange (Barlowe and Schekman, 1993; Peyroche *et al.*, 1996). In the course of nucleotide exchange, which occurs on membranes, a highly hydrophobic stretch, which is buried in a pocket formed out of a hairpin loop in the GDP-bound form, becomes inserted in the outer layer of a membraneous bilayer leading to the tight membrane association of the GTPase (Antonny *et al.*, 1997; Paris *et al.*, 1997). Arf-GTP is able to

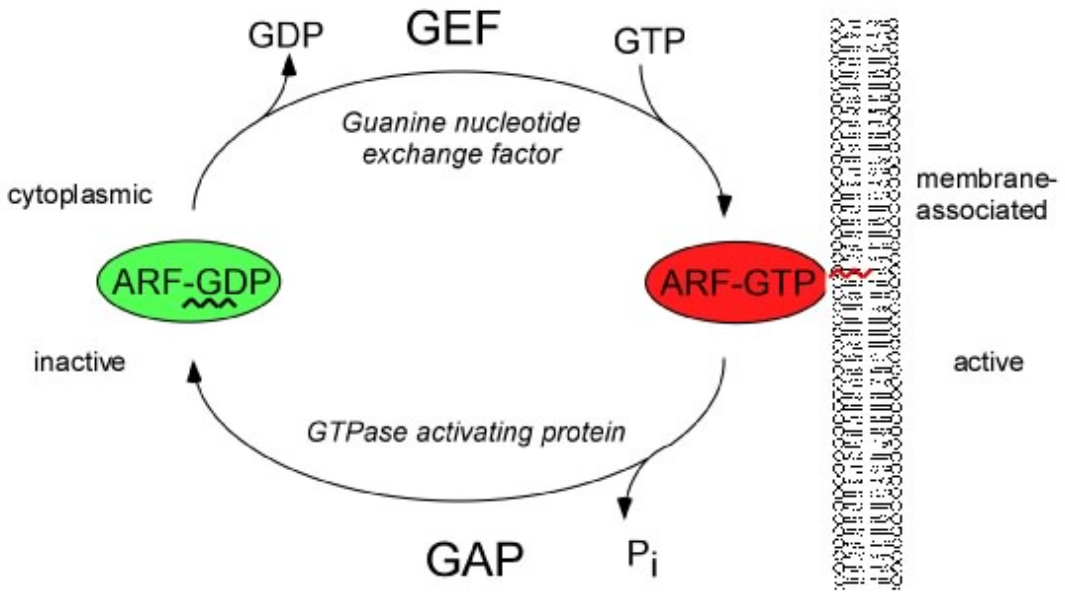

Figure 3: GTPase cycle of Arf1p. Arf1p-GDP is inactive and cytosolic. A guanine-nucleotide exchange factor (GEF) catalyzes the nucleotide exchange and thus mediates activation and stable membrane association Arf1p. To hydrolyze GTP and become inactivated again, Arf1p-GTP requires the interaction with a GTPase-activating protein (GAP).

recruit effectors like coat proteins to the membrane. In order to become inactivated again, ArfGTPases require GTPase-activating proteins (GAPs) (Makler *et al.*, 1995; Randazzo and Kahn, 1994).

3.2.2 ArfGAPs facilitate GTP hydrolysis

Currently six ArfGAPs are known in yeast, three of them Glo3p, Gcs1p and Age2p have been characterized in more detail: Glo3p provides GAP activity in retrograde transport from the Golgi to the ER and for intra-Golgi traffic (Poon *et al.*, 1999). Age2p functions in the late secretory pathway and in the endosomal system (Poon *et al.*, 2001). Gcs1p is thought to have a more general function, carrying out redundant functions in early and late steps along the secretory pathway. Gcs1p can substitute for Glo3p and Age2p, whereas Age2p cannot complement the loss of Glo3p and vice versa (Poon *et al.*, 2001). Moreover, Gcs1p is involved in actin remodeling. The genome of mammalian cells encodes as many as sixteen ArfGAP proteins in several subfamilies (reviewed by Randazzo and Hirsch, 2004). Mammalian ArfGAPs regulate Arf proteins in vesicular transport and additionally have Arf-dependent and independent functions in the regulation of the actin cytoskeleton and in integrin signaling (Krugmann *et al.*, 2002; Mazaki *et al.*, 2001; Miura *et al.*, 2002).

All ArfGAPs share an approximately 60 residues long GAP domain comprising the catalytic site. For most ArfGAPs, the GAP domain resides in the N-terminus of the protein. The active site is composed out of a Zn^{2+}-finger in which the zinc ion is coordinated by four cystein residues in a distinct spacing ($CXXCX_{16}CXXC$) (Cukierman *et al.*, 1995; Ireland *et al.*, 1994). Most GAPs for small GTPases of the Ras superfamily act by a so-called arginine-finger mechanism (Ahmadian *et al.*, 1997; Scheffzek *et al.*, 1997). An arginine residue of the GAP is positioned in the catalytic site of the GTPase. The arginine provides a positive charge that can compensate negative charges that occur during the transition state; thus, the GAP is able to promote GTP hydrolysis. Based on structural data for human ArfGAP1 (Goldberg, 1999) it was suggested that the coatomer complex is required for efficient GTP-hydrolysis that occurs in a tripartite complex composed out of ARF1, ArfGAP1 and coatomer.

3.2.3 Cargo contributes to vesicle generation

ArfGTPases have been show to be recruited first to the membranes by low-affinity interactions before nucleotide exchange occurs (Aridor *et al.*, 2001; Giraudo and Maccioni, 2003). ArfGAPs can induce a conformational change in SNARE (soluble NSF-attachment protein receptor) proteins, which are important for vesicle fusion (Rein *et al.*, 2002). SNAREs in the activated conformation recruit Arf1p. As there was no discrimination between the GDP- or GTP-bound form of the GTPase, SNAREs can serve as initial membrane-receptors for Arf1p. Members of the p24 cargo receptor family have even a higher affinity for the GDP-bound form of Arf, than for Arf-GTP (Gommel *et al.*, 2001). Several lines of evidence suggest that cargo sorting into vesicles requires GTP hydrolysis by Arf GTPases (Lanoix *et al.*, 1999; Nickel *et al.*, 1998; Pepperkok *et al.*, 2000). Sorting is at least in part achieved by regulation of ArfGAP membrane localization and activity (Aoe *et al.*, 1997; Lanoix *et al.*, 2001; Majoul *et al.*, 2001; Yang *et al.*, 2002).

3.2.4 Vesicle coats

Vesicle coats are required as scaffolds during budding. They aid cargo capture and GTP hydrolysis. Coat proteins shape a vesicle and determine its size which ranges from 60 to 150 nm dependent on the coat (Kirchhausen, 2000b). Currently three major vesicle coats are well characterized (reviewed in Kirchhausen, 2000b). COPII-coated vesicles mediate anterograde transport from the ER to the Golgi apparatus. Vesicles carrying a COPI coat function in retrieval from the Golgi to the ER as well as in intra-Golgi trafficking. From the *trans*-Golgi network (TGN) a diverse set of vesicles can bud that subsequently fuses with compartments of the endosomal/lysosomal system or the plasma membrane. Clathrin, which also plays a prominent role in endocytosis, serves as major coat component for most of these vesicles.

3.2.4.1 COPII-coated vesicles

COPII-coated vesicles transport newly synthesized secretory proteins from the ER to the *cis*-Golgi apparatus in yeast or the ER-Golgi intermediate compartment (ERGIC) in higher eukaryotes (Barlowe, 1994; Kuge, 1994; Scales, 1997). In principle, COPII-coated vesicles can transport all lumenal and transmembrane cargo proteins required in downstream compartments of the secretory pathway as well as GPI-anchored proteins

(Belden and Barlowe, 2001; Otte and Barlowe, 2004; reviewed in Barlowe, 2003; Lee *et al* , 2004).

To bud a COPII-coated vesicle, Sar1p-GTP recruits the Sec23/24p heterodimer from the cytoplasm to form a pre-budding complex (Antonny *et al.*, 2001; Springer and Schekman, 1998; Springer *et al.*, 1999). In a subsequent step Sec13/31p are recruited to the ER membrane forming a membrane-distal layer, which serves as a scaffold for the COPII-coated vesicle (Bi *et al.*, 2002; Lederkremer *et al.*, 2001; Matsuoka *et al.*, 2001). Sec24p seems to be the major cargo binding subunit (Miller *et al.*, 2003; Mossessova *et al.*, 2003). To shed the COPII coat, Sec23p acts as a GAP on Sar1p (Yoshihisa *et al.*, 1993).

3.2.4.2 COPI-coated vesicles

The second vesicle coat involved in transport in the ER-Golgi shuttle is the COPI coat (Orci *et al.*, 1993; Waters *et al.*, 1991). COPI vesicles retrieve chaperones which escaped the lumen of the ER and recycle SNARE proteins (Letourneur *et al.*, 1994; Pelham *et al.*, 1988). Furthermore, COPI-coated vesicles retrieve *cis*- and medial-Golgi-resident proteins which reach later Golgi compartments during cisternal maturation of the Golgi apparatus (reviewed by Glick and Malhotra, 1998; Pelham, 2001). Yet, there is also evidence for a role of COPI in late secretory trafficking (Aniento *et al.*, 1996; Gu *et al.*, 1997; Gu and Gruenberg, 2000; Robinson *et al.*, 2006; Whitney *et al.*, 1995).

To bud a COPI-coated vesicle, Arf-GDP is recruited to the donor membrane first. After nucleotide exchange, Arf-GTP is able to recruit the coatomer complex from the cytoplasm (Figure 4). Coatomer is a stable heptameric complex with a half-life of several hours (Lowe and Kreis, 1996). However, upon high salt treatment, two subcomplexes appear, the membrane-proximal F-COP subcomplex, consisting of the β-, γ-, δ- and ζ-COP subunits (respectively Sec26p, Sec21p, Ret2p and Ret3p in yeast) and the membrane-distal B-COP subcomplex composed out of α-, β'- and ε-COP (Sec33p, Sec27p and Sec28p in yeast) (Eugster *et al.*, 2000; Gaynor and Emr, 1997; Hara-Kuge *et al.*, 1994; Waters *et al.*, 1991). Arf is recognized by the membrane-proximal F-COP subcomplex, additionally most cargo binding sites reside within the β- and γ-COP subunits (Eugster *et al.*, 2000; Goldberg, 1999). Similar to Sec23p in COPII vesicles, the ArfGAP can be an intrinsic part of the coat (Lewis *et al.*, 2004; Yang *et al.*, 2002).

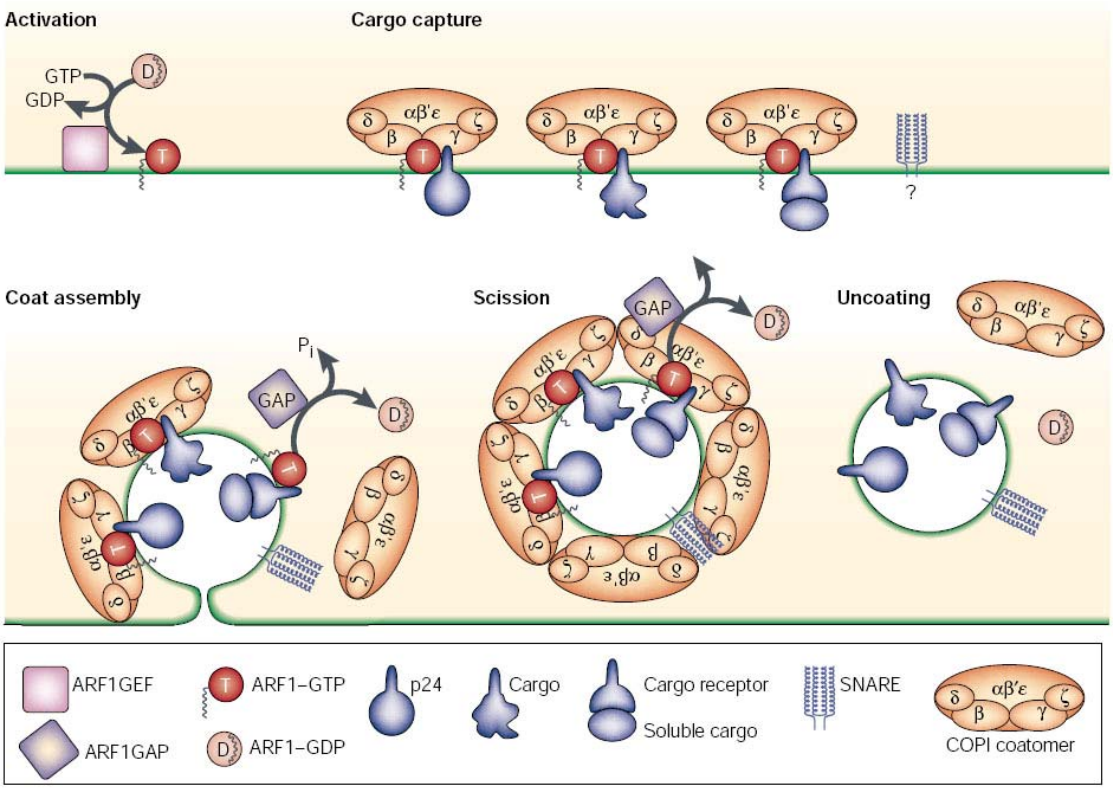

Figure 4: Key steps in the formation of COPI coated vesicles. Arf1p-GDP is recruited to the membrane, nucleotide exchange by a GEF mediates stable membrane anchoring. Initiation complexes are formed when Arf1p-GTP recruits its effector coatomer and cargo sorting is initiated. Coat polymerization on the membrane leads to membrane deformation and finally budding. Uncoating is initiated when GTP-hydrolysis is stimulated by an ArfGAP (taken from Kirchhausen, 2002).

3.2.4.3 Clathrin-coated vesicles

Clathrin-coated vesicles function in late secretory trafficking and early stages of the endocytic pathway. Clathrin co-operates with heterotetrameric adaptor protein complexes (APs) or the monomeric GGA proteins to form vesicles (for review see Boehm and Bonifacino, 2001; Kirchhausen, 2000a; Kirchhausen, 2000b).

First the AP complex is recruited to the membrane forming a membrane-proximal layer of the coat, later clathrin is bound forming a membrane-distal layer (Smythe *et al.*, 1992). The involvement of Arf1p in formation of clathrin-coated vesicles is less clear defined. So far, an Arf1p-GTP requirement on the membrane has only been shown for AP-1 and GGA (Crottet *et al.*, 2002; Hirst *et al.*, 2000). For the budding of AP-2/clathrin-coated vesicles from the plasma membrane, the membrane phospholipid composition as well as the actin cytoskeleton has been shown to be crucial (Honing *et al.*, 2005). As Arf3p is involved in regulating both processes, it might the ArfGTPase essential for the AP-2 complex. Vesicle fission requires the GTPase dynamin (reviewed by Sever, 2002). Coat

shedding is more complex than for COPI- and COPII-coated vesicles; it requires auxiliary cytoplasmic factors like Hsc70 and auxilin (reviewed in Kirchhausen, 2000a; Lemmon, 2001).

3.3 Molecular mechanism of vesicle consumption

After the vesicle coat is shed, fusion with a target membrane can occur. To ensure the successful fusion of a vesicle, the process follows an orchestrated set of events in distinct steps (Figure 2). First, priming resolves remnants of previous fusion events. In a next step, tethering that establishes an initial specific contact between the two membranes, is carried out. During the docking stage of vesicle fusion, cognate SNARE proteins residing on vesicle (v-SNAREs) and target membrane (t-SNAREs) form *trans*-complexes, which can zipper up progressively from the membrane-distal end pulling vesicle and target membranes into close proximity. Finally, membrane fusion and content mixing occurs. The order of events is best studied monitoring homotypic organelle fusion as well as vesicle fusion events at the Golgi and the neuronal synapse (reviewed by Jahn *et al.*, 2003; Mayer, 2001; Sudhof, 2004; Wickner and Haas, 2000). Most studies were carried out using isolated vesicles and organellar membranes *in vitro*, which allows the addition of purified proteins or inhibiting agents such as specific antibodies.

3.3.1 Rab-GTPases and tethering factors

Tethering is controlled by largely compartment-specific small GTPases of the Rab protein family and tethering factors. Like ArfGTPases, the RabGTPases belong to the Ras-superfamily of small GTPases (for review see Segev, 2001; Takai *et al.*, 2001; Zerial and McBride, 2001). They also cycle between an active GTP-bound form, that is able to recruit effectors to membranes and an inactive GDP-bound form, which is extracted from membranes by GDI (guanine-nucleotide-dissociation-inhibitor) (reviewed by Pfeffer *et al.*, 1995). RabGTPases are implicated in vesicle formation and cargo concentration (Carroll *et al.*, 2001; Pagano *et al.*, 2004) as well as in vesicle and organelle transport along actin cables and microtubules (Nielsen *et al.*, 2001). The most prominent role of RabGTPases is the recruitment of tethering factors from the cytoplasm. Membrane-localized tethering factors then can recruit vesicles to the vicinity of the target membrane. Thus, they establish a first specific contact between the vesicle and the target membrane. Based on their structure, tethering complexes are divided into two subgroups: large

coiled-coil containing tethers like the yeast protein Uso1p, which has been implicated in fusion of COPII vesicles with the Golgi or the mammalian EEA1, which is involved in endosome fusion. Multimeric tethering complexes like the COG, TRAPP, GARP and HOPS complexes have been implicated in most other vesicle fusion events studied so far (reviewed by Lupashin and Sztul, 2005; Whyte and Munro, 2002). Although for many fusion events in the cell, the tethering complexes involved have been identified, the mechanistic details of tethering and how downstream events are induced remain in large part elusive. Some tethering factors and Rab proteins can bind unpaired SNAREs. Thus, they are maybe involved in regulating the assembly of new SNARE complexes (Collins *et al.*, 2005; Shorter *et al*., 2002).

3.3.2 SNARE proteins

SNAREs have been implicated in the docking stage of all vesicle and organelle fusion events in the secretory and endocytic pathways (for reviews see Chen and Scheller, 2001; Jahn *et al.*, 2003; Mayer, 2001). SNARE proteins undergo a well defined cycle during membrane fusion (Figure 5). In the docking stage of vesicle fusion, four SNARE domains contributed by the v- and t-SNAREs form *trans*-complexes that can zipper up progressively from the membrane-distal end pulling vesicle and target membranes into close proximity, which is a prerequisite for fusion to occur (Fiebig *et al.*, 1999). Energy is released as the initially unstructured SNARE domains interact and form a highly structured α-helical bundle (Figure 6) (Fasshauer *et al.*, 1998; Poirier *et al.*, 1998b). The energy released during zippering is mainly responsible for overcoming the repulsive forces between the two hydrated membranes and thus is responsible for vesicle fusion being a vectorial process (Fasshauer *et al.*, 1997; Poirier *et al.*, 1998b; Sutton *et al.*, 1998). Due to the fusion event, SNARE complexes reside in the target membrane and are referred to as *cis*-SNARE complexes, or according to their sedimentation coefficient, 7S complexes. In order to recycle SNAREs for new fusion events, *cis*-SNARE complexes are resolved by α-SNAP (soluble NSF attachment protein) and NSF (N-ethylmaleimide-sensitive factor) (Sec17p and Sec18p in yeast, respectively). This allows v-SNAREs to be packaged into vesicles traveling to the donor compartment and t-SNAREs to engage in *trans*-complexes with v-SNAREs on newly arrived vesicles.

Studying vesicle fusion events at the neuronal synapse (Sollner *et al.*, 1993b) has led to division of the SNARE proteins in three sub-families: the syntaxin-like SNAREs, the SNAP-25-like SNAREs, and the synaptobrevins (Weimbs *et al.*, 1998). One member of

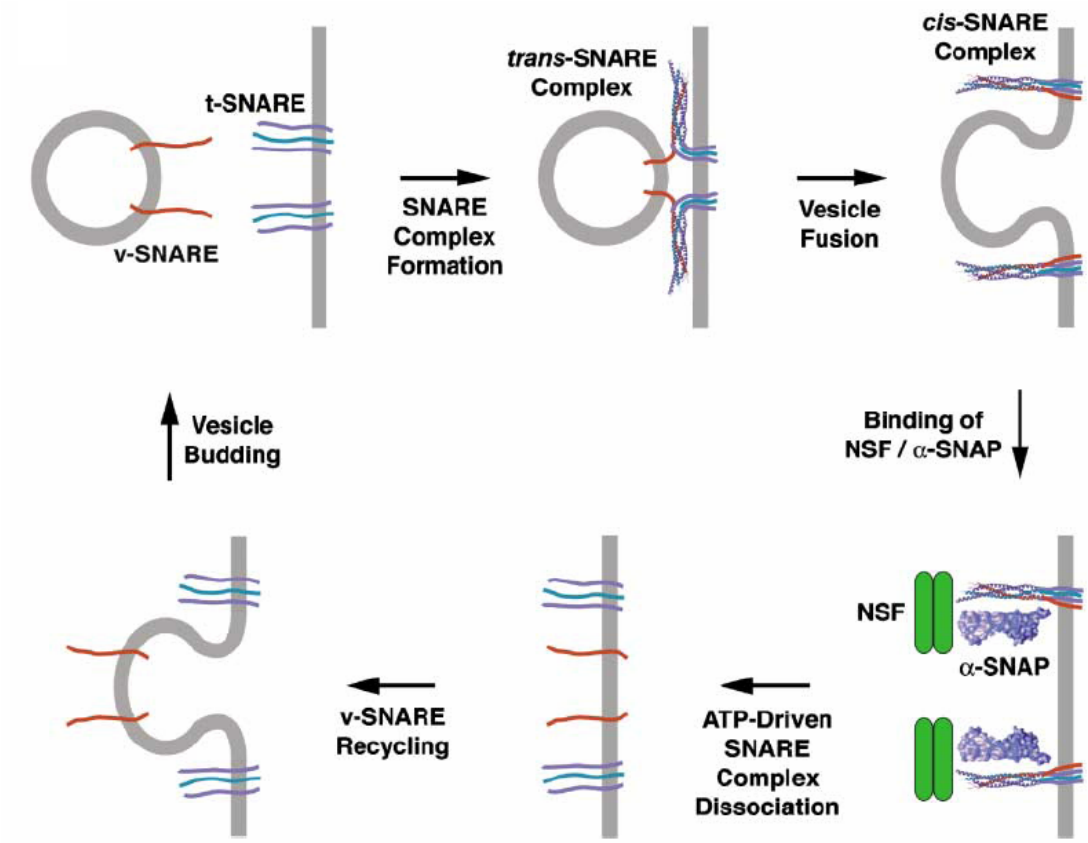

Figure 5: SNARE cycle. During membrane fusion SNARE proteins residing n the vesivle (v-SNAREs) and on the target membrane (t-SNAREs) form *trans*-complexes, bridging both membranes. During fusion the *trans*-compexbis converted to a very stable *cis*-complex, as both, v-SNAREs and t-SNAREs reside in the same membrane. The recycle SNARE proteins for future fusion events, α-SNAP and NSF bind the *cis*-SNARE complex. ATP-hydrolysis by NSF allows the resolving of the SNARE complex. v-SNAREs can be recycled to the donor membrane and t-SNAREs are able to engage with v-SNAREs on newly delivered transport vesicles (from Bonifacion and Gloick, 2004).

each sub-family is required for the formation of a SNARE complex. Unconventionally, SNAP-25 contributes two SNARE motifs to the SNARE complex in neuronal exocytosis. In most other fusion events, the four SNARE motifs reside within four different proteins (Hay, 2001). SNARE proteins show a similar domain structure (for reviews see Chen and Scheller, 2001; Hay, 2001). Only the syntaxins and some synaptobrevins have N-terminal extensions, which are capable to assume an independent folding. These have autoregulatory functions and serve as platform for binding of SNARE-regulating proteins. The middle part of the SNARE is governed by the SNARE motif, which is composed of specialized heptad repeats (Weimbs *et al.*, 1998). The SNARE motif takes part in forming the core SNARE complex. C-termini of SNARE proteins are responsible for membrane anchoring. Most SNAREs are so-called tail-anchored proteins, which post-translationally insert their C-terminal transmembrane domain in the ER membrane (Jantti *et al.*, 1994; Kutay *et al.*, 1995). Other SNAREs use hydrophobic modifications like

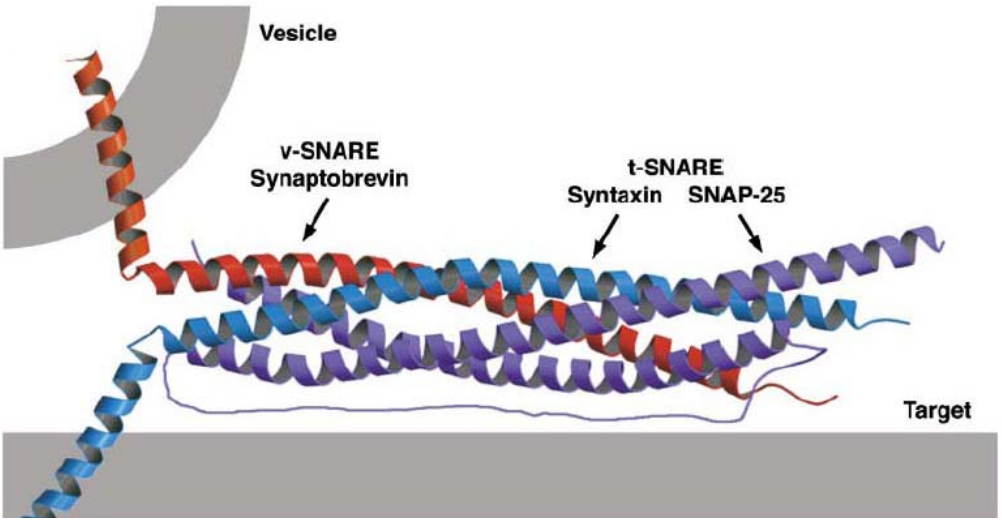

Figure 6: SNARE complex structure. Crystal structure of a synaptic *trans*-SNARE complex drawn after Sutton et al. (1998). The structures of the two membrane anchors and of the peptide that links the two SNAP-25 α-helices are hypothetical (taken from Bonifacino and Glick, 2004).

palmitoylation or phosphoinositide-binding domains for reversible membrane localization (Cheever *et al.*, 2001; Dietrich *et al.*, 2005; McNew *et al.*, 1997).

Structure determination of only distantly related SNARE complexes have demonstrated the existence of a common SNARE core complex (Antonin et al., 2002; Sutton *et al.*, 1998). The outer surface of the coiled-coil is composed mainly of charged and polar residues. The central part of the SNARE complex is composed out of sixteen layers, these layers orient perpendicular to the coiled-coil. Each layer is made out of four largely hydrophobic residues, contributed by the different SNARE proteins. The only exception is the central, `zero´ layer. Three of the polypeptdide chains contribute a glutamine (Q) residue, one chain contributes an arginine (R) residue. Due to conflicting reports whether a particular SNARE is required on the vesicle or on the target membrane as well as to avoid ambiguity in homotypic fusion events, Fasshauer *et at.* (1998) suggested to refer to SNAREs as R- or Q-SNAREs based on the residue in the zero layer. The α-helical SNARE complex bundle is stabilized by a large number of interactions, like hydrophobic interactions within the coiled-coil, hydrogen-bonds between the arginine and the glutamine residues that form the zero layer as well as size-complementary interactions. Furthermore, interhelical salt-bridges serve to stabilize the SNARE complex (Lauer *et al.*, 2006). Thus, SNARE complexes can only be denatured *in vitro* by a combination of SDS and heat, whereas either treatment alone leaves SNARE complexes intact. Unlike unpaired SNAREs, SNARE complexes are insensitive to limited proteolysis as well as to

some bacterial toxins, which have been shown to cleave non-complexed SNAREs (Fasshauer *et al.*, 1998; Hayashi *et al.*, 1994; Poirier *et al.*, 1998a; Yang *et al.*, 1999).

3.3.3 α-SNAP and NSF function during priming

The cell has invented a sophisticated system involving two proteins, α-SNAP and the AAA-ATPase NSF (Sec17p and Sec18p in yeast, respectively), to resolve SNARE complexes in a process termed priming (Fasshauer *et al.*, 1998; Mayer *et al.*, 1996; Sollner *et al.*, 1993a; Wilson *et al.*, 1992). *In vitro* assays used to dissect the events during vesicle fusion placed this reaction upstream of other fusion events, as it does not require the two membranes to be in close proximity.

To disassemble a helical bundle, rotational force is required (Muller *et al.*, 2002a). This force is generated by NSF and transduced to the SNARE complex by α-SNAP. NSF is a homohexamer with a barrel-like structure in six-fold rotational symmetry (Furst *et al.*, 2003). Each protomer is composed out of three distinct domains. The N-domain is essential for interaction with the SNARE complex via α-SNAP. Nucleotide binding motifs reside in the D1 and the D2 domain. The high ATPase activity of the D1 domain is used to generate the force that is required to dissolve *cis*-SNARE complexes. ATP binding in the D2 domain is required for hexamer formation, the structural ATP is not hydrolyzed (Furst *et al.*, 2003). α-SNAP is arch-shaped, the N- and the C-terminus are required for SNARE complex binding. The interaction with NSF is established by the C-terminus of α-SNAP. Binding to SNARE complexes occurs via basic patches on the concave surface, which match the slightly negative charge on the surface of SNARE complexes (Marz *et al.*, 2003; Rice and Brunger, 1999). First three molecules of α-SNAP bind one 7S *cis*-SNARE complex, covering it in a sheath-like manner (Rossi *et al.*, 1997; Wimmer *et al.*, 2001). 20S complexes are formed when NSF binds in a second step. These complexes have been visualized by electron microscopy showing that α-SNAP binding to the SNARE complex occurs laterally, while the NSF hexamer resides on top of the SNARE complex at the membrane-distal end. (Hanson *et al.*, 1997; Hohl *et al.*, 1998; Wimmer *et al.*, 2001). 20S complexes are transient intermediates which are rapidly disassembled in presence of Mg^{2+} and ATP (Barnard *et al.*, 1997; Clary *et al.*, 1990; Sollner *et al.*, 1993a; Wilson *et al.*, 1992).

Although it is known for more than fifteen years, that α-SNAP and NSF can resolve *cis*-SNARE complexes, the exact mechanism is still under debate. Initial experiments

showed that α-SNAP can stimulate ATP hydrolysis by NSF only when α-SNAP was absorbed on a SNARE complex (Barnard *et al.*, 1997; Clary *et al.*, 1990). Structures of NSF, showing the ATPase in its ATP and ADP bound forms, indicated that there are large conformational changes in the course of ATP hydrolysis (Hanson *et al.*, 1997). Several models for SNARE complex disassembly were brought up and are reviewed in May *et al.* (2001). An initial model for SNARE complex disassembly was based on the idea that SNAREs are fixed in the membrane by their C-terminal membrane anchors. Therefore, providing a rotational force on the membrane-distal end of the complex, should be sufficient to dissolve SNARE complexes. However, SNARE complexes can be dissolved in solution with the same kinetics as on membranes. Thus, Muller *et al.* (2002a) postulated, that NSF needs to be in a direct, fixed contact with the N-terminus of one SNARE. The rotationary force would then be carried out by α-SNAP on the core of the SNARE complex, also leading to helix unwinding. A third model proposed, that the helical bundle collapses as soon as one single SNARE is removed from the coiled-coil. However, there is not enough experimental data to finally prove one of these models.
An indication why specifically *cis*-SNARE complexes are resolved by α-SNAP/NSF is lacking. Partially assembled SNARE complexes, which are intermediates during zippering of the *trans*-SNARE complex, should not serve as bona fide receptors for α-SNAP (Weber *et al.*, 2000). Likely steric hindrance by the vesicle and target membrane, which, at the endpoint of zippering, have approached to a distance of only 2 nm, plays a role in reducing the accessibility for the large NSF hexamer (Hohl *et al.*, 1998). Furthermore, it was also speculated that *trans*-SNARE complexes can oligomerize during membrane fusion, which would also restrict access for the ATPase (Han *et al.*, 2004).

3.3.4 SNARE Master (SM) proteins

The role of the essential SM proteins in vesicle fusion is still under debate. One reason for this is, that in different model systems the binding mode and the effect of a SM protein varies (Dulubova *et al.*, 2003; Kosodo *et al.*, 2002; for review see Jahn, 2000). At the synapse Munc-18 has been shown to negatively regulate subsequent fusion by keeping the syntaxin t-SNARE in a closed conformation. Thus, Munc-18 is able to inhibit *trans*-SNARE complex formation (Dulubova *et al.*, 1999; Yang *et al.*, 2000). The best characterized SM protein in intracellular fusion events is Sly1p. Sly1p has a positive

effect on SNARE complex formation and confers specificity to complex assembly (Kosodo *et al.*, 2002; Peng and Gallwitz, 2002; Peng and Gallwitz, 2004). A dominant active mutation of Sly1p allows fusion to proceed when an essential, compartment-specific RabGTPase was replaced by a different one (Ballew *et al.*, 2005). Thus, one possible role for SM proteins would be signaling correct tethering to downstream components of the fusion machinery.

3.3.5 LMA1

In vitro reactions reconstituting homotypic vacuole fusion or the fusion of COPII vesicles with the *cis*-Golgi (Cao *et al.*, 1998; Xu *et al.*, 1997) highlighted the requirement of another factor for the fusion process in yeast: LMA1 (low molecular weight activity 1). LMA1 stimulates Sec18p activity together with Sec17p (Cao *et al.*, 1998; Xu *et al.*, 1997). It was demonstrated for vacuolar fusion that LMA1 binds to Sec18p in a complex with Sec17p and the SNAREs Vam3p and Nyv1p (Xu *et al.*, 1998). In order to block reformation of *cis*-SNARE complexes, LMA1 is transferred to the t-SNARE Vam3p to stabilize the unpaired SNARE. In fusion events at the mammalian Golgi GATE-16 acts as functional analog of LMA1 (Sagic, 2000).

3.3.6 Membrane fusion

Two models for fusion of biological membranes exist: The proximity model proposes, that it is sufficient to bring membranes in close proximity to drive fusion. In contrast, in an alternative model proteinacious pores are established first, before lipid mixing occurs (reviewed by Jahn and Grubmuller, 2002; Mayer, 2001). Both models differ in the contribution of proteins to achieve bilayer mixing. Although in proximity models proteins like SNAREs are required to bring both membranes in close contact, bilayer mixing is achieved first by a lipid stalk formed by the outer layers of the opposing membranes before fusion of the inner lipid layer and content mixing occurs. Pore models suggest, that an initial contact is established by a fusion pore protein or complex and subsequently, due to radial opening or complex disassembly, lipid mixing occurs. Pore models were brought up as mitochondria, which do not fuse in a SNARE-dependent mechanism, have membrane channels which have the capacity to open laterally. These channels were implicated in fusion events of these organelles (reviewed by Griffin *et al.*, 2006). A candidate for such a fusion pore in SNARE-mediated fusion is the V_0 sector of

the vacuolar H^+-ATPase (Bayer *et al.*, 2003; Muller *et al.*, 2002b; Muller *et al.*, 2003). This channel travels the secretory pathway and is therefore present on all compartments of the secretory and endocytic pathways including the plasma membrane (Harvey and Wieczorek, 1997). Fusion culminates in membrane and content mixing according to both models.

3.3.7 The hemifusion state and the importance of the SNARE transmembrane segment

One of the best studied fusion events is the fusion of membrane-enclosed viruses with their host cells using specialized type I viral fusion proteins. Here, initially a fusion peptide is inserted into the host cell membrane, then both membranes are pulled together by zippering up of a linker. The zippered linker forms a trimeric coiled-coil bundle resembling a SNARE complex (reviewed by Jahn *et al.*, 2003; Skehel and Wiley, 2000; Sollner, 2004). Fusion occurs via a hemifusion state, in which the outer membrane layer has fused. The inner layer initially stays intact so that a stalk is formed. Upon fusion of the inner lipid layer of the membrane, the stalk is resolved and content mixing can occur. Until recently experimental evidence for a hemifusion state during intracellular membrane fusion events was lacking.

Reese *et al.* (2005) demonstrated that lipid flow is possible before content mixing occurrs. Both events are separable by a step sensitive to non-hydrolyzable GTPγS. In addition, truncations of the SNARE transmembrane segments caused an arrest of membrane fusion at a hemifusion state (Xu *et al.*, 2005). Furthermore, Han *et al.* (2004) replaced residues within the transmembrane segment of SNAREs by the bulky hydrophobic residue tryptophane, leading to a reduced conductance of the initial fusion pore. These data highlight an importance of not only the coiled-coil domain, but also the transmembrane segment of SNAREs for the fusion process and might provide evidence for a SNARE-lined fusion pore.

3.3.8 Regulation of membrane fusion: Calcium, Ca^{2+}-binding proteins and protein phosphatase 1

Local calcium fluxes have been implicated in triggering the final fusion event at the neuronal synapse as well as in intracellular fusion (reviewed in Sudhof, 2004). Two proteins have been implicated in transducing the Ca^{2+}-signal: synaptotagmin and

calmodulin (Chen *et al.*, 1999; Chen *et al.*, 2001; Colombo *et al.*, 1997; Davis *et al.*, 1999; Peters and Mayer, 1998; Rizo and Sudhof, 1998). Synaptotagmin functions in fast neuronal exocytosis. Before the Ca^{2+}-stimulus, synaptotagmin is able to prevent membrane fusion by clamping a zippered *trans*-SNARE complex (Chapman *et al.*, 1995; Li *et al.*, 1995). Ca^{2+}-binding to synaptotagmin induces a conformational change that releases synaptotagmin from the SNARE complex and allows fusion to proceed (Fernandez-Chacon *et al.*, 2001; Wang *et al.*, 2002). In yeast, calmodulin binds the V_0 sector of the vacuolar H^+-ATPase dependent on Ca^{2+}-efflux from the vacuolar lumen (Peters *et al.*, 2001; Peters and Mayer, 1998). A complex out of V_0, calmodulin and protein phosphatase 1 is enriched in vertex regions at which fusion is thought to start. Dephosphorylation of a yet unknown factor is thought to mediate the final steps of vacuole fusion leading to lipid and content mixing (Peters *et al.*, 1999; Peters and Mayer, 1998; Xu *et al.*, 1998).

4 Aim of this study

The function of the small GTPase Arf1p is regulated by guanine-nucleotide-exchange factors (GEFs) and GTPase-activating-proteins (GAPs). The latter class of proteins is also involved in cargo recruitment and vesicle formation. Yet, not all of these functions require deactivation of Arf1p. Previous results from our lab showed that ArfGAP induced a conformational change in SNARE proteins, which are essential for the correct targeting and fusion of transport vesicles. This conformational change promoted Arf1p´s recruitment and may represent a mechanism by which SNARE proteins are efficiently recruited into nascent vesicles. The aim to this project was to learn more about the function of this conformation switch by identifying other proteins that would bind to the "activated" conformation and to determine, what influence GAP proteins may have on SNARE traffic in the cell. Furthermore, we characterized novel interactors of Arf1p and examined their role in the late secretory pathway.

5 Results and discussion

5.1 Interaction of SNAREs with ArfGAPs precedes recruitment of Sec18p/NSF

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Transport along the secretory and endocytic pathway is in most part achieved by small vesicles that bud from a donor compartment and fuse with an acceptor organelle. SNARE (soluble NSF attachment protein receptor) proteins are important membrane-anchored fusion factors. Thus, they have to be included in vesicles already at the time-point of budding. Previously, our lab showed that vesicle SNAREs (v-SNAREs) specifically recruit stoichiometric amounts of the small GTPase Arf1p, which is a component of the COPI vesicle coat. This interaction was dependent on a conformational switch, which was catalytically induced by Arf1p GTPase-activating proteins (GAPs), Glo3p and Gcs1p. As no activation of Arf1p was required, SNAREs might serve as membrane receptors for Arf1p early in vesicle budding (Rein *et al.*, 2002). Moreover, SNAREs in the altered conformation would be automatically taken up in newly generated vesicles. In this project, we aimed to gain further insight into the ArfGAP-induced SNARE conformation and how it might affect traveling of SNARE proteins and SNARE-mediated vesicle fusion.

We established first that Gcs1p and Glo3p were also able to induce the conformational switch in target-membrane SNAREs (t-SNAREs). Furthermore, the pre-incubation of SNAREs with ArfGAP enhanced SNARE complex formation *in vitro*. SNARE complex formation is an essential step in vesicle fusion. This finding implies that SNAREs are already fusion-competent when they are incorporated in transport vesicles. We therefore tested whether factors involved in the fusion process preferentially interact with SNAREs in the altered conformation. During the first step in vesicle fusion, Sec17p and the AAA-ATPase Sec18p (the yeast homologs of α-SNAP and NSF, respectively) cooperate to resolve *cis*-SNARE complexes in an ATP-dependent manner. *Cis*-SNARE complexes are remnants of previous fusion events. Sec17p and Sec18p were able to resolve SNARE complexes, which had been formed with the help of Gcs1p before. This dissolution was

dependent on ATP-hydrolysis, because an ATP hydrolysis-deficient mutant of Sec18p (Sec18E350Qp) was not able to resolve these SNARE complexes. Even after the SNARE complex was resolved, we were able to detect Sec17p and Sec18p on the t-SNARE Sed5p. Thus, we tested whether Sec17p and Sec18p are able to bind SNAREs, which are not engaged in SNARE complexes and found this to be the case. We next tested, whether both factors require each other for binding to single SNAREs. Both, Sec17p and Sec18p, bound independently from each other to v- and t-SNAREs that were pre-incubated with ArfGAP. This surprising finding demonstrates that binding of Sec18p to SNAREs is possible in the absence of the Sec18p co-factor Sec17p. The binding sites of Sec17p and Sec18p were mapped and overlapped at least partially with the Arf1p binding site in the SNARE domain. Remnants of the COPI vesicle coat might be required for an initial recognition by the target membrane, but they might hinder efficient vesicle fusion to take place. Therefore, we tested, whether Arf1p is displaced from SNAREs by Sec17p or Sec18p. We found that Sec18p successfully displaced Arf1p from the SNARE Bet1p. This displacement was independent from ATP-hydrolysis as well as from Sec18p´s co-factors Sec17p. This second function of Sec18p, in addition to the well described function in *cis*-SNARE complex disassembly, would allow vesicle fusion to proceed as the single SNAREs would be freed from residual coat and could engage efficiently in new *trans*-SNARE complexes.

Interaction of SNAREs with ArfGAPs precedes recruitment of Sec18p/NSF

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Running title: ArfGAP promotes Sec18p recruitment

Keywords: Sec18p, NSF, SNAREs, Arf1p, membrane fusion, ArfGAP

Abstract

SNARE proteins are key components of the fusion machinery in vesicular transport and in homotypic membrane fusion. We previously found that ArfGAPs promoted a conformational change on SNAREs, which allowed recruitment of the small GTPase Arf1p in stoichiometric amounts. Here we show that the ArfGAP Gcs1p accelerates v-t-SNARE complex formation in vitro, indicating that ArfGAPs may act as folding chaperones. These SNARE complexes were resolved in the presence of ATP by the yeast homologues of α-SNAP and NSF, Sec17p and Sec18p, respectively. In addition, Sec18p and Sec17p also recognized the 'activated' SNAREs even when they were not engaged in v-t-SNARE complexes. Here again, the induction of a conformational change by ArfGAPs was essential. Surprisingly, recruitment of Sec18p to SNAREs did not require Sec17p or ATP hydrolysis. Moreover, Sec18p displaced prebound Arf1p from SNAREs, indicating that Sec18p may have more than one function: first to ensure that all vesicle coat proteins are removed from the SNAREs before the engagement in a trans-SNARE complex and secondly to resolve cis-SNARE complexes after membrane fusion has occurred.

Introduction

Secretion is an essential process for eukaryotic cells. Communication between different organelles in the cell is mostly mediated by vesicles that are formed at a donor compartment and are consumed by a specific target compartment (Kirchhausen, 2000; Bonifacino and Glick, 2004). Factors involved in these processes show a high degree of conservation between such distantly related species as yeast and mammals. To bud a vesicle, small GTPases of the Arf-family are first recruited to the donor membrane. Arf1p is activated through interaction with an ARF guanine nucleotide exchange factor (ArfGEF) at the membrane (Donaldson and Jackson, 2000). Subsequently, coatomer is recruited and forms a complex with Arf1p, cargo or SNARE and an ARF-GTPase activating protein (ArfGAP), a so-called primer (Springer *et al.*, 1999; Spang, 2002). This interaction does not require the deactivation of Arf1p by a GAP. If more such priming complexes are formed, the coat polymerizes laterally and deforms the membrane until the donor membrane is severed from the nascent vesicle. Thus, ArfGAPs are not only important to stimulate GTP hydrolysis on Arf1p, but are also important at a much earlier step in vesicle biogenesis.

Prior to vesicle fusion the coat has to be shed to expose the v-SNAREs on the vesicle. Four SNARE domains residing in both vesicle and target membrane interact in trans and zipper up into a coiled-coil bundle pulling the membranes into close proximity (Nichols *et al.*, 1997; Ungermann *et al.*, 1998; Weber *et al.*, 1998). Then a fusion pore is created that opens up and finally membrane fusion takes place. Through the fusion event, the trans-SNARE complex becomes a cis-SNARE complex, which needs to be resolved to recycle the SNAREs for the next round of fusion. Therefore, α-SNAP and the AAA ATPase NSF

(Sec17p and Sec18p in *S. cerevisiae,* respectively) are recruited to cis-SNARE complexes and catalytically unwind the helix bundle (Sollner *et al.*, 1993; Morgan *et al.*, 1994; Mayer *et al.*, 1996). The driving force to overcome the strong interaction within the cis-SNARE complex is provided through ATP hydrolysis by NSF (Whiteheart *et al.*, 1994). The involvement of α-SNAP and NSF in the priming step of vesicle fusion with the target membrane has been shown to be essential for all vesicle fusion events examined so far, as well as for homotypic vacuole fusion in yeast (Wickner, 2002; Morgan and Burgoyne, 2004; Sollner, 2004). However, it remains unclear whether unwinding the remains of the previous fusion event satisfies the Sec18p/NSF requirement before fusion.

Other factors that intimately interact with SNAREs are the ArfGAPs Gcs1p and Glo3p, which have been shown to catalytically induce a conformational change in SNAREs involved in ER-Golgi trafficking (Rein *et al.*, 2002). SNAREs in this altered conformation were able to recruit Arf1p. This recruitment step required neither other factors nor the activation of the GTPase by a GEF. The interaction between Arfp and SNAREs might provide a mechanism by which each vesicle carries at least one SNARE protein and would therefore be capable of fusing with the target membrane (Rein *et al.*, 2002). However, membrane-anchored cargo proteins may also provide a platform for Arf1p binding.

Previously, we reported that the ArfGAPs Glo3p and Gcs1p induce a conformational change in v-SNAREs involved in ER-Golgi and post-Golgi trafficking (Rein *et al.*, 2002; Robinson *et al.*, 2006). In this paper we extended these results to t-SNAREs (target-SNAREs on the target membrane) and show that Gcs1p accelerates v-t-SNARE complex formation in vitro. The ArfGAPs may promote the conversion of a high energy to a low energy state in SNAREs and thereby help to form SNARE complexes. These v-t-SNARE complexes were

not dead-end complexes because they could be resolved by the action of Sec17p and Sec18p in the presence of ATP. Furthermore, we revealed an additional function of Sec18p as it could displace Arf1p from single SNAREs in vitro. Surprisingly, this binding to single SNAREs was independent from ATP hydrolysis and Sec17p. Thus, Sec18p may have an additional role during the priming step in vesicle fusion: to ensure that the SNAREs are free to engage in v-t- SNARE complexes, and that they are not masked by coat proteins bound to the SNAREs.

Results

The ArfGAPs Glo3p and Gcs1p recruit Arf1ΔN17p to v- and t-SNAREs

We reported previously that the ARFGAPs Gcs1p and Glo3p catalytically induce a conformational change in v-SNAREs involved in fusion events in the early secretory pathway and in exo- and endocytosis (Rein *et al.*, 2002; Robinson *et al.*, 2006). SNAREs in this primed conformation are able to recruit Arf1p. We aimed to test whether this ArfGAP activity is limited to v-SNAREs or could be extended to t-SNAREs as well. To test this, we used recombinantly expressed ArfGAP and Arf1ΔN17p-Q71L. Arf1ΔN17p lacks the N-terminal 17 amino acids that are strongly hydrophobic and carry the myristoylation site and thus would interfere with purification and in vitro assays. The Q71L point mutant reflects the predominantly activated form of Arf1p. We have shown previously that Arf1p does not have to be activated to bind to v-SNAREs (Rein *et al.*, 2002). Despite this finding, we used in all experiments Arf1ΔN17p-Q71L and we refer to it as Arf1ΔN17p.

We performed GST pull-down assays with SNARE proteins (Table 1) that carried a GST fusion either at the N-terminus or which replaced the C-terminal transmembrane domain. Similarly to what we reported previously (Rein *et al.*, 2002), the ArfGAPs Gcs1p and Glo3p were able to induce a conformational change in all SNARE proteins tested, albeit with varying efficiencies (Fig. 1). ArfGAPs promoted Arf1ΔN17p binding to the v-SNAREs Bet1p, Bos1p, Sec22p and Snc1p (Rein *et al.*, 2002; Robinson *et al.*, 2006) without themselves binding in stoichiometric amounts to the SNAREs. Furthermore, ArfGAPs allowed recruitment of the small GTPase on the prenylated v-SNARE Ykt6p and on the t-SNARE Sed5p. Arf1ΔN17p was also recruited in significant amounts to the GST fusions of

the syntaxin t-SNAREs Vam3p, Tlg2p, Sso1p and Sso2p (Fig. 1C and data not shown). Despite the difference in the level of Arf1pΔN17p recruitment by the two ArfGAPs, both GAPs were able to induce a conformational change on the SNAREs tested. In contrast, a third ArfGAP, Age2p, did not lead to the recruitment of stoichiometric amounts of Arf1ΔN17p to SNAREs (data not shown). The GTPase Arf1p is involved not only in the generation of COPI coated vesicles at the ER-Golgi interface, but also in the formation of several clathrin and non-clathrin coated vesicles. Thus, Arf1p recruitment to SNARE proteins in the 'primed' conformation occurs throughout the secretory and endocytic pathway. These interactions seem to provide a mechanism to include SNAREs into nascent vesicles and could provide the means by which not only v-SNAREs but also t-SNAREs reach their final compartment. Furthermore, the conformational change induced by ArfGAPs could be important for vesicle fusion at the target membrane.

ArfGAPs promote SNARE complex formation

Since ArfGAPs induced a conformational change on both v- and t-SNAREs, we wondered whether ArfGAPs could promote v/t-SNARE complex formation in vitro. GST-Sed5p was incubated with His-tagged versions of Bet1p, Bos1p and Sec22p in the presence or absence of Gcs1p (Fig. 2 A-C). The reaction was stopped at the indicated time points and followed immediately by extensive washing. Upon addition of Gcs1p, the formation of SNARE complexes was strongly accelerated and more complexes were formed compared to the experiment in which Gcs1p was omitted. A similar result was observed using a C-terminal fusion of GST to Sed5p (Fig. 2D-F). Thus, the conformational change induced by ArfGAPs catalyzes the zippering process of SNAREs. Since this reaction did not require

energy, these results also indicate that ArfGAPs may change the conformation of the SNAREs from a high energy state to a low energy state, which would enable an efficacious engagement into SNARE complexes in vitro that may reflect the fusion competent SNARE complexes in vivo.

If the SNARE complexes formed in the presence of Gcs1 were indeed functional SNARE complexes, one would expect that addition of Sec17p (the yeast homologue of α-SNAP) and Sec18p (the yeast homologue of NSF) but not of an ATPase-deficient Sec18p (Sec18(E350Q)p (Steel *et al.*, 1999) should dissolve these complexes again, and that this process should require energy. This mutant can still bind ATP and thus forms the characteristic hexameric complex, yet it is deficient in ATP hydrolysis. Indeed, Sec17p, together with Sec18p but not with mutant Sec18(E350Q)p, disassembled SNARE complexes (Fig. 2G, compare lines 2 and 3 and lines 7 and 8), implying that ArfGAPs may render SNAREs fusion competent already upon inclusion into transport vesicles. Furthermore, these results strongly suggest that ArfGAPs can indeed promote functional SNARE complex formation, perhaps by acting like a chaperone.

ArfGAPs Glo3p and Gcs1p recruit Sec17p and Sec18p to SNAREs involved in ER-Golgi and post-Golgi trafficking

If our hypothesis that ArfGAPs could render v-SNAREs fusion competent upon inclusion into transport vesicles was correct, then Sec17p and Sec18p may already bind to the single SNARE proteins, which are not engaged in SNARE complexes. Therefore, SNARE-GST fusions were pre-treated with the ArfGAP Gcs1p as before and after extensive washing equimolar amounts of Sec17p and Sec18p, in the presence or absence of ATP and

GTP, were added to the reaction. We found that both Sec17p and Sec18p bound independently of the presence of nucleotides in the reaction mix but rather required the pre-incubation of SNAREs with ArfGAP (Fig. 3A). Again, no binding of Gcs1p was detected on the gel. More Sec17p (Fig. 3B) and Sec18p (Fig. 3C) bound to the v-SNARE Bet1p compared to the t-SNARE Sed5p. Thus the Sec17p/Sec18p binding mode to SNARE tails might be similar to that to SNARE complexes. Therefore, we could use this much simpler model to investigate the binding of Sec17p/Sec18p to SNAREs.

Next, we explored whether Sec17p and Sec18p require each other for binding to SNAREs. Sec17p bound SNAREs involved in ER-Golgi and post-Golgi trafficking almost exclusively in the 'primed' conformation that is induced by the ArfGAPs Gcs1p or Glo3p (Fig. 3D and data not shown). Strong Sec17p binding occurred on Bet1p, Bos1p and Sed5p, whereas binding to Sec22p, Snc1p and Ykt6p was much weaker. Surprisingly, even in the absence of Sec17p, Sec18p was recruited to SNAREs in the primed SNARE conformation (Fig. 3E). However, in contrast to Sec17p binding to SNAREs, Sed5p recruited much less Sec18p compared to Bet1p or Bos1p. Similarly to the binding to the entire SNARE complex, Sec18p binding occurred in absence of additional ATP in the reaction. Thus, Sec18p and Sec17p can bind to single SNAREs. Furthermore, our data demonstrate that Sec18p does not require Sec17p for binding individual SNAREs, which may point to a function of Sec18p unrelated to unwinding of SNARE complexes.

Gcs1p enhances Sec18p binding to microsomal membranes

We wanted to extend our in vitro findings that Sec18p bound to SNAREs in an ArfGAP-dependent manner, to conditions that would better reflect the in vivo situation.

Therefore, we tested whether Sec18p would be recruited to microsomal membranes in an ArfGAP-dependent fashion. Microsomal membranes were incubated with Sec18p and ATP in the presence or absence of Gcs1p. After incubation at 30°C, the membrane fraction was isolated and analyzed by immunoblot (Fig. 4). Although Sec18p binding to membranes did not necessitate the presence of ArfGAP, Gcs1p strongly enhanced the interaction of Sec18p with SNAREs on microsomal membranes. Sec17p is still present on the microsomal membranes and may recruit Sec18p in the absence of ArfGAP. It is very unlikely that SNARE complex formation was induced by Gcs1p, especially since there are no transport vesicles formed under these conditions. Therefore, it seems more likely that Gcs1p permits binding of Sec18p to single SNAREs. Taken together, our data demonstrate an ArfGAP-dependent Sec18p recruitment to SNAREs and that this recruitment might not correlate to the Sec18p function to resolve SNARE complexes.

Sec17p and Sec18p bind to the SNARE domain

We have shown previously that Arf1p is recruited to the membrane-proximal region of the SNARE domain of Bet1p (Rein *et al.*, 2002). We next asked where Sec17p and Sec18p might bind to Bet1p. Fragments of the cytoplasmic domain of Bet1p (Fig. 5A) were used to determine the binding sites of Sec17p and Sec18p. Sec17p bound most efficiently to the coiled-coil SNARE domain comprised in Bet1p(residues 41-119) and to a smaller fragment of the membrane-proximal region, Bet1p(79-119) (Fig 5B). However strong binding also occurred to the membrane-distal region of the SNARE domain (Bet1p(41-79)). Because Sec17p migrates at the same position on the gel as the N-terminal fragment Bet1p(1-65), Sec17p binding to Bet1p(1-65), was determined instead by semi-quantitative

immunoblot and was found to be at background levels (data not shown). Thus, Sec17p binds to the SNARE domain of Bet1p. Sec18p also bound to the SNARE domain; although a slight preference for the membrane-proximal part was detectable (Fig 5C and D).

We also confirmed these results by determining the binding of Sec17p and Sec18p to truncations of the vacuolar SNAREs Vam3p and Nyv1p. The Vam3p(Δ1-144)-GST construct contained the SNARE domain, which was deleted in the Nyv1p(1-136)-GST construct. While Sec18p and Sec17 bound the full-length Vam3p-GST and Nvy1p-GST as well as to the SNARE domain of Vam3p(Δ1-144)-GST alone, no binding was observed to Nyv1p(1-136)-GST construct lacking the SNARE domain (data not shown). Thus, both Sec17p and Sec18p bind to the SNARE domains of different SNAREs.

Sec18p competes with Arf1ΔN17p for binding sites on SNARE domains

We have shown previously that Arf1p also binds to the SNARE domain, preferentially to the membrane-proximal region. Because of the overlap of the binding sites of Sec18p, Sec17p and Arf1p, we wanted to test whether these proteins would compete with each other for binding sites on SNAREs. Therefore, we first formed a complex of Arf1ΔN17p and Bet1p-GST with the help of Glo3p. After extensive washing this complex was then incubated with increasing concentrations of Sec18p. Arf1ΔN17p was partially displaced from the SNARE at high Sec18p concentrations (Fig 6A). However, the amount of Sec18p recruited to the Arf1ΔN17p-SNARE complex was reduced compared to ´primed´ SNAREs alone. When we reversed the order of the addition of Arf1ΔN17p and Sec18p, binding of Arf1ΔN17p was strongly reduced in the presence of stoichiometric amounts of Sec18p on the SNAREs (Fig. 6B). We repeated the assays with an intermediate Sec18p

concentration (Fig. 6C-F). Under these conditions, Sec18p displaced about 50% of Arf1ΔN17p from Bet1p-GST, while in the converse experiment Arf1ΔN17p could not compete with Sec18p for binding sites on Bet1p (Fig.6, compare C with F). Yet, not all binding sites could be exchanged; pre-incubation of Bet1p with Arf1ΔN17p reduced Sec18p binding by about 50%, and a similar effect was observed when the order of addition was inverted (Fig. 6D and E). Furthermore, Arf1ΔN17p was unable to chase Sec18p off Bet1p-GST (Fig. 6F). The Sec18p-mediated loss of Arf1ΔN17p from the SNARE was independent of nucleotide in the reaction mix for all conditions tested. Taken together, Sec18p could displace prebound Arf1ΔN17p from Bet1p-GST, and this displacement did not require energy.

Displacement of Arf1p by Sec18p is independent of ATP hydrolysis

Since recombinant Sec18p is in the ATP-bound state on both the catalytic and the structural ATP binding sites, Sec18p could potentially undergo at least one round of ATP hydrolysis even in the absence of ATP from the reaction mixture. To exclude effects of ATP hydrolysis in the competition experiments we used the ATPase deficient mutant Sec18(E350Q)p (Steel *et al.*, 1999). Sec18(E350Q)p bound the SNARE Bet1p even stronger than wild-type (Fig. 7A). Addition of equal amounts of Sec18p or Sec18(E350Q)p led in both cases to a loss of 50% of the Arf1ΔN17p bound to Bet1p-GST (Fig. 7B). Furthermore, the ability of Sec18(350Q)p to bind to microsomes was similar to that of Sec18p (Fig. 7C). Therefore, the competition of Arf1ΔN17p and Sec18p for binding sites on the SNARE protein is most likely not due to unwinding of SNARE complexes, but rather involves steric hindrance by Sec18p.

Discussion

In this manuscript we show that the transient interaction of ArfGAPs with v- and t-SNAREs does not only allow the recruitment of Arf1p and therefore promote the inclusion of SNAREs into vesicles, but also strongly accelerate the formation of functional SNARE complexes. This led us to propose that ArfGAP might have a chaperone-like function to help the SNAREs to assume a low energy conformation. Furthermore, these findings indicate that the v-SNAREs might adopt a conformation that will allow rapid engagement in trans-SNARE complexes, which might play a role in determining the velocity of vesicle fusion.

We tested three different ArfGAPs for their ability to recruit Arf1ΔN17p to SNARE proteins as indication for the conformational change. Two of them, Glo3p and Gcs1p, but not Age2p promoted Arf1ΔN17p binding. We have previously demonstrated that the transient interaction Glo3p with the v-SNAREs Sec22p and Bet1p renders the SNAREs more protease resistant (Rein *et al.*, 2002). This conformational change may promote the inclusion of SNAREs into vesicles, and they might even serve as primers for vesicle formation (Springer *et al.*, 1999; Spang, 2002).

Gcs1p and Glo3p are the orthologues of mammalian ARFGAP1 and ARFGAP3, respectively. At least for ARFGAP1 a similar role in the inclusion of the v-SNARE membrin into vesicles has been reported (Honda *et al.*, 2005). ARFGAP1 contains a domain that senses membrane curvature via an amphiphatic helix (Bigay *et al.*, 2003). This domain could be as well responsible for the catalytic change on SNAREs. This exact domain is not conserved in Glo3p. However, Glo3p contains a coiled-coil region, which could fulfill a similar function. Age2p contains neither an amphiphatic helix nor a coiled-coil domain. The

difference in the structural organization of the ArfGAPs may explain the variation in the ability to act as chaperone on SNAREs. Whether this chaperone-like function is limited to SNAREs or if ArfGAPs are generally involved in cargo recruitment remains to be established. The KDEL receptor Erd2 and members of the p24 family of proteins, however, require ARFGAP1 for uptake into COPI coated vesicles (Aoe *et al.*, 1999; Lanoix *et al.*, 2001).

Both v- and t-SNAREs were positively affected by the interaction with ArfGAPs. One explanation for this observation is, that this interaction provides the mechanism by which t-SNAREs reach their final compartment and that the conformational change serves as switch form a high to a low energy state, which promotes efficient inclusion of also t-SNAREs into transport vesicles. v-SNAREs seem to use this pathway for efficient inclusion into at least COPI-coated vesicles (Rein *et al.*, 2002; Robinson *et al.*, 2006). Alternatively, the conformational change on t-SNAREs may only take place on the target membrane and may accelerate trans-SNARE complex formation.

We found that ArfGAP accelerated the formation of functional SNARE complexes, and that these complexes were resolved by the yeast homologues of NSF and α-SNAP, Sec18p and Sec17p, respectively. Surprisingly, Sec18p and Sec17p also bound independently of each other to SNAREs. This interaction was strictly dependent on the induced conformational switch on SNAREs by ArfGAPs. Moreover, Sec18p competed with Arf1ΔN17p for binding sites on SNAREs. Since this interaction did not require nucleotide hydrolysis, this function of Sec18p is most likely unrelated to the well-established unwinding activity of the AAA-ATPase required to dissolve the four-helix bundle of cis-SNARE complexes. Why would Sec18p bind to SNAREs and displace Arf1p? A transport

vesicle arrives at the target membrane and although it is generally thought that all coat has been shed at this point, experimental proof is lacking. The SNAREs on the vesicles must be exposed to engage in trans-SNARE complexes with the SNAREs on the target membrane. Sec18p could bind to SNAREs and displace coat proteins, especially Arf1p (Fig. 8). We have shown previously that binding of Arf1ΔN17p binds to SNAREs in a nucleotide-independent manner. Therefore, it is conceivable that Arf1p-GDP might still stick to SNARE proteins after GTP hydrolysis has occurred and most of the coat proteins have left the transport vesicle. Displacement of Arf1p by Sec18p would provide a mechanism, which would ensure the availability of the SNAREs for complex formation. Since Sec17p is not required at this step, Sec18p is unable to unwind the four-helix bundle and efficient membrane fusion could proceed. This function of Sec18p would also be independent of the action of tethering complexes. Tethering complexes may actually require the presence of residual coat components as determinants for the tethering activity (Malsam *et al.*, 2005).

This is the first report demonstrating binding of Sec18p/NSF in stoichiometric amounts to SNAREs in the absence of Sec17p/α-SNAP. Supposedly, Sec18p/NSF only binds to SNAREs after Sec17p/α-SNAP recruitment to cis-SNARE complexes (Sollner *et al.*, 1993; Morgan *et al.*, 1994). We used, however, single SNARE molecules and therefore we likely provide a different molecular environment compared to the four-helix bundle of the cis-SNARE complex. The binding of Sec17p to single SNARE molecules has been shown previously, and this binding was independent of ArfGAP (Rossi *et al.*, 1997). However, in the experimental setup of Rossi *et al.* (1997) only sub-stiochiometric amounts of Sec17p were recruited, which were detected by immunoblot. ArfGAP-mediated binding of Sec17p to SNAREs was stiochiometric and detectable by Coomassie blue staining,

despite a 5 fold lower concentration of Sec17p in the assay. Therefore, the ArfGAP induced conformational change greatly enhances the binding of Sec17p to SNAREs. Furthermore, Sec18p binding to single SNAREs was probably undetected because of the low affinity of Sec18p to the single SNARE in the high-energy state.

We mapped the binding sites of Sec18p and Sec17p on the SNAREs. Sec17p and Sec18p bound to the SNARE domain and Sec18p had within this domain a slight preference for the membrane proximal part. These data seem to somewhat disagree with previously published reports, where Sec18p/NSF and Sec17p/α-SNAP bind to the N-terminal (membrane distal) part of cis-SNARE complexes (Hanson *et al.*, 1997; Hohl *et al.*, 1998). However, our mapping data were performed on single SNAREs and the binding of Sec18p was determined in the absence of Sec17p. Sec18p binding to cis-SNARE complexes is strictly dependent on Sec17p. Therefore, the mode of interaction of Sec18p with single SNAREs and with SNAREs complexes could be different. However, one could probably expect a high affinity of Sec18p to SNARE domains, and that is exactly what we observe. These data also support our model of dual function of Sec18p in membrane traffic (Fig. 8): first Sec18p ensures that the single SNAREs can engage in trans-SNARE complexes by displacing residual coat components, and secondly Sec18p, together with Sec17p, unwinds cis-SNARE complexes to free the SNAREs for another round of transport.

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Material &Methods

Plasmids

C-terminal GST-tagged SNAREs were expressed using pETGEXCT (Sharrocks, 1994). GST-Sed5p was expressed from pGEX2T, GST-Snc1p from pGEX4T1, GST-Vam3p and GST-Nyv1p from pGEX4T3 and GST-Tlg2p from pGEX6p-1. His-tagged SNAREs were obtained by expression using vector pET24b (Novagen). Sec17p and Sec18p were expressed from pQE9 (Qiagen), Sec18E350Qp was expressed from pQE30 (Qiagen). The vectors for expression of Gcs1p, Glo3p and Age2p and Arf1ΔN17p have been described elsewhere (Poon *et al.*, 1996; Poon *et al.*, 1999; Poon *et al.*, 2001; Rein *et al.*, 2002).

Protein purification

The different SNARE-GST fusion proteins were expressed in *E.coli* BL21* and purified over glutathione-agarose. Cells from 1 L culture were resuspended in 20 ml STE (25% (w/v) sucrose, 50 mM Tris-Cl, pH 8.0, 40 mM EDTA). After lysozyme treatment (1 mg/ml), 8 ml 50 mM Tris-Cl pH 8.0, 0.2% TritonX-100, 100 mM $MgCl_2$ were added and the suspension was subjected to sonication. The cell lysate was cleared by centrifugation (10.000 x g, 20 min). The supernatant was bound to 1 ml glutathione agarose beads (Sigma) for 2 h at 4°C. Beads were washed several times in PBS and transferred into a polyprep column (Biorad). Protein was eluted with 50 mM glutathione, 150 mM Tris pH 8.0, 120 mM NaCl, 5 mM DTT, 1 mM EDTA, 1 mM PMSF. Protein-containing fractions were pooled and dialyzed against PBS, 5% glycerol. His_6-tagged SNARE proteins and His_6-Sec17p were expressed in *E. coli* BL21* and purified using Ni^{2+}-NTA-agarose (Qiagen) according to the

manufacturer's instructions. Arf1ΔN17p was purified as described (Rein *et al.*, 2002). His_6-Sec18p and His_6-Sec18E350Qp were purified according to Whiteheart *et al.* (1994) with the following modifications: Ni^{2+}-NTA-agarose with bound proteins were washed in batch twice with 10 column volumes of wash buffer 20 mM Hepes-KOH, pH 6.8, 400 mM KOAc, 0.5 mM ATP, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10% glycerol, 2x 10 column volumes of wash buffer20, 2x 10 column volumes of wash buffer50 (wash buffer containing 20 and 50 mM imidazole, respectively). After the last wash, beads were resuspended in wash buffer100 (wash buffer containing 100 mM imidazole), transferred into a polyprep column and washed with 5 column volumes of wash buffer100. Protein was eluted with elution buffer (wash buffer containing 250 mM imidazole). Eluted protein was dialyzed against 20 mM Hepes pH 6.8, 150 mM KOAc, 5 mM $Mg(OAc)_2$, 15% glycerol, 1 mM ATP, 1 mM DTT, 1 mM PMSF. ArfGAPs were purified and the GAP activity was determined as described (Huber *et al.*, 2001; Rein *et al.*, 2002).

SNARE binding assay

SNARE binding assays were performed as described previously (Rein *et al.*, 2002). 5 µM Arf1ΔN17p was used in the reaction. When indicated, the reaction contained 20 nM Gcs1p, Glo3p or Age2p. Sec17p and Sec18p were used at concentrations of 0.6 µM His_6-Sec17p, 0.6 µM His_6-Sec18p or 0.6 µM His_6-Sec18(E350Q)p. For the competition assays with Arf1ΔN17p, 50 nM to 3.5 µM His_6-Sec18p was used. 1 mM ATP and 0.5 mM GTP was added were indicated. Incubation steps with Arf1ΔN17p or ArfGAP were performed for 1h at 4°C, while His_6-Sec17p, His_6-Sec18p or His_6-Sec18(E350Q)p were incubated for 1h at RT. The recruitment of Arf1ΔN17p to SNAREs was visualized by Coomassie blue staining.

SDS-PAGE containing assays performed with His_6-Sec17p or His_6-Sec18p were stained with SyproRed (Molecular Probes). Images were acquired with a Storm PhosphorImager (GE healthcare). Bands were quantified using the ImageQuant software.

Assembly and disassembly of SNARE complexes

SNARE complex assembly was performed for indicated times at 4°C as described (Peng and Gallwitz, 2002) with minor modifications: 5 µg GST-Sed5p immobilized on glutathione-agarose beads was incubated with 10 µg of Bet1p-His_6, 20 µg Bos1p-His_6 and 20 µg Sec22-His_6 in a total assay volume of 200 µl at 4°C, in the presence or absence of 20 nM Gcs1p. The reaction was stopped at indicated time points by centrifugation, followed by 3 washes in buffer C (25 mM Tris-Cl pH 7.5, 150 mM KCl, 10% glycerol, 1% TX-100, 2 mM ß-mercaptoethanol) and a final wash in 20 mM Tris-Cl, pH 7.5. Bound proteins were dissolved in sample buffer by heating the beads 10 min at 65°C. The proteins were separated by SDS-PAGE and visualized by Fairbanks staining. Band intensities were determined using an Odyssey imaging system (LICOR). To dissolve SNARE complexes, they were formed as described above for 18-22 h, washed twice in buffer C and once in disassembly buffer containing 30 mM Hepes-KOH pH 7.4, 70 mM KCl, 5 mM $MgCl_2$, 2.5 mM EGTA, 0.5 mM DTT, 2 mM ATP. SNARE complex disassembly was performed by addition of 1.35 µM His_6-Sec17p and 1.5 µM His_6-Sec18p in an assay volume of 100 µl. The assay was incubated for 1 h at RT and then stopped immediately by the addition of 1 ml ice-cold disassembly buffer. The beads were settled 30 min on ice. Unbound protein was removed by 2 washes in disassembly buffer and a final wash in 20 mM Hepes, pH 7.4. Bound protein was analyzed as described above.

Microsome binding assay

Preparation of microsomal membranes and the assay for binding to microsomal membranes was carried out as described previously (Rein *et al.*, 2002), with the following modifications: The membranes containing approx. 8 µg protein were incubated with 8 nM Gcs1p, 12 nM His_6-Sec18p in the presence of 0.5 mM ATP in a total volume of 50 µl with gentle agitation for 30 min at 30°C. Unbound protein was removed by centrifugation. After washing, microsomal membranes were dissolved in sample buffer containing 8 M urea, resolved by SDS-PAGE and analyzed by immunoblot.

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Figure legends

Figure 1: The ArfGAPs Glo3p and Gcs1p mediate binding of Arf1ΔN17p to the cytoplasmic domains of SNAREs. SNARE-GST fusion proteins were immobilized onto glutathione-agarose beads and incubated with Arf1ΔN17p. Where indicated Glo3p **(A)** or Gcs1p **(B**, **C)** was added to the reaction. Bound proteins were separated by SDS-PAGE and bands were visualized by Coomassie blue staining. **(A)** and **(B)** Analysis of *in vitro* binding of Arf1ΔN17p to Bet1p-GST (lanes 1 and 2), Bos1p-GST (lanes 3 and 4), Ykt6p-GST (lanes 5 and 6), GST-Snc1p (lanes 7 and 8), Sec22p-GST (lanes 9 and 10) and GST-Sed5p (lanes 11 and 12). **(C)** In vitro binding of Arf1ΔN17p to Sso1p-GST (lanes 1 and 2), GST-Tlg2p (lanes 3 and 4), GST-Vam3p (lanes 5 and 6) GST-Sed5p (lanes 7 and 8) and GST. 10% of the Arf1ΔN17p present in the incubation is shown in lane 11.

Figure 2: ArfGAP accelerates functional SNARE complex formation in vitro. **(A)** N-terminally tagged GST-Sed5p was immobilized onto glutathione-agarose beads and incubated with Bet1p-His_6, Bos1p-His_6 and Sec22-His_6. 10% of the input of the different proteins is shown in lanes 11-13. Gcs1p was added to the incubation in lanes 6-10. The reaction was stopped at indicated time-points by extensive washing. Bound proteins were eluted in sample buffer, separated by SDS-PAGE and visualized by Fairbanks staining. **(B)** and **(C)** Band intensities from **(A)** were determined using an Odyssey imaging system. Data for Bos1p-His_6 and Sec22-His_6 are shown in **(B)** and for Bet1p-His_6 in **(C)**. **(D)** C-terminally tagged Sed5p-GST was immobilized and the SNARE complex formation was performed as in **(A)**. **(E)** and **(F)** contain the quantification of the experiment shown in **(D)**. **(G)** SNARE complexes were formed over night in the absence (lanes 1-5) or presence (lanes 6-10) of

Gcs1p were incubated with His_6-Sec17p (lanes 2, 3, 7 and 8), His_6-Sec18p (lanes 3, 5, 8 and 10) or His_6-Sec18E350Qp (lanes 2, 4, 7 and 9). The assay was stopped on ice and samples were processed as described in **(A)**.

Figure 3: Sec17p and Sec18p binding to SNARE proteins depend on ArfGAP. **(A)** Bet1p-GST (lanes 1-4), GST-Sed5p (lanes 5-8) and GST (lanes 9-12) were immobilized onto glutathione-agarose beads and were incubated with Gcs1p. After washing, 0.6 μM His_6-Sec17p and 0.6 μM His_6-Sec18p were added to each sample and nucleotides were added as indicated. Lane 13 and 14 show 25% and 10% of the input of His_6-Sec17p and His_6-Sec18p, respectively. The reactions were stopped by extensive washing. Bound proteins were eluted in sample buffer, resolved by SDS-PAGE followed by SyproRed staining. **(B)** and **(C)** Quantification of **(A)** for His_6-Sec17p and His_6-Sec18p, respectively. Binding of 0.6 μM His_6-Sec17p **(D)** or 0.6 μM His_6-Sec18p **(E)** to a variety of single SNAREs is dependent on pretreatment with ArfGAP Glo3p. The experiment was performed as in **(A)** except that Glo3p instead of Gcs1p was used to induce the conformational change.

Figure 4: Gcs1p mediates Sec18p recruitment to microsomal membranes. Buffer-washed microsomal membranes were incubated with His_6-Sec18p and ATP in the absence (lane 2) or presence (lane 3) of Gcs1p. After extensive washing the membrane fraction was solubilized and analyzed by immunoblot. Lane 1 contains the microsomal membranes incubated with buffer. 10% of the Gcs1p and 2% of Sec18p present in the incubation shown in lanes 4 and 5, respectively.

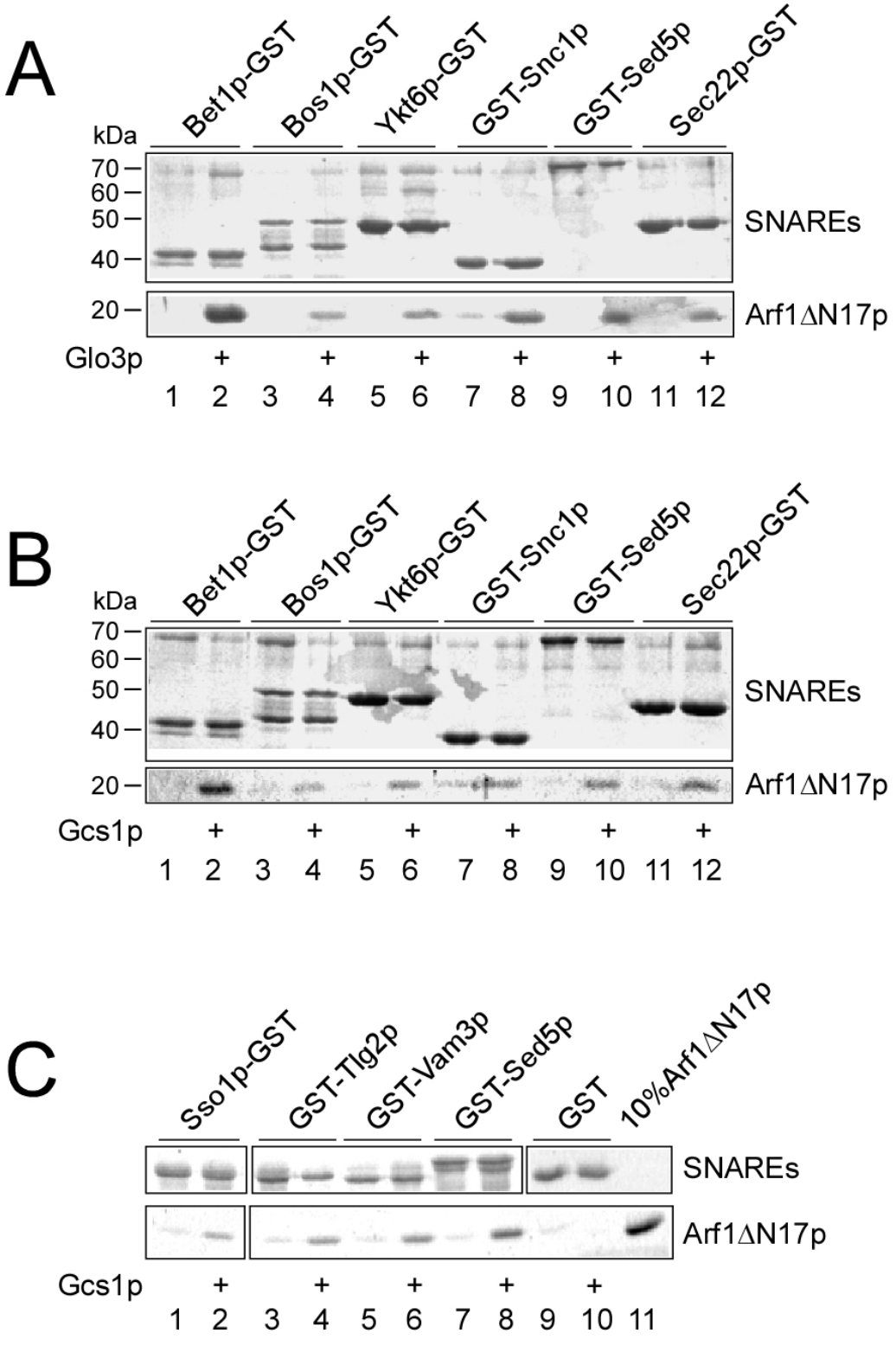
Figure 5: Binding sites of Sec17p and Sec18p on Bet1p-GST. **(A)** Schematic depiction of Bet1p and GST fusions of Bet1p. **(B)** and **(C)** GST fusions were immobilized onto glutathione-agarose beads and incubated with Gcs1p. After washing **(B)** His_6-Sec17p or **(C)** His_6-Sec18p and 1 mM ATP were added. Unbound proteins were removed and bound proteins were analyzed as described in Fig. 3. **(D)** represents the quantification of the experiment in **(C)**.

Figure 6: Sec18p and Arf1ΔN17p compete for binding sites on Bet1p-GST. (A) Bet1p-GST was immobilized on glutathione agarose beads. The immobilized SNARE was incubated with Glo3p and Arf1ΔN17p (lanes 8-15) After removal of unbound proteins, increasing amounts of His_6-Sec18p, 1 mM ATP and 0.5 mM GTP were added in lanes 1-7 and 9-15. After the incubation, unbound proteins were removed and bound proteins were analyzed as described above. Binding of Arf1ΔN17p and His_6-Sec18p to Bet1p-GST was quantified as before and is shown in **(C)** and **(E). (B)** Immobilization and induction of the conformational change were performed as in **(A)**. In the second incubation step, increasing amounts of His_6-Sec18p were added. After incubation, the unbound Sec18p was removed and Arf1ΔN17p was added. Binding of Arf1ΔN17p and His_6-Sec18p to Bet1p-GST was quantified as before and is shown in **(D)** and **(F)**.

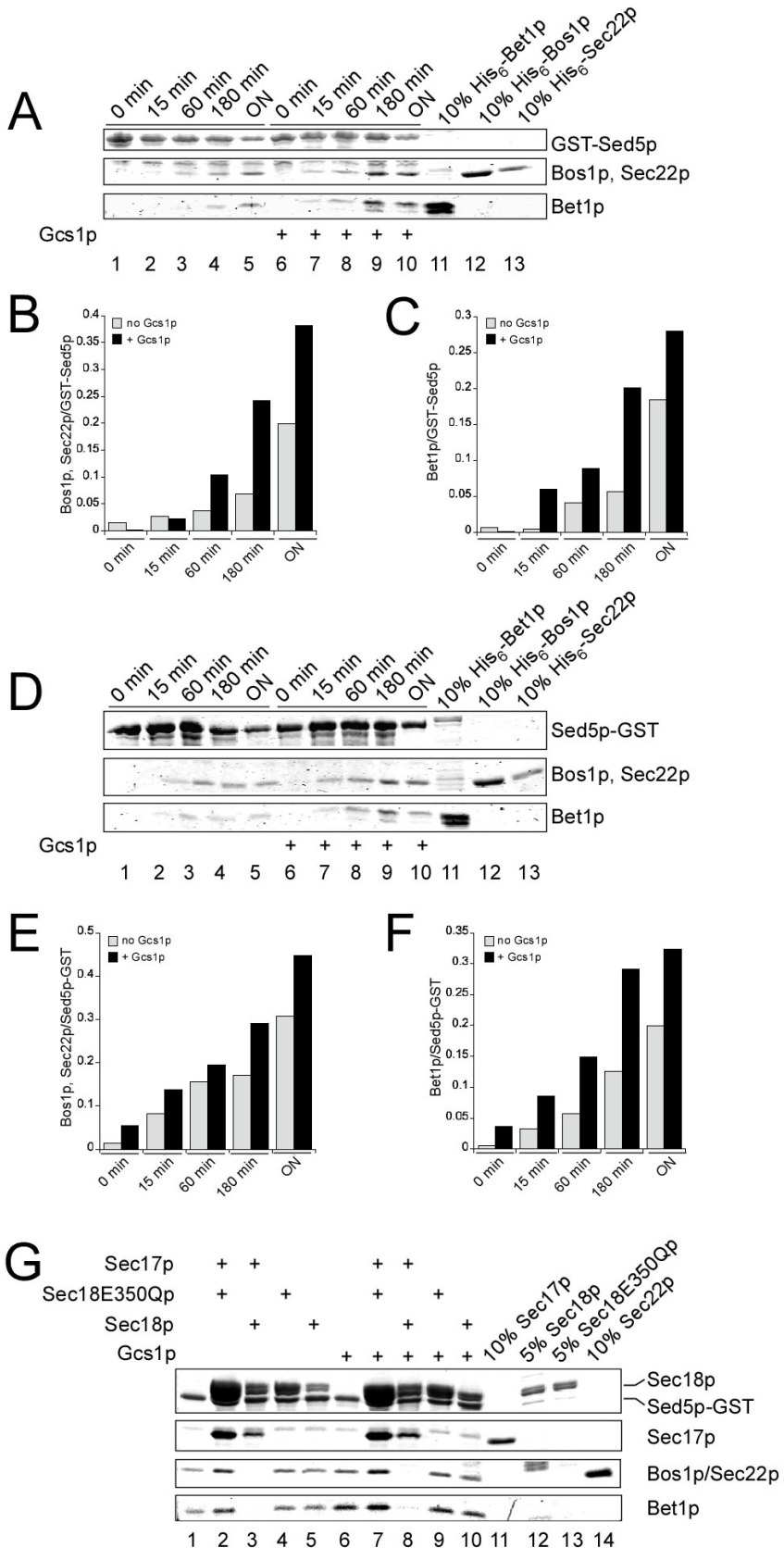
Figure 7: Displacement of Arf1ΔN17p by Sec18p does not require ATP hydrolysis by Sec18p. **(A)** Bet1p-GST was immobilized on glutathione agarose beads. The beads were incubated with Gcs1p in the presence of 1 mM ATP and 0.5 mM GTP. Arf1ΔN17p was present in lanes 1, 4 and 5. After removal of unbound proteins, His_6-Sec18p (lanes 2 and 4)

or His_6-Sec18E350Qp (lanes 3 and 5) were added in the presence of 1 mM ATP and 0.5 mM GTP. After the incubation, unbound proteins were removed and the bound proteins were analyzed as described above. 10% of the input of Arf1ΔN17p (lane 6), His_6-Sec18p (lane 7) and His_6-Sec18E350Qp (lane 8) are shown. **(B)** Quantification of loss of Arf1ΔN17p from Bet1p-GST in the presence of His_6-Sec18p and His_6-Sec18E350Qp. **(C)** Quantification of Arf1ΔN17p effect on subsequent His_6-Sec18p and His_6-Sec18E350Qp binding.

Figure 8: Schematic drawing on the action of ArfGAP on SNAREs and the displacement of Arf1p by Sec18p. For details see text.



Schindler and Spang, Figure 1



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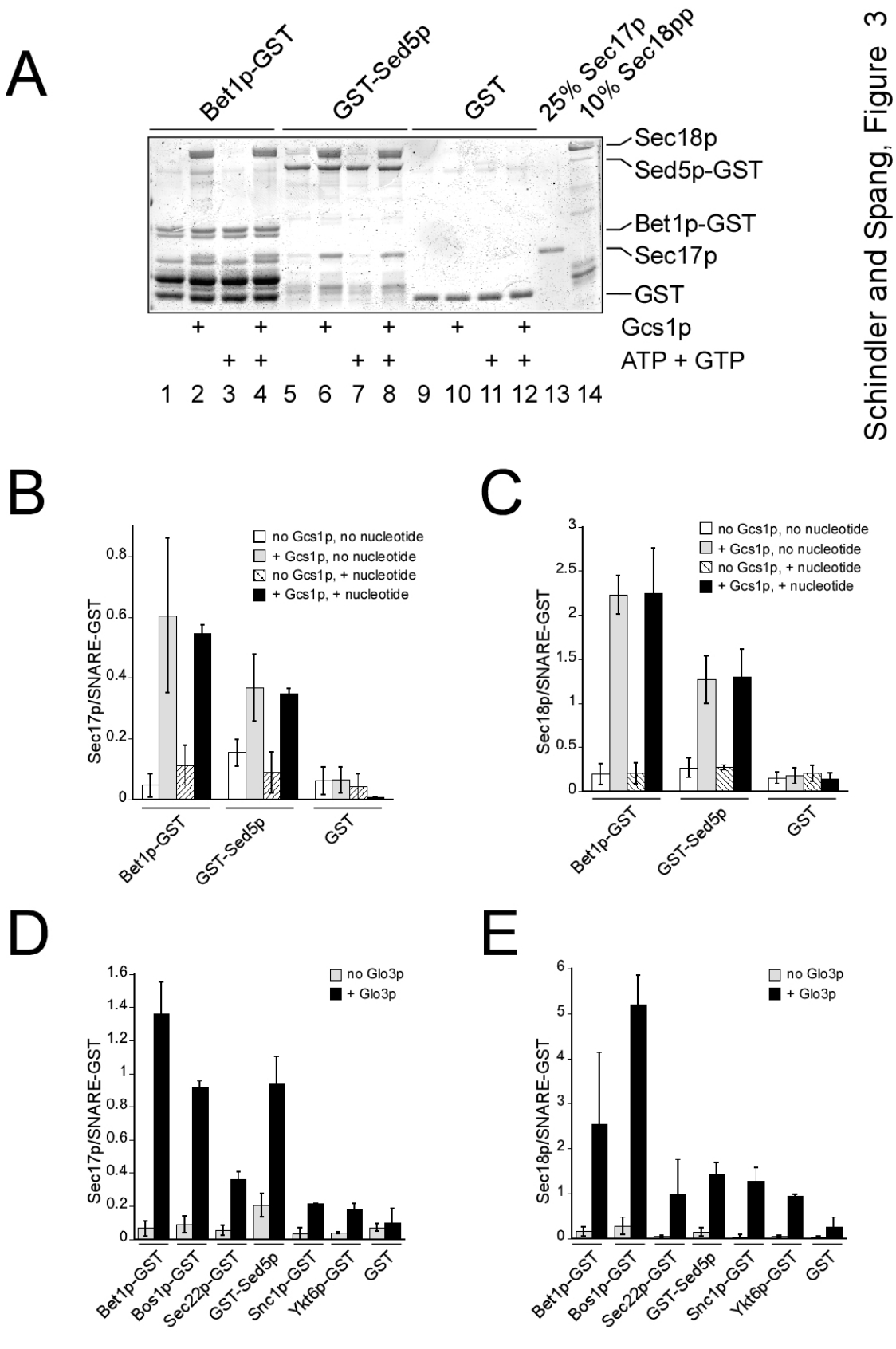
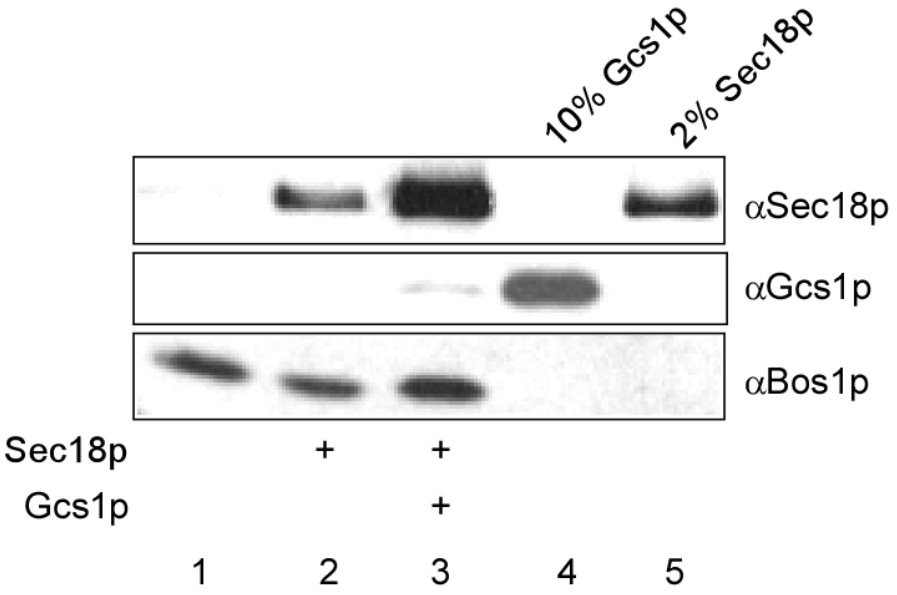
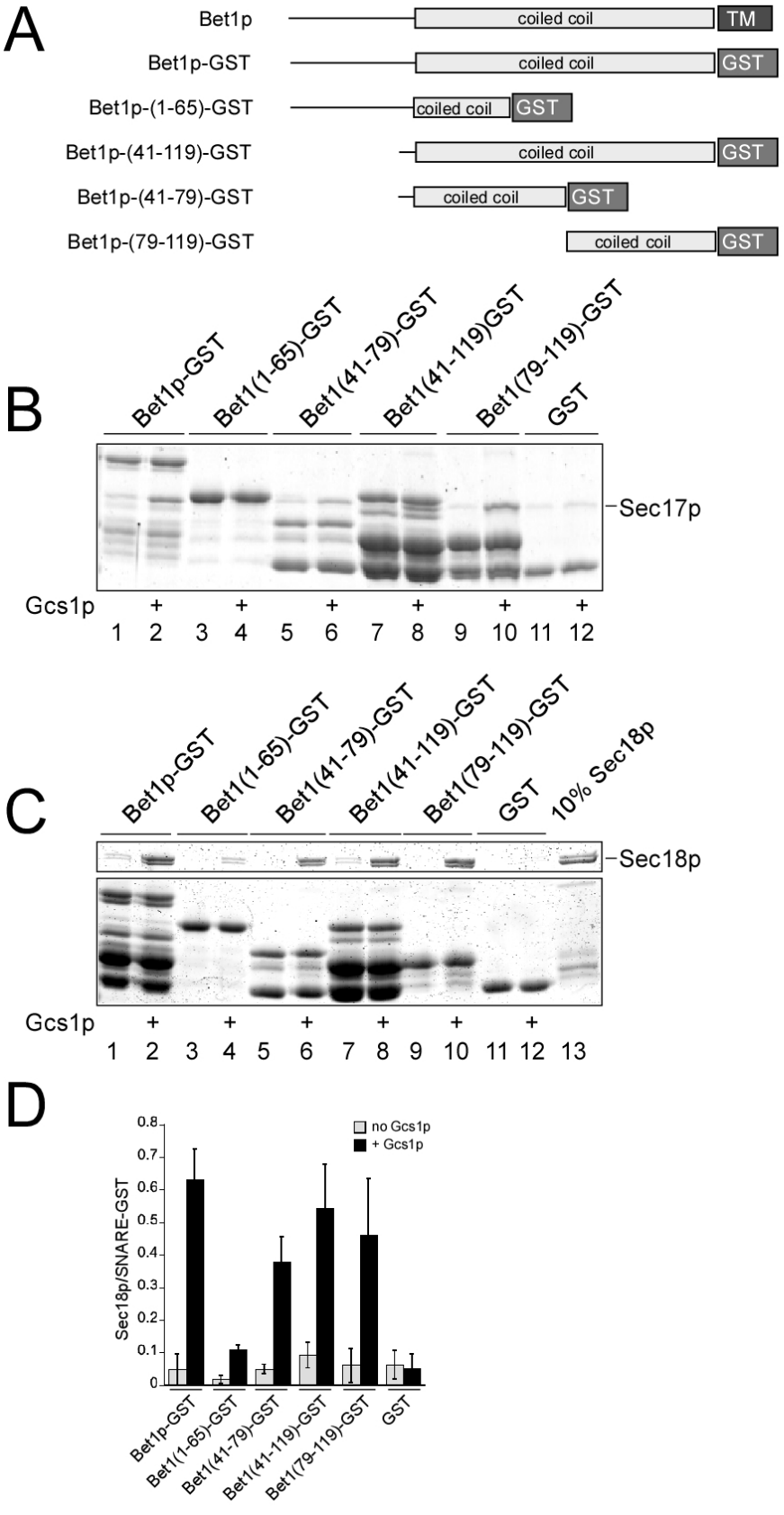
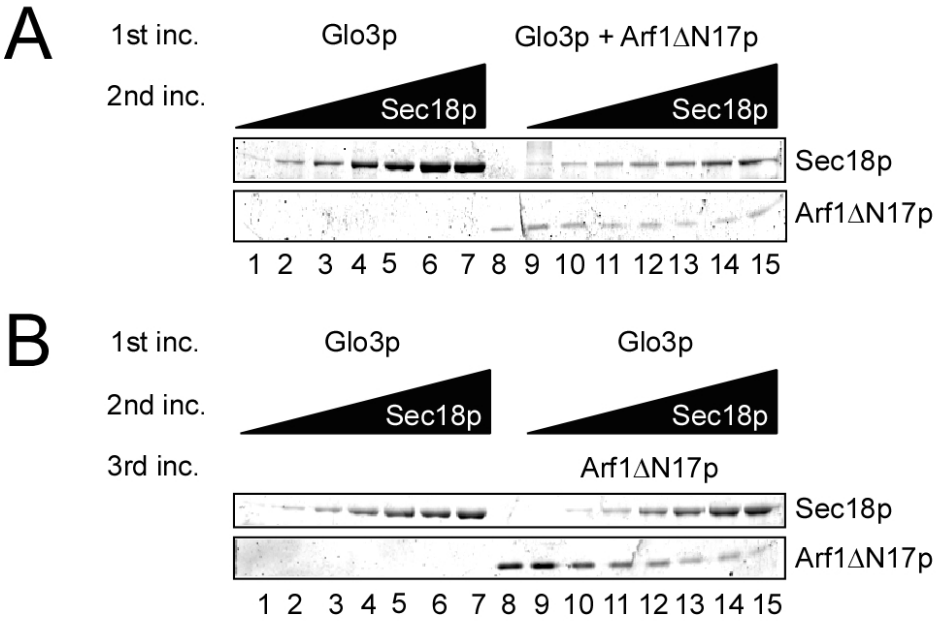


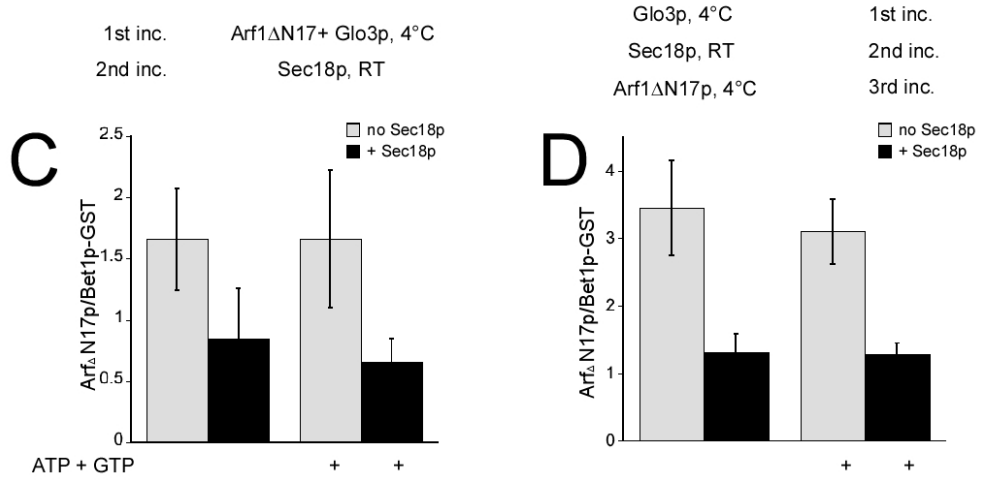
Schindler and Spang, Figure 3

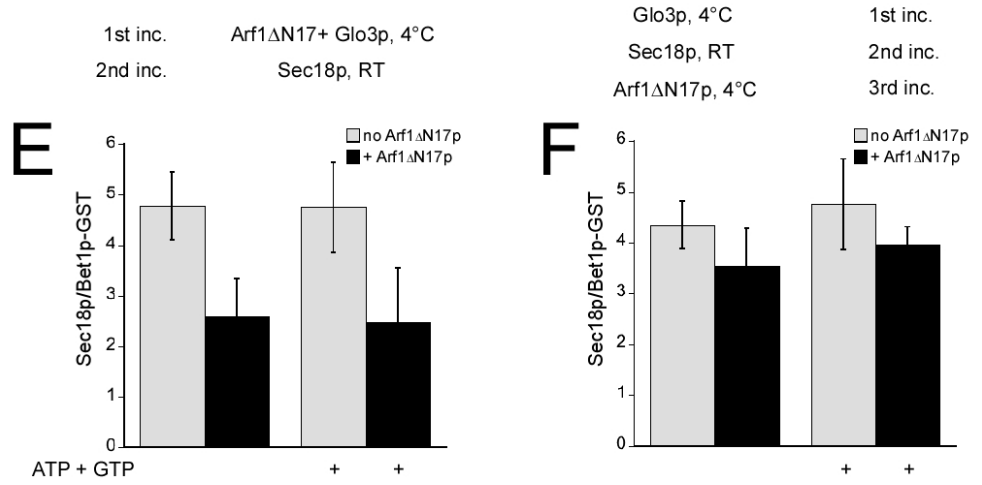
Schindler and Spang, Figure 4




A
Bet1p
coiled coil
TM
Bet1p-GST
GST
Bet1p-(1-65)-GST
Bet1p-(41-119)-GST
Bet1p-(41-79)-GST
Bet1p-(79-119)-GST
B
Bet1p-GST
Bet1(1-65)-GST
Bet1(41-79)-GST
Bet1(41-119)GST
Bet1(79-119)-GST
GST
Sec17p
Gcs1p
1 2 3 4 5 6 7 8 9 10 11 12
C
Bet1p-GST
Bet1(1-65)-GST
Bet1(41-79)-GST
Bet1(41-119)-GST
Bet1(79-119)-GST
GST
10% Sec18p
Sec18p
Gcs1p
1 2 3 4 5 6 7 8 9 10 11 12 13
D
no Gcs1p
+ Gcs1p
Sec18p/SNARE-GST
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Bet1p-GST
Bet1(1-65)-GST
Bet1(41-79)-GST
Bet1(41-119)-GST
Bet1(79-119)-GST
GST







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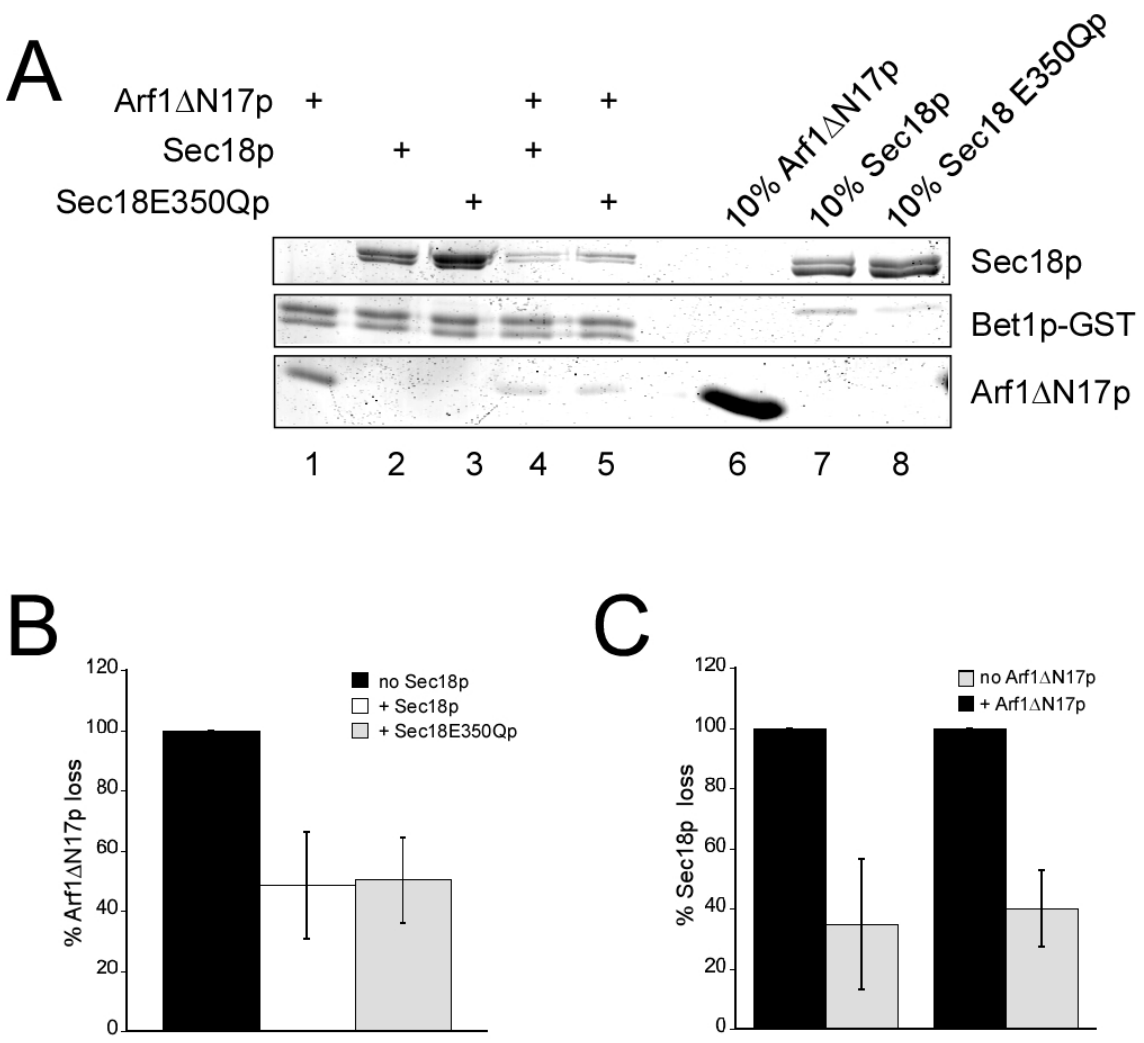
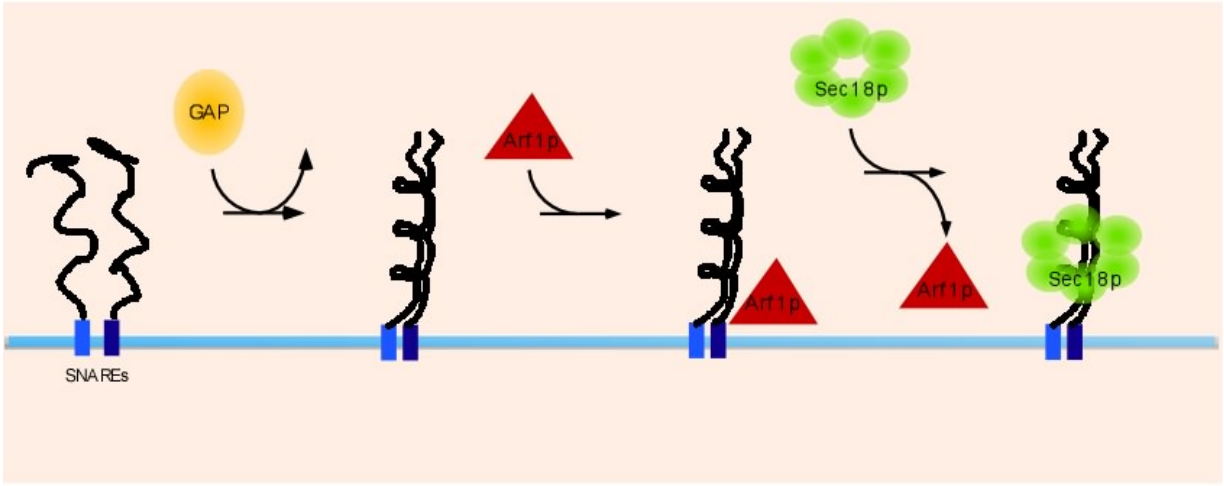


Schindler and Spang, Figure 7



Schindler and Spang, Figure 8

Table 1: SNAREs used in this study

SNARE	v-/t-SNARE	localisation
Bet1p	v	ER-Golgi
Bos1p	v	ER-Golgi
Nyv1p	v	vacuole
Sec22p	v	ER-Golgi
Sed5p	t	ER-Golgi
Snc1p	v	plasma membrane, endosome
Sso1p	t	plasma membrane
Sso2p	t	plasma membrane
Tlg2p	t	endosomes
Vam3p	t	vacuole
Ykt6p	v	Golgi

5.2 The Gcs1 Arf-GAP Mediates Snc1,2 v-SNARE Retrieval to the Golgi in Yeast

Micah Robinson, Pak Phi Poon, **Christina Schindler**, Lois E. Murray, Rachel Kama, Galina Gabriely, Richard E. Singer, Anne Spang, Gerald C. Johnston, and Jeffrey E. Gerst

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The heptameric coatomer complex and the small GTPase Arf1p constitute the essential components of the COPI vesicle coat. Coatomer is a stable complex with a half-life of several hours. Under high-salt conditions coatomer separates into the membrane-proximal F-COP subcomplex (consisting out of Sec21p, Sec26p, Ret2p and Ret3p) and the membrane-distal B-COP subcomplex (composed out of Sec33p, Sec27p and Sec28p). The COPI vesicle coat has been implicated mainly in the formation of vesicles that mediate retrograde transport within the Golgi apparatus and in the ER-Golgi shuttle. Furthermore, a function of at least some COPI components, especially of the B-COP subcomplex, was also found in endosomal trafficking and endosome maturation in mammalian cells (Aniento *et al.*, 1996; Daro *et al.*, 1997; Gu *et al.*, 1997; Whitney *et al.*, 1995). In budding yeast, a function of coatomer subunits in endosomal trafficking had not yet been elucidated.

Initially, we found a 2-hybrid interaction of the GTPase-activating-protein for Arf1p (ArfGAP) Gcs1p with the SNARE protein (soluble NSF attachment protein receptor) Snc2p and its homolog Snc1p. Both SNAREs share essential functions in exo- and endocytosis. Using truncated forms of Snc2p and Gcs1p in 2-hybrid analysis, we showed that the interaction requires the non-catalytic domain of the ArfGAP and the SNARE-domain of Snc2p. Furthermore, we established a genetic interaction between the *SNC* v-SNAREs and *GCS1*. A yeast strain, which was depleted for both Snc proteins is severely impaired in growth. When *GCS1* is additionally deleted, the resulting strain is unable to grow at all. Microscopic analysis of GFP-tagged Snc2p or Snc1p and RFP-Gcs1p showed a partial localization overlap at the *trans*-Golgi-network (TGN) and the endosomes. Co-immunoprecipitation experiments indicated that Gcs1p and Snc2p

interact weakly as the observed interaction was sensitive to increased amounts of salt. Next, we tested if the purified proteins were able to interact directly, which was indeed the case. Thus, we wanted to establish a function for this interaction and tested whether the Gcs1p pre-incubation of the SNAREs allows subsequent interaction with components of the COPI vesicle coat. We found that the recruitment of Arf1p was dependent on the pre-treatment of the SNAREs with ArfGAP. Based on previous findings from our lab (Rein *et al.*, 2002), we hypothesized that this interaction could mediate the sorting of the SNAREs into COPI-coated vesicles. After exocytosis, the Snc v-SNAREs recycle through the early endosomes back to the Golgi apparatus, where they are packaged into new vesicles directed to the plasma membrane. In mutant cells lacking *GCS1*, this recycling was severely disturbed. The distribution of GFP-Snc1p in *Δgcs1* was similar to distribution of the protein in *Δrcy1*, a strain which is known to be affected in endosome-to-Golgi trafficking. Furthermore, Gcs1p is present in a complex with Snx4p, a sorting nexin required for the retrieval of Snc1p from the endosomes to the Golgi. To test whether COPI-coated vesicles are involved in recycling *in vivo*, we monitored the GFP-Snc1p localization in several transport mutants. GFP-Snc1p was mislocalized in the B-COP mutants *sec27-1* and *Δsec28*. To further extend our knowledge on COPI-mediated recycling from the endosomes, we tested whether overexpression of the *SNC* v-SNAREs has an effect on coatomer mutants. Under permissive conditions these mutants grew fine. However, transport defects leading to a growth defect were obvious, when the cells were forced to cope with higher trafficking rates upon overexpression of the “cargo” proteins Snc1p or Snc2p. A strong reduction in growth was observed in *sec33-1* and *sec27-1*; both are components of the B-COP subcomplex. Furthermore, co-overexpression of *GCS1* further reduced viability of the *sec33-1* strain. This result indicates that the interaction of Gcs1p and the *SNC* v-SNAREs is required for the uptake of SNAREs in COPI-coated vesicles.

The findings presented in this report demonstrate a previously unknown function of the COPI vesicle coat in endosomal trafficking in budding yeast. Our results are consistent with the studies in mammalian cells which highlighted a special importance of the membrane-distal B-COP subcomplex of coatomer for endosome function and sorting in the endosomal pathway.

Own contribution

For this publication, I cloned the C-terminal GST-tagged versions of Snc1p and Snc2p. I purified all proteins that were required to demonstrate the direct interaction between the GST-tagged SNAREs Snc1p and Snc2p with the ArfGAP Gcs1p in GST-pulldown assays, which I performed. Furthermore, I did the GST-pulldown assays, showing that Arf1p is specifically recruited to GST-tagged Snc1p dependent on Gcs1p.

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The Gcs1 Arf-GAP Mediates Snc1,2 v-SNARE Retrieval to the Golgi in Yeast

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Gcs1 is an Arf GTPase-activating protein (Arf-GAP) that mediates Golgi–ER and post-Golgi vesicle transport in yeast. Here we show that the Snc1,2 v-SNAREs, which mediate endocytosis and exocytosis, interact physically and genetically with Gcs1. Moreover, Gcs1 and the Snc v-SNAREs colocalize to subcellular structures that correspond to the *trans*-Golgi and endosomal compartments. Studies performed in vitro demonstrate that the Snc-Gcs1 interaction results in the efficient binding of recombinant Arf1Δ17N-Q71L to the v-SNARE and the recruitment of purified coatomer. In contrast, the presence of Snc had no effect on Gcs1 Arf-GAP activity in vitro, suggesting that v-SNARE binding does not attenuate Arf1 function. Disruption of both the *SNC* and *GCS1* genes results in synthetic lethality, whereas overexpression of either *SNC* gene inhibits the growth of a distinct subset of COPI mutants. We show that GFP-Snc1 recycling to the *trans*-Golgi is impaired in *gcs1*Δ cells and these COPI mutants. Together, these results suggest that Gcs1 facilitates the incorporation of the Snc v-SNAREs into COPI recycling vesicles and subsequent endosome–Golgi sorting in yeast.

INTRODUCTION

Protein and lipid transport between intracellular compartments is required for the functional and structural integrity of organelles in eukaryotic cells. This transport is mediated by carrier vesicles generated by protein-based coat complexes. The COPI coat consists of the Arf1 small GTPase and coatomer and confers intra-Golgi and Golgi-to-ER retrograde transport (Kreis *et al.*, 1995; Kirchhausen, 2000; Spang, 2002; Nie *et al.*, 2003). Coatomer consists of seven subunits: αCOP (Sec33, 160 kDa); βCOP (Sec26, 110 kDa); β′COP (Sec27, 102 kDa); γCOP (Sec21, 98 kDa); δCOP (60 kDa); ϵCOP (Sec28, 35 kDa); and ζCOP (20 kDa), which are conserved from yeast to mammals. Coatomer subunits can be divided into two subcomplexes: the B subcomplex (COPI B) composed of the α, β′, and ϵ subunits; and the F subcomplex (COPI F), consisting of the β, δ, γ, and ζ subunits (Eugster *et al.*, 2000; McMahon and Mills, 2004). Interestingly, the γ subunit of COPI F shows structural similarity to components of the clathrin adaptor, AP2 (Hoffman *et al.*, 2003), whereas COPI B has been suggested to be clathrinlike (McMahon and Mills, 2004).

Arf GTPases undergo a cycle of GTP binding and hydrolysis to regulate vesicle formation from a variety of intracellular compartments, such as the Golgi and endosomes (Kreis *et al.*, 1995; Spang, 2002; Nie *et al.*, 2003). In the GTP-bound and myristoylated form, Arf1 becomes membrane-bound and drives COPI vesicle formation in vitro from Golgi membranes and defined liposomes (Spang *et al.*, 1998). Membrane-localized GTP-bound Arf1 recruits coatomer to create a molecular platform capable of deforming the lipid bilayer and leading to vesiculation. Thus, the regulation of Arf function is critical for the initial steps that lead to vesicle biogenesis.

GTP binding and hydrolysis on Arf is mediated by guanine nucleotide exchange factors and GTPase-activating proteins, known as Arf-GEFs and Arf-GAPs, respectively. Because vesicle uncoating is a prerequisite for fusion, Arf-GAP activity is thought to fulfill a role in the removal of coatomer, although roles in cargo packaging and vesicle formation have also been shown (Nickel *et al.*, 1998; Pepperkok *et al.*, 2000; Lanoix *et al.*, 2001; Rein *et al.*, 2002). Although in vitro studies suggest that myristoylated Arf1 and coatomer are sufficient to drive vesicle formation (Spang *et al.*, 1998; Springer *et al.*, 1999), these studies utilized amounts of protein that are unlikely to be physiological. Thus, other factors may be needed for Arf recruitment to membranes. These factors include p23 family members and SNAREs (Gommel *et al.*, 2001; Rein *et al.*, 2002). Recent work demonstrated that yeast Arf1 interacts directly with the SNAREs involved in ER–Golgi transport in a manner requiring Arf-GAP catalytic activity (Rein *et al.*, 2002; Randazzo and Hirsch, 2004). Moreover, Arf-GAP function was shown to be sufficient for COPI coat recruitment even in the absence of activated Arf (Reinhard *et al.*, 1999; Rein *et al.*, 2002; Yang *et al.*, 2002; Lee *et al.*, 2004). Thus, it has been suggested that Arf-GAP is a component of coatomer (Yang *et al.*, 2002; Lewis *et al.*, 2004) and that its catalytic activity is necessary for coat formation and vesicle production (Lee *et al.*, 2004).

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Table 1. Yeast strains used in this study

Strain	Genotype	Source
AH109	*MAT***a** *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1UAS-GAL1TATA-HIS3 GAL2UAS-GAL2TATA-ADE2 URA3::MEL1UAS-MEL1TATA-lacZ*	Clontech
Y153	*MAT***a** *gal4 gal80 his3 trp-902 ade2-101 ura3-52 leu2-3,-112 URA3::GAL-lacZ LYS2::Gal-HIS3*	S. Elledge
RH268-1C	*MAT***a** *can1 his4 leu2 trp1 bar1-1 end4-1*	H. Riezman
GWK8A	*MAT***a** *can1 his3 leu2 trp1 ade2 gcs1::URA3*	P. Poon
GWK8A pRS1-1	*MAT***a** *can1 leu2 trp1 ade2 gcs1::URA3* pRS1-1	P. Poon
RSY1309	*MAT***a** *his3-Δ leu2-3,112 lys2-801 suc2-9 sec21-2*	A. Spang
RSY1312	*MAT***a** *leu2-3,112 trp1 ura3-52 sec27-1*	A. Spang
RDY241	*MATα leu2 ura3 trp1 ade2 his3 lys2 sec28Δ::HIS3*	R. Duden
RDY260	*MATα leu2 ura3 sec33-1*	R. Duden
JG8 T15:85 (*sncΔ*)	*MAT***a** *can1 his3 leu2 snc1::URA3 snc2::ADE8* pTGAL-SNC1	J. Gerst
SP1	*MAT***a** *can1 his3 leu2 trp1 ura3 ade8*	J. Gerst
SP1-SEC7RFP	*MAT***a** *can1 his3 leu2 ura3 ade8 trp1::TRP1::TPI1-SEC7RFP*	J. Gerst
MRY1	*MAT***a** *can1 his3 leu2 trp1 ura3 ade8 gcs1::LEU2*	This study
MRY2	*MAT***a** *can1 his3 leu2 trp1 ura3 ade8 gcs1::URA3*	This study
MRY3	*MAT***a** *can1 his3 leu2 lys2 trp1 ura3 ade2 gcs1::LEU2*	This study
MRY4	*MAT***a** *can1 his3 leu2 lys2 trp1 ura3 ade2 gcs1::URA3*	This study
MRY5 (*sncΔ gcs1Δ*)	*MAT***a** *can1 his3 snc1::URA3 snc2::ADE8 gcs1::LEU2* pTGAL-SNC1	This study
PPY169-4	*MATα leu2Δ-0 lys2Δ-0 his3Δ1 ura3Δ-0 gcs1Δ::Nat-R mfa1ΔMFApr-HIS*	This study
W303-1a	*MAT***a** *can1 his3 leu2 lys2 trp1 ura3 ade2*	J. Hirsch
rcy1Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rcy1Δ::kanMX*	Euroscarf

Rein *et al.* (2002) found that yeast COPI (e.g., Arf1 and coatomer) bound to the Bet1, Bos1, and Sec22 ER–Golgi SNAREs in vitro (Rein *et al.*, 2002). This binding occurred only after preincubation of the SNAREs with either of the two Arf-GAPs known to facilitate ER–Golgi transport (e.g., Gcs1 and Glo3; Poon *et al.*, 1999). As SNAREs are central components of the vesicle docking and fusion machinery (reviewed in Chen and Scheller, 2001), Arf1-SNARE-coat interactions may be required to generate SNARE-equipped fusion-competent vesicles in vivo.

Because Arf1-GTP and coatomer binding to the SNAREs is Arf-GAP–dependent, it implies that Gcs1 and Glo3 may have two distinct functions. The first is to initiate Arf1-GTP binding, presumably by inducing a conformational change in SNARE structure and allowing for coat association. The second function is to uncoat the vesicle after budding has occurred by catalyzing GTP hydrolysis on Arf1. This additional role presumably allows the uncoated vesicles to undergo docking and fusion at the appropriate acceptor compartment.

We have been studying the Snc1 and Snc2 v-SNAREs that participate in both exocytosis and endocytosis in yeast (Protopopov *et al.*, 1993; Gurunathan *et al.*, 2000). These v-SNAREs partner with the Sso1,2 and Sec9 t-SNAREs to mediate exocytic functions (Brennwald *et al.*, 1994; Couve and Gerst, 1994) and with the Tlg1,2 and Vti1 t-SNAREs to mediate endocytic functions (Bryant and James, 2003). Thus, the Snc v-SNAREs, which are members of the synaptobrevin/VAMP family, engage in multiple transport steps and recycle continually between the plasma membrane and *trans*-Golgi via early endosomes (Lewis *et al.*, 2000; Hettema *et al.*, 2003). Efficient Snc1 recycling to the early endosome requires the sorting nexin, Snx4, which is involved in protein retrieval from endosomes to the Golgi (Hettema *et al.*, 2003). Here we show that the Snc1,2 v-SNAREs and Gcs1 Arf-GAP interact physically and genetically, leading to v-SNARE recycling to the *trans*-Golgi. This recycling process appears to involve coatomer, as well as Snx4, and thus, may represent a novel trafficking pathway from sorting endosomes back to the Golgi.

MATERIALS AND METHODS

Media, DNA, and Genetic Manipulations

Yeast were grown in standard growth media containing either 2% glucose or 3.5% galactose. Synthetic complete (SC) and drop-out media were prepared similar to that described (Rose *et al.*, 1990). Standard methods were used for the introduction of DNA into yeast and the preparation of genomic DNA (Rose *et al.*, 1990).

Growth Tests

Yeast were grown on synthetic and rich growth media (Rose *et al.*, 1990). For cold-sensitive growth tests on plates, yeast were grown to stationary phase, normalized for optical density, diluted serially, and plated by drops onto solid medium preincubated at different temperatures. For growth tests involving *sncΔ* or *sncΔ gcs1Δ* cells, which carry a galactose-inducible form of *SNC1*, cells were first grown to stationary phase on galactose-containing synthetic medium. Next, a portion of the cells was shifted to glucose-containing medium for 24 h to induce the *sncΔ* phenotype. Cultures were then normalized for optical density, diluted serially, and plated by drops onto solid medium preincubated at different temperatures. For temperature-sensitive growth of COPI mutants, cells were grown to midlog phase on synthetic medium before normalization, serial dilution, and plating onto solid medium preincubated at different temperatures. Calcofluor resistance was measured by adding between 50 and 150 μg/ml fluorescent brightener 28 (Sigma, St. Louis, MO) to plates and plating serial dilutions of the different strains by drops.

Yeast Strains and Plasmids

Yeast strains are listed in Table 1. Vectors included: pRS313 (*CEN, TRP1*); pRS315 (*CEN, LEU2*); pRS316 (*CEN, URA3*); YCp50 (*CEN, URA3*); pAD11 (*CEN, HIS3*); pRS426 (2μ, *URA3*); pAD4Δ (2μ, *LEU2, ADH1* promoter); and pAD54 and pAD6 (same as pAD4Δ, but containing sequences encoding the HA or Myc epitopes, respectively). Previously described *SNC* expression plasmids included: pADH-SNC1 (Gerst *et al.*, 1992); pADH-SNC2, pADH-HASNC1, and pTGAL-SNC1 (Protopopov *et al.*, 1993); and pADH-mycSNC2 and pADH-HASNC2 (Lustgarten and Gerst, 1999). Previously described plasmids for the bacterial expression of Gcs1 and Arf1 included pPPL21 and pET-Arf1H, respectively (Poon *et al.*, 1996). A plasmid expressing recombinant *N*-myristoyltransferase in bacteria, pACY177/ET3d/yNMT (Haun *et al.*, 1993), was used to create myristoylated Arf1, as described (Poon *et al.*, 1996). Plasmid YIplac204-T/C-SEC7-dsRED.T4 was generously provided by B. Glick (University of Chicago, IL).

Plasmids created for this study are listed in Table 2. Sequences of the oligonucleotides used will be provided on request. Disruption constructs for *GCS1* that do not interfere with adjacent open reading frames were created by amplifying a region corresponding to 1999 bp upstream of the start codon and 1455 bp downstream of the stop codon of *GCS1* from genomic DNA. This PCR product was cloned into pGEM-T-Easy to give pGEM-GCS1. Next, a fragment containing either *URA3* or *LEU2* was cloned into the PmeI-XbaI sites of *GCS1* in pGEM-GCS1. Digestion with PmeI and XbaI resulted in the removal of

Table 2. Expression plasmids used in this study

Plasmid name	Gene	Backbone	Sites	Type	Selectable marker	Created by
pAD54-cSNC1	*cSNC1* (*SNC1* cDNA)	pAD54	SalI-SacI	2μ	*LEU2*	M. Robinson
pAD54-GFP-cSNC1	*GFP* (w/o ATG and STOP)	pAD54-cSNC1	SalI-SacI	2μ	*LEU2*	M. Robinson
pAD54-GFP-SNC2	*GFP* (w/o ATG and STOP)	pADH-HASNC2	SalI-SacI	2μ	*LEU2*	M. Robinson
pGADT7-SNC1	*SNC1*[a]	pGADT7	EcoRI-SacI	2μ	*LEU2*	M. Robinson
pGADT7-SNC2	*SNC2*[a]	pGADT7	EcoRI-SacI	2μ	*LEU2*	M. Robinson
pHADH-mycSNC1	*SNC1*	pAD11	BamHI	*CEN*	*HIS3*	Gerst Lab
pHADH-mycSNC2	*SNC2*	pAD11	BamHI	*CEN*	*HIS3*	Gerst Lab
pRS426-HA-cSNC1	*HA-cSNC1*	pRS426	BamHI	2μ	*URA3*	M. Robinson
pRS426-HA-SNC2	*HA-SNC2*	pRS426	BamHI	2μ	*URA3*	M. Robinson
pRS315-HA-GFP-cSNC1	*HA-GFP-cSNC1*	pRS315	BamHI	*CEN*	*LEU2*	M. Robinson
pRS315-HA-GFP-SNC2	*HA-GFP-SNC2*	pRS315	BamHI	*CEN*	*LEU2*	M. Robinson
pRS316-HA-mRFP-cSNC1	*HA-mRFP-cSNC1*	pRS316	BamHI	*CEN*	*URA3*	R. Kama
pPP381-39	$SNC2^{21\text{-}348}$[a]	pGAD424	BamHI-Sau3A	2μ	*LEU2*	P. Poon
pPPL92	$SNC2^{1\text{-}92}$	pET32mlic				P. Poon
pAD54-GCS1	*GCS1*	pAD54	SalI-SacI	2μ	*LEU2*	M. Robinson
pAD54-DsRedT4-GCS1	*DsRedT4*	pAD54-GCS1	SalI-SalI	2μ	*LEU2*	M. Robinson
pAD54-GFP-GCS1	*GFP* (w/o ATG & STOP)	pAD54-GCS1	SalI-SalI	2μ	*LEU2*	M. Robinson
YCp50-GCS1-DsRedT4	*GCS1-DsRedT4*	YCp50	BamHI	*CEN*	*URA3*	M. Robinson
pRS315-GFP-GCS1	HA-GFP-GCS1	pRS315	BamHI	*CEN*	*LEU2*	M. Robinson
pRS426-HA-GCS1	HA-GCS1	pRS426	BamHI	2μ	*URA3*	M. Robinson
pSH4	*GCS1*	pRS315		*CEN*	*LEU2*	P. Poon
pGBKT7-GCS1	*GCS1*[b]	pGBKT7	NcoI-SalI	2μ	*TRP1*	M. Robinson
pLM60	*GCS1*[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM61	$GCS1^{1\text{-}6782}$	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM62	$GCS1^{145\text{-}1059}$[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM63	$GCS1^{349\text{-}1059}$[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM64	$GCS1^{409\text{-}1059}$[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pLM65	$GCS1^{1\text{-}417}$[b]	pGBD-C2	EcoRI-ClaI	2μ	*TRP1*	L. Murray
pSP10C	$SNC2^{153\text{-}348}$[a]	pGAD-C3	ClaI	2μ	*LEU2*	L. Murray
pGEM-GCS1-LEU2	*gcs1::LEU2*	pGEM-T-Easy	PmeI-XbaI		*LEU2*	M. Robinson
pGEM-GCS1-URA3	*gcs1::URA3*	pGEM-T-Easy	PmeI-XbaI		*URA3*	M. Robinson
pPP269	*GCS1*[b]			2μ	*TRP1*	P. Poon
pPP329	*GCS1*	YEp352		2μ	*URA3*	P. Poon
pRS315-GFP-TLG2	*GFP-TLG2*	pRS315	BamHI	*CEN*	*LEU2*	M. Robinson
YCp50-DsRedT4-AGE2	*DsRedT4-AGE2*	YCp50	BamHI	*CEN*	*URA3*	M. Robinson
pRS313-GFP-YIF1	*HA-GFP-YIF1*	pRS313	BamHI	*CEN*	*TRP1*	R. Kama
pSE1112	*SNF1*[b]			2μ	*TRP1*	P. Poon
pCL1	*GAL4*			2μ	*LEU2*	Clontech

[a] Fused with transactivating domain of Gal4.
[b] Fused with DNA-binding domain of Gal4.

nucleotides 213–592 from the coding region of *GCS1*. Subsequent insertion of either the *URA3* or *LEU2* selectable marker gave plasmids pGCS1::URA3 and pGCS1::LEU2, respectively. A ~6-kb disruption fragment was excised from pGCS1::URA3 or pGCS1::LEU2 by digestion with NotI and was used to transform both wild-type and *snc*Δ null cells.

Synthetic Genetic Analysis

For synthetic genetic analysis (SGA), a query strain (PPY169-4) was constructed by replacing the *GCS1* gene with a nourseothricin-resistance cassette (Goldstein and McCusker, 1999) via homologous recombination. The cassette was created by PCR amplification using oligonucleotides bearing sequences flanking the *GCS1* coding region and plasmid p4339 as a template (Tong *et al.*, 2001). The replacement of *GCS1* in PPY169-4 was verified by PCR analysis. An automated approach was then used to cross strain PPY169-4 with the yeast gene-deletion collection, and the resulting haploid double segregants were screened for synthetic-lethal combinations as described previously (Tong *et al.*, 2001).

Microscopy

GFP and RFP fluorescence in strains expressing the appropriate GFP- and DsRedT4/mRFP-tagged fusion proteins was visualized by confocal microscopy (Bio-Rad, Hercules, CA).

Immunoprecipitation

Protein–protein interactions were monitored by the immunoprecipitation (IP) from cell extracts, as described in (Couve and Gerst, 1994) except that a 10 mM Tris (pH 7.5), 1 mM EDTA buffer was substituted for phosphate-buffered saline. Monoclonal antisera included anti-myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-HA antibodies (gift of Michael Wigler, Cold Spring Harbor Laboratory). Anti-myc antibodies were used for IP (4 μl per reaction) and detection (1:1000). Anti-HA antibodies were also used for IP (0.8 μl per reaction) and detection (1:7000). Polyclonal antibodies included anti-Gcs1 antibodies (1:2500). Samples of TCLs and immunoprecipitates were resolved by electrophoresis and detected by Western blotting. Detection was performed using enhanced chemiluminescence (Amersham).

Two-Hybrid Assay

Assessment of the Snc v-SNARE and Gcs1 Arf-GAP interaction in β-galactosidase assays (Figure 1, A and D) was performed using Y153 cells in the yeast two-hybrid assay (Durfee *et al.*, 1993). Transformants were patched onto selective synthetic medium, before being subjected to lifts onto nitrocellulose filters and lysis in liquid nitrogen. β-Galactosidase assays were performed using standard procedures. Assessment of the Snc v-SNARE and Gcs1 Arf-GAP interaction in drop tests (Figure 1B) was performed using AH109 cells in the yeast two-hybrid assay, as described by Durfee *et al.* (1993). Transformants were grown in liquid selective medium, diluted serially, and plated by drops onto solid medium lacking histidine and containing 0–2 mM 3-aminotriazole.

In Vitro Arf-GAP Activity Assays

To assess the effects of Snc2 on Gcs1 Arf-GAP activity in vitro, recombinant His6-tagged Gcs1 (plasmid pPPL21) and His6-tagged Snc2 (plasmid pPPL92), which lacks the transmembrane domain, were expressed in *Escherichia coli*

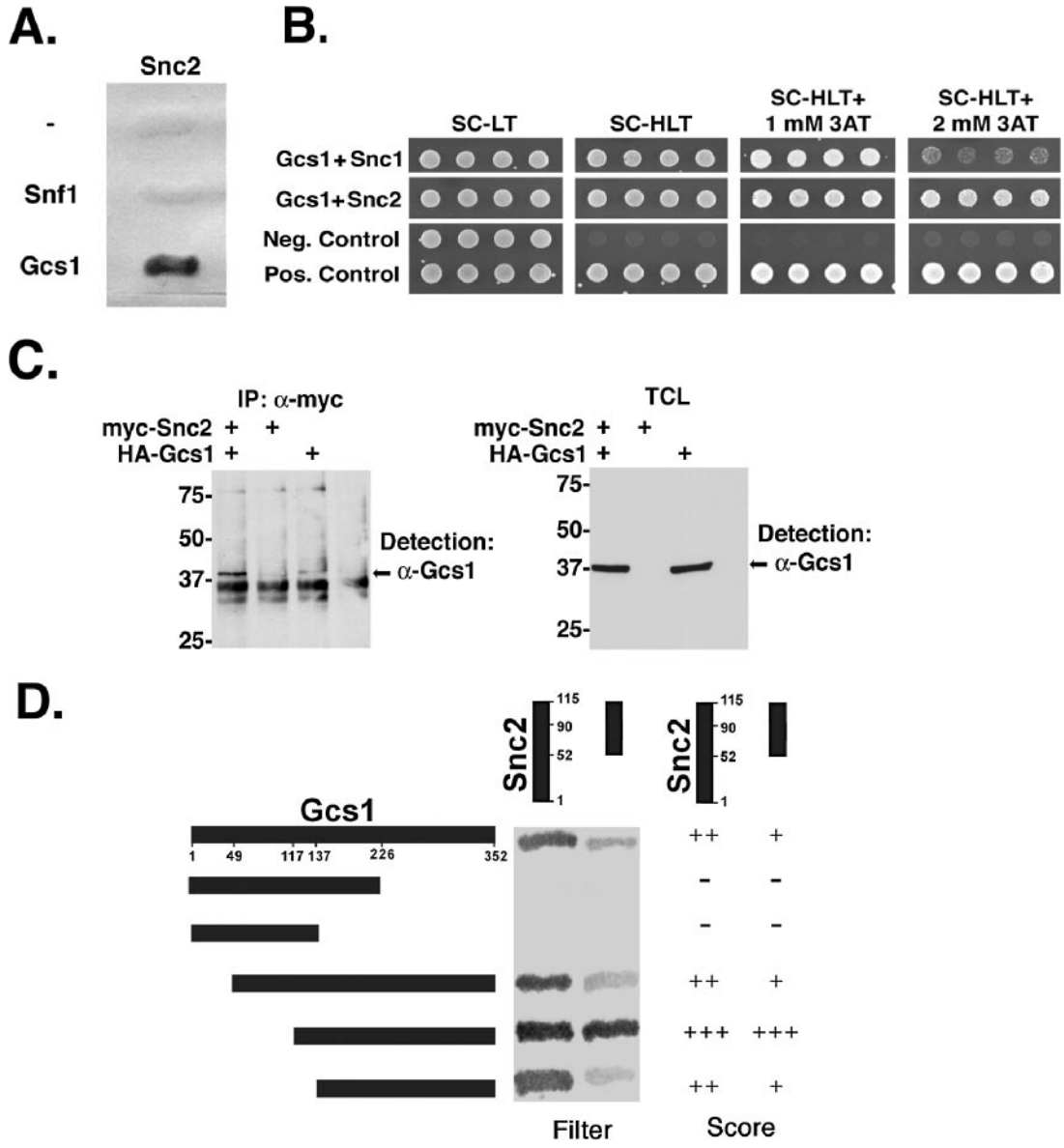

Figure 1. The Snc v-SNAREs interact physically with Gcs1. (A) Snc2 interacts with Gcs1, as assayed using the two-hybrid *lac*Z detection assay. Yeast (Y153) were transformed with a prey plasmid expressing Snc2 fused to the transactivating domain of Gal4 ($Snc2^{8-115}$; plasmid pPP381-39) and bait plasmids, including vectors expressing the DNA-binding domain of Gal4 alone (−; plasmid pGBT9), or fused with Snf1 (Snf1; plasmid pSE1112) or Gcs1 (Gcs1; plasmid pPP269). Cells were grown in patches on selective medium, replica plated onto nitrocellulose filters, lysed in liquid nitrogen, and measured for β-galactosidase activity using standard techniques. (B) Snc1 and 2 interact with Gcs1, as assayed using the two-hybrid 3-AT growth assay. Bait plasmids expressing the Gal4-transactivating domain fused to either Snc1 or Snc2 (Snc1 or Snc2; plasmids pGADT7-SNC1 and pGADT7-SNC2, respectively), along with a plasmid expressing the Gal4 DNA-binding domain fused to Gcs1 (Gcs1; pGBKT7-GCS1), were transformed into AH109 cells and examined for their ability to grow on medium lacking histidine and containing 3-aminotriazole (3-AT). Cells were plated by serial dilution on selective medium (SC-LT), selective medium lacking histidine (SC-HLT), and the same medium with or without the addition of either 1 mM or 2 mM 3-AT. Negative control (Neg. control) consisted of empty vectors expressing the DNA-binding and TA domains alone. A positive control consisted of an empty vector plus a plasmid (pCL1) expressing full-length Gal4. Cells were grown for 48-72 h at 30°C. (C) HA-Gcs1 coimmunoprecipitates with myc-Snc2. *gcs1*Δ (MRY4) cells bearing: (1) a multicopy plasmid expressing HA-tagged Gcs1 (pAD54-GCS1) and a single-copy plasmid expressing myc-tagged Snc2 (pHADH-mycSNC2); (2) either expression plasmid (e.g., pAD54-GCS1 or pHADH-mycSNC2) alone along with the appropriate control vector (e.g., pAD54 or pAD11); or (3) both control vectors (pAD54 and pAD11) were grown to log phase, lysed, and processed for coIP with anti-myc antibodies. The immunoprecipitation lanes (IP) and total cell lysate (TCL) shown were detected with anti-Gcs1 antibody (1:2500). (D) The Arf-GAP domain of Gcs1 and amino terminus of Snc2 are dispensable for the Gcs1-Snc2 two-hybrid interaction. Yeast (Y153) was transformed with prey plasmids expressing either Snc2 or a truncated form of Snc2, $Snc2^{52-115}$, fused to the TA domain of Gal4 (plasmids pPP381-39 and pSP10C, respectively) and bait plasmids expressing Gcs1 ($Gcs1^{1-352}$) or truncated forms of Gcs1 (e.g., $Gcs1^{1-139}$; $Gcs1^{1-226}$, $Gcs1^{49-352}$; $Gcs1^{117-352}$; and $Gcs1^{137-352}$; plasmids pLM65, pLM61, pLM62, pLM63, and pLM64, respectively) fused to the DNA-binding domain of Gal4. Cells were grown in patches on selective medium, replica plated onto nitrocellulose filters, lysed in liquid nitrogen, and measured for β-galactosidase activity using standard techniques. Shown are a representative filter after β-galactosidase detection (Filter) and a qualitative assessment of β-galactosidase activity (Score).

BL21 and purified in native form using standard protocols (Poon *et al.*, 2001). First, 50 ng of purified Gcs1 was mixed with varying amounts of His6-Snc2, ranging from 50 ng to 25 μg, and incubated on ice in 80 μl of 12.5% glycerol, 0.125% bovine serum albumin (fraction V), 1.25 mM DTT, 1.25 mM ATP, 1.25 mM MgCl, 187 mM KOAc, and 31.3 mM MOPS buffer at pH 7.5. After 3 h of incubation, GAP activity was assayed by the addition of 20 μl γ-^{32}P-GTP-bound myristoylated Arf1, incubation at 30°C for 15 min, and subsequent assessment of GTP-hydrolysis, as previously described (Poon *et al.*, 2001).

In Vitro Arf- and Coatomer-binding Assays

Truncated genes encoding SNAREs lacking their transmembrane domains were cloned into vector pETGEXCT (Sharrocks, 1994). N- and C-terminal GST-tagged SNARE fusion proteins were expressed in *E. coli* and purified using standard procedures. Gcs1 and Arf1ΔN17-Q71L expression in *E. coli* and purification were performed as described (Rein *et al.*, 2002). Coatomer was purified from yeast as described (Hosobuchi *et al.*, 1992). Pulldown assays employing immobilized SNARE-GST fusion proteins were performed essentially as described (Rein *et al.*, 2002). In brief, 5 μg SNARE-GST fusion proteins were immobilized onto GSH-agarose (Sigma) and subsequently incubated for 1 h at 4°C with 20 nM recombinant Gcs1 in a total reaction volume of 100 μl in BBP (25 mM HEPES, pH 6.8, 300 mM KOAc, 1 mM DTT, 0.5 mM $MgCl_2$, and 0.2% Triton X-100). Gcs1 was removed from the reaction by three washes with BBP. The beads were incubated with 40 nM coatomer and 7.3 μM recombinant Arf1ΔN17-Q71L for 1 h at 4°C, washed three times with BBP and once with 20 mM HEPES, pH 6.8. The proteins bound to the beads were separated by SDS-PAGE, visualized by Fairbanks staining, and visualized using the Odyssey system (Li-Cor).

RESULTS

The Snc2 v-SNARE Interacts with the Gcs1 Arf-GAP in the Two-Hybrid Assay

The yeast two-hybrid screen was used to identify proteins that interact with the Gcs1 Arf-GAP. Full-length Gcs1 fused to the DNA binding domain of Gal4 was used as bait and screened with a yeast genomic library fused to the transactivating domain of Gal4. One of the candidate prey genes identified in this assay encoded the yeast synaptobrevin/VAMP ortholog, Snc2, as shown in Figure 1A. Interestingly, a systematic genome-wide two-hybrid screen also identified Snc2 as interacting with Gcs1 (Ito *et al.*, 2001). Thus, independent two-hybrid screens suggest that this Arf-GAP and a post-Golgi v-SNARE interact physically. This interaction could be of consequence for post-Golgi vesicular transport as the Snc v-SNAREs mediate both endo- and exocytosis (Protopopov *et al.*, 1993; Gurunathan *et al.*, 2000) and cycle between the plasma membrane and the Golgi (Lewis *et al.*, 2000). Indeed, a role for Gcs1 in post-Golgi vesicle biogenesis has already been proposed (Poon *et al.*, 2001).

To further extend our observations using two-hybrid analysis, we assessed protein–protein interactions with either full-length Snc1 or Snc2 (e.g., Snc1$^{2\text{-}116}$ and Snc2$^{2\text{-}115}$) fused to the transactivating domain of Gal4 and Gcs1 fused to the DNA-binding domain (Figure 1B). When tested for the ability to confer growth in the absence of histidine and in the presence of a metabolic inhibitor of His3 (3-aminotriazole [3-AT]), we found that either v-SNARE could do so in the presence of Gcs1. Thus, both Snc1 and Snc2 interact with this Arf-GAP. However, the Snc1-Gcs1 interaction was more sensitive to higher concentrations of 3-AT (Figure 1B), indicating that it may be weaker than that of Snc2-Gcs1. Thus, both lines of experimentation verify an interaction between the Gcs1 Arf-GAP and the Snc1,2 v-SNAREs.

Snc v-SNAREs Coimmunoprecipitate with Gcs1

Because the Snc v-SNAREs interact physically with Gcs1 in the two-hybrid assay (Figure 1, A and B), we next examined whether these proteins coimmunoprecipitate. In the absence of a functional copy of *GCS1*, yeast cells are unable to reenter the cell cycle at 14°C and are rendered cold-sensitive (Ireland *et al.*, 1994). For the coimmunoprecipitation experiments, we used an HA-tagged version of Gcs1 that was deemed functional by virtue of its ability to confer cold-resistant growth to *gcs1-1* and *gcs1*Δ mutant cells (our unpublished observations). We found that HA-tagged Gcs1 coprecipitated with myc-tagged Snc2 from lysates prepared from wild-type cells expressing these proteins (Figure 1C). A single band corresponding to a molecular mass of about 40 kDa was observed in precipitates from cells expressing both proteins. This signal was specific, but weak, being eliminated by relatively low concentrations of salt (i.e., 130 mM NaCl; our unpublished observations). Thus, these proteins interact physically, but perhaps not tightly, in vivo. Similar results were obtained with myc-Snc1 and HA-Gcs1 (our unpublished observations).

The Arf-GAP Domain of Gcs1 Is Not Required for the Interaction with Snc2

To determine which regions of Gcs1 and Snc2 are required for their physical association, truncated forms of the proteins were tested by two-hybrid analysis (Figure 1D). Deletions in the amino terminus of Gcs1 (Gcs1$^{49\text{-}352}$, Gcs1$^{117\text{-}352}$, and Gcs1$^{137\text{-}352}$), which effectively remove the Arf-GAP domain (amino acid residues 8-129) or portions thereof, did not abolish and even enhanced the interaction with Snc2 isolated in the initial screen (e.g., Snc2$^{8\text{-}115}$). In contrast, the amino terminus of Gcs1 alone (Gcs1$^{1\text{-}139}$ and Gcs1$^{1\text{-}226}$) conferred no β-galactosidase activity when coexpressed with Snc2. This lack of interaction was not due to insufficient protein expression because Western blot analysis indicated that the amino terminal Gcs1 fusion protein was as abundant as the full-length Gcs1 fusion (our unpublished observations). These results suggest that the Arf-GAP domain of Gcs1 is dispensable for the interaction with Snc2.

Deletion of the first 51 amino acids of Snc2 did not abolish the interaction with either full-length Gcs1 or its amino terminus–truncated forms. Thus, the Gcs1-interacting domain resides in the carboxy terminus of the v-SNARE, which encompasses the SNARE domain (Fasshauer *et al.*, 1998). As access to the transmembrane region of Snc2 is unlikely in vivo, this result implies that Gcs1 binds to the t-SNARE–interacting domain of the protein.

A Genetic Interaction between the SNC and GCS1 Genes

We next assessed the significance of the Gcs1-Snc interaction in vivo using a genetic approach. We examined whether the combined disruption of the *SNC* and *GCS1* genes leads to synthetic defects, which would indicate a related function in post-Golgi transport. We first disrupted *GCS1* in the same genetic background (SP1 wild-type cells) as that used to generate the *snc*Δ strain (Protopopov *et al.*, 1993). The disruption of *GCS1* led to cold sensitivity on synthetic and rich medium (YPD) at 15°C in the SP1 background (Figure 2A and our unpublished results), as shown previously in the W303 background (Ireland *et al.*, 1994). The disruption of *GCS1* in SP1 cells also resulted in an inhibition of growth at 37°C on rich medium (our unpublished results).

We examined whether overexpression of the *SNC* genes or reexpression of *GCS1* could confer cold-resistant growth to *gcs1*Δ cells. We found that *HA-GCS1* expression from a multicopy plasmid or *GCS1-RFP* expression from a single copy plasmid could confer growth at 15°C to *gcs1*Δ cells. In contrast, overexpression of the *SNC* genes had no effect and could not confer growth at 15°C (Figure 2A). This verified that the *GCS1* expression constructs are functional and could restore cold-resistant growth to *gcs1*Δ cells. We note that overexpression of either *HA-GCS1* (Figure 2A) or native *GCS1* (our unpublished observations) from multicopy plasmids has a mild inhibitory

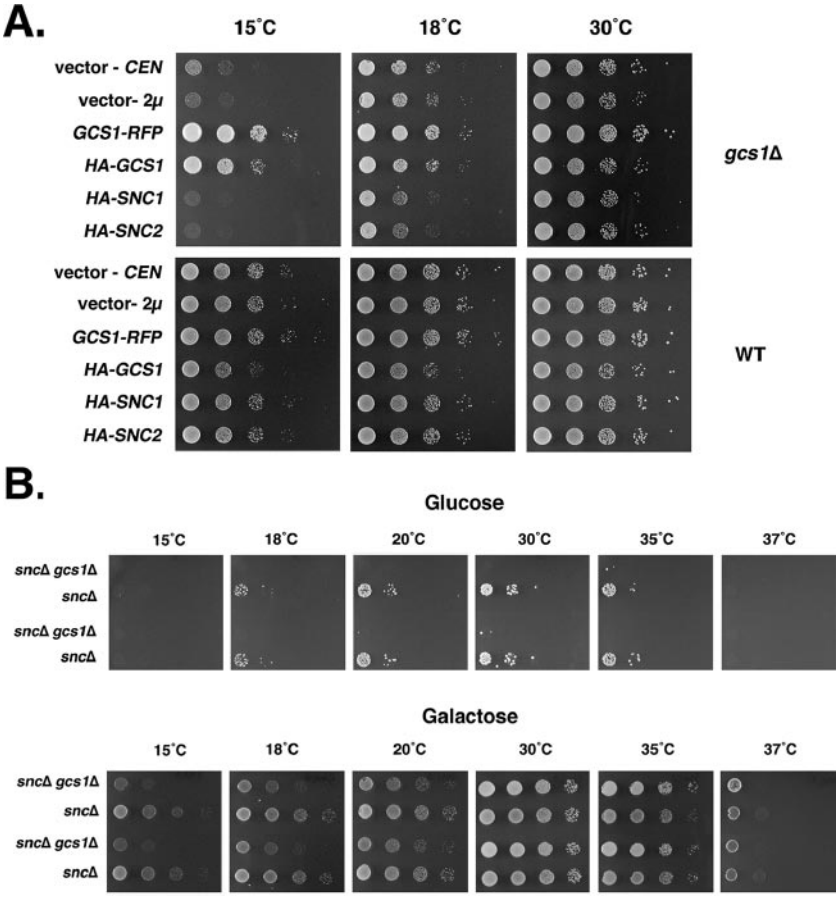

Figure 2. Deletion of both the *SNC1,2* and *GCS1* genes results in lethality. (A) *gcs1*Δ cells are cold-sensitive and are rescued by plasmids expressing *GCS1*. SP1 wild-type cells and a *gcs1*Δ disruption strain (MRY1) were transformed with multicopy plasmids expressing *SNC1* (pRS426-HA-cSNC1), *SNC2* (pRS426-HA-SNC2), *GCS1* (pRS426-HA-GCS1), or a single copy plasmid expressing *GCS1* fused to *RFP* (YCp50-GCS1-DsRedT4) were grown to stationary phase (72 h), plated serially on selective medium at various temperatures, and incubated for 2-11 d. Cells bearing empty multicopy (pRS426; vector: 2μ) or single copy (YCp50; vector: *CEN*) plasmids were used as controls. *gcs1*Δ cells were grown at 15°C for 11 d; at 18°C for 6 d; and at 30°C for 2 d. Wild-type cells were grown at 15°C for 8 d; at 18°C for 5 d; and at 30°C for 2 d. (B) Combined *snc*Δ and *gcs1*Δ null mutations are synthetically lethal. *snc*Δ (JG8 T15:85) or *snc*Δ *gcs1*Δ (MRY5) cells, which both bear plasmid pTGAL-SNC1, were grown to stationary phase (48 h) on galactose-containing medium (which induces expression from the *GAL*-inducible *SNC1* gene). Cells were diluted to 1 OD_{600}/ml either in galactose-containing medium or glucose-containing medium (to deplete Snc1) for 24 h, before being serially diluted and plated onto either galactose-containing (Galactose) or glucose-containing (Glucose) solid medium. Cells were grown for 4-11 d on glucose at various temperatures: 15°C, 11 d; 18°C, 8 d; 20°C, 7 d; 30°C, 4 d; 35°C, 4 d; and 37°C, 6 d. Cells were grown for 3-9 d on galactose at various temperatures: 15°C, 9 d; 18°C, 6 d; 20°C, 3 d; 30°C, 3 d; 35°C, 3 d; and 37°C, 4 d.

effect on wild-type cells at lower temperatures (15 and 18°C; Figure 2A). This is likely to result from a general inhibitory effect the Arf-GAP has on Arf function.

Next, we disrupted *GCS1* in *snc*Δ null cells, which are temperature-sensitive on synthetic medium and unable to grow on amino acid–rich medium (Protopopov *et al.*, 1993). To maintain viability, *SNC1* was expressed in the *snc*Δ *gcs1*Δ strain under the control of an inducible *GAL* promoter and the cells grown on galactose-containing medium. On shifting the cells to glucose-containing medium the *snc*Δ phenotype becomes apparent after 12 h (Protopopov *et al.*, 1993). We grew *snc*Δ and *snc*Δ *gcs1*Δ cells to stationary phase and examined their growth upon plating onto solid medium at different temperatures. Cells lacking the *SNC* genes alone were both cold- and temperature-sensitive for growth on glucose-containing medium, as previously shown (Protopopov *et al.*, 1993 and our unpublished results), but could grow slowly at 18–35°C. In contrast, cells disrupted in both the *SNC* v-SNARE genes and the *GCS1* Arf-GAP gene were unable to grow on glucose-containing medium at any temperature (Figure 2B). However, both *snc*Δ and *snc*Δ *gcs1*Δ cells were fully viable when maintained on galactose-containing medium (Figure 2B), whereon Snc1 is expressed. Thus, synthetic lethality is observed between the *gcs1*Δ and *snc*Δ mutations and suggests that these gene products provide related functions that allow for an essential transport activity.

Gcs1 Colocalizes with the Snc v-SNAREs

As the Snc v-SNAREs and Gcs1 interact both physically and genetically, we examined whether these proteins colocalize in yeast (Figure 3). We used functional (Figure 2A and our unpublished observations) green and red fluorescent protein (GFP and RFP, respectively) derivatives expressed from low copy plasmids. Both GFP-Snc1 and GFP-Snc2 strongly labeled the yeast plasma membrane and some cytoplasmic structures and weakly labeled vacuolar membranes (Figure 3A). This corresponds well with the pattern of labeling described for GFP-Snc1 (Gurunathan *et al.*, 2000; Lewis *et al.*, 2000). The cytoplasmic structures observed earlier with GFP-Snc1 included the *trans*-Golgi and endosomes (Lewis *et al.*, 2000; Galan *et al.*, 2001). Importantly, these v-SNAREs have been demonstrated to recycle back to the *trans*-Golgi via early endosomes in a manner dependent on the involvement of Rcy1, Ric1, and Ypt6, which mediate endosome–Golgi transport (Lewis *et al.*, 2000; Galan *et al.*, 2001; Lafourcade *et al.*, 2004). Rcy1 is an F-box protein involved in recycling at endosomes (Wiederkehr *et al.*, 2000), whereas Ric1 is part of the GEF complex for the rab-GTPase Ypt6 at the TGN (Siniossoglou *et al.*, 2000). In our colabeling experiments, we found that both GFP-Snc1 and GFP-Snc2 localized with Gcs1-RFP at a subset of large punctate structures present in the cytoplasm, but not at the plasma membrane (Figure 3A). The intracellular localization of Gcs1 was previously unknown, though its role in post-Golgi vesicle biogenesis (Poon *et al.*, 2001) suggested a possible Golgi or endosomal localization. Thus, the internal compartments colabeled by Gcs1-RFP and GFP-Snc1 (or GFP-Snc2) are likely to be late Golgi or endosomal in nature.

To better address the intracellular localization of Gcs1, we examined the location of Gcs1 tagged either at the carboxy terminus with RFP or at the amino terminus with GFP along with markers of other endomembrane compartments or

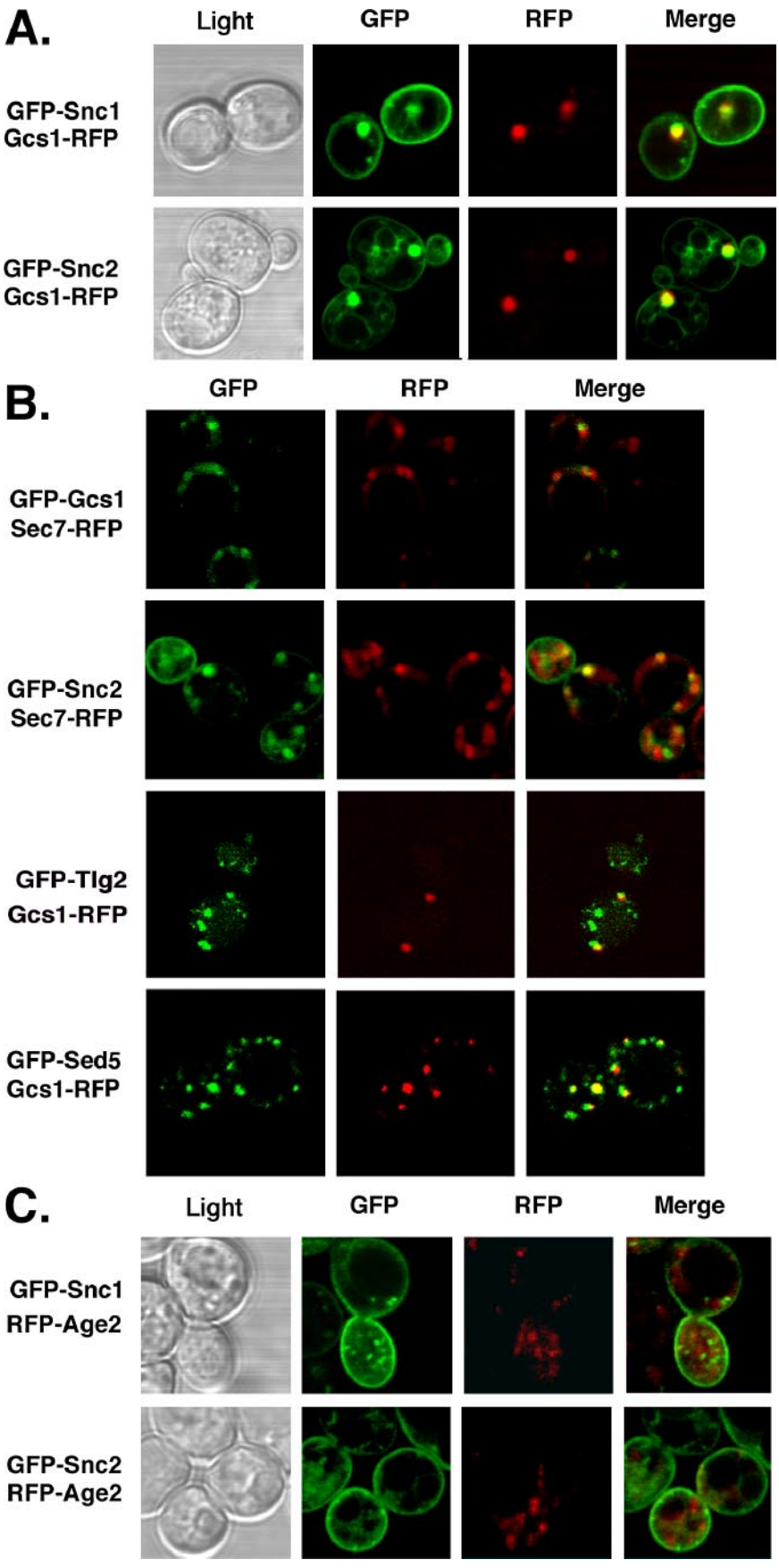

Figure 3. Snc v-SNAREs and Gcs1 colocalize to late Golgi and endosomal compartments. (A) A subset of internalized Snc1,2 v-SNAREs colocalizes with Gcs1. Single-copy plasmids producing Snc1 or Snc2 tagged with GFP at their amino termini (GFP-Snc1; pRS315-GFP-cSNC1 and GFP-Snc2; pRS315-GFP-SNC2) were transformed into wild-type yeast having a single-copy plasmid expressing Gcs1 tagged at its carboxy terminus with RFP (Gcs1-RFP; YCp50-GCS1-DsRedT4). Cells were visualized by confocal microscopy to show excitation at the appropriate wavelengths ("GFP" and "RFP" windows, respectively). "Merge" represents the merger of the windows. (B) Gcs1 colocalizes with Golgi and early endosomal markers. Wild-type cells bearing an integrated copy of Sec7-RFP (SP1-SEC7RFP), a late Golgi marker, were transformed with single-copy plasmids expressing Gcs1 or Snc2 tagged at the amino terminus with GFP (GFP-Gcs1; pRS315-GFP-GCS1 and GFP-Snc2; pRS315-GFP-SNC2, respectively) and examined for fluorescent marker colocalization. Similarly, cells bearing a single-copy plasmid expressing Gcs1-RFP (YCp50-GCS1DsRedT4) were transformed with plasmids expressing GFP-tagged Tlg2 (GFP-Tlg2; pRS315-GFP-TLG2) or Sed5 (GFP-Sed5; pRS315-GFP-SED5) and examined for colocalization. (C) The Snc1,2 v-SNAREs do not colocalize with Age2. Single-copy plasmids expressing GFP-Snc1 (pRS315-GFP-cSNC1) or GFP-Snc2 (pRS315-GFP-SNC2) and Age2 tagged at the amino terminus with RFP (RFP-Age2; YCp50-DsRedT4-AGE2) were transformed into wild-type cells and visualized by confocal microscopy.

structures (Figure 3B). We found that Gcs1 colocalized in part with both Sed5 and Sec7, which are early and late Golgi markers, respectively (Franzusoff *et al.*, 1991; Hardwick and Pelham, 1992). This colocalization was limited to a subset of punctate structures and correlated well with the known functional overlap between Gcs1 and the Glo3 and Age2 Arf-GAPs, which facilitate Golgi–ER and post-Golgi transport, respectively (Poon *et al.*, 1999, 2001). In addition, Gcs1 also colocalized to a subset of compartments labeled by Tlg2 (Figure 3B), a t-SNARE involved in endocytosis and the delivery of proteins to endosomes and the vacuole (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998; Seron *et al.*, 1998). In contrast, Gcs1 did not colocalize with a preautophagosomal marker, Aut7/Apg8 (Kim *et al.*, 2001; our unpublished observations). Together, these results imply that Gcs1 resides in both Golgi and endosomal compartments.

We also found that Snc2 colocalized well with Sec7 (Figure 3B), as predicted (Lewis *et al.*, 2000; Galan *et al.*, 2001). This colocalization was observed at numerous cytoplasmic structures that are thought to correspond to the *trans*-Golgi (Lewis *et al.*, 2000; Galan *et al.*, 2001), but which may include early endosomes. In contrast, there was little to no colocalization between GFP-Snc1 (or GFP-Snc2) and the Age2 Arf-GAP (Figure 3C). This implies that the site of interaction between the Snc v-SNAREs and Gcs1 may be distinct from the intracellular locale governed by Age2. Although we cannot exclude the possibility that fluorescent protein-tagged Gcs1, as well as the tagged organellar markers, do not induce changes in the morphology and distribution of intracellular trafficking compartments, our results are consistent with Gcs1 and Snc v-SNAREs colocalizing at late or post-Golgi (endosomal) structures.

Snc-Gcs1 Interactions Do Not Alter Arf-GAP Activity In Vitro

Because the Snc v-SNAREs and Gcs1 interact and colocalize at an endosomal compartment, we examined the functional consequences of this interaction. First we examined whether Snc2 v-SNARE binding to Gcs1 alters its ability to activate GTP hydrolysis by Arf1. Recombinant His_6-Snc2, lacking the transmembrane domain, was mixed with His_6-tagged Gcs1 in the presence of recombinant myristoylated Arf1 prebound to GTP and subsequent GTP hydrolysis was measured in vitro. It was found that the presence of Snc2 had no effect on GTP hydrolysis by Arf1 (Figure 4A). This suggests that the Snc v-SNAREs do not alter Gcs1-mediated GTP-hydrolysis.

Snc-Gcs1 Interactions Promote Arf1 Binding and Coatomer Recruitment In Vitro

Because the Snc v-SNAREs physically interact with Gcs1 (Figure 1), but do not regulate Gcs1 GAP activity in vitro (Figure 4A), we examined whether Gcs1 modulates the binding of Arf1 to the v-SNARE. Previous work suggested that Arf-GAP-SNARE interactions prime the v-SNARE to bind Arf1 and allow for subsequent coat recruitment (Rein *et al.*, 2002). Specifically, the catalytic interaction of either Glo3 or Gcs1 with the ER–Golgi v-SNAREs (e.g., Bet1, Bos1, and Sec22) resulted in the binding of Arf1ΔN17-Q71L and the acquisition of coat in a nucleotide- and GAP activity–independent manner in vitro. Thus, Arf-GAP-SNARE interactions have been proposed to recruit both SNAREs and coat proteins to the sites of vesicle formation (Rein *et al.*, 2002).

To determine whether Gcs1 fulfills a similar role with the Snc v-SNAREs, we substituted Snc1 or Snc2 for the ER–Golgi v-SNAREs in this in vitro binding assay. First, we examined whether GST-tagged Snc1,2 v-SNAREs could precipitate Gcs1 in vitro (Figure 4B). We found that Snc1-GST and Snc2-GST could precipitate recombinant Gcs1 as well as Sec22-GST (Figure 4B). Next, when used in the in vitro binding assay either GST-Snc1 or Snc1-GST was readily able to recruit purified coatomer in a Gcs1-dependent manner (Figure 4C; lanes 4, 6, 10, and 12). This reaction does not appear to require Arf1, as COPI was recruited in its absence (see lanes 4 and 10, Figure 4C). This property was observed previously with the ER–Golgi SNAREs (Rein *et al.*, 2002). Arf1ΔN17-Q71L binding to Snc1 or to Snc2 (our unpublished observations), however, was dependent on the addition of Gcs1 to the in vitro assay (Figure 4C; lanes 2, 6, 8, and 12). In contrast, GST alone was unable to recruit either Arf1ΔN17-Q71L or coatomer (Figure 4D). Thus, the Snc v-SNAREs interact with both Arf and coatomer, which is suggestive of a post-Golgi role for this complex in vivo.

Synthetic Lethal Interactions with gcs1Δ

To identify factors that facilitate Gcs1-mediated post-Golgi transport, we used a nonbiased screen to select for gene deletions that are synthetically lethal in combination with *gcs1Δ*. By exhaustive screening against a yeast deletion library of nonessential genes, we found that a number of deletions are synthetically lethal in combination with the *gcs1Δ* mutation. These included deletions in genes encoding the other known Arf-GAPs, *GLO3* and *AGE2* (Poon *et al.*, 1999, 2001). In addition, the screen identified numerous genes encoding factors involved in Golgi–endosome transport, including: *TLG2*, *VPS1*, *VPS51*, and *YPT31* (see Table 3). These findings are consistent with a role for Gcs1 in post-Golgi transport.

Snc v-SNARE Overexpression Inhibits the Growth of Certain Coatomer Mutants

Because the Snc v-SNAREs recruit COPI coat components in a Gcs1-dependent manner in vitro (Figure 4C), we examined the significance of this in vivo. Overexpression of the *SNC* genes is known to rescue mutations affecting partner t-SNAREs from the plasma membrane (i.e., *sec9-4* and *sso2-1*) as well as a mutation in a SNARE regulator (*sec1-1*; Couve and Gerst, 1994; Gerst, 1997). We decided to examine the effect of *SNC1* and *SNC2* overexpression in mutants of coatomer (i.e., *sec21-2*, *sec27-1*, and *sec33-1*). Interestingly, the growth of *sec27-1* and *sec33-1* cells, which express mutated components of the clathrinlike B subcomplex of COPI (McMahon and Mills, 2004), was significantly inhibited by the overexpression of either *SNC1* or *SNC2* (Figure 4E). In contrast, the overexpression of either *SNC1* or *SNC2* in *sec21-2* cells, which expresses a mutated component of the adaptor-like F subcomplex of COPI (McMahon and Mills, 2004), had no deleterious effect (Figure 4F). Thus, we could identify genetic interactions between the Snc v-SNAREs and components of the COPI B subcomplex. Because Gcs1 interacts physically with the Snc proteins, we addressed the possibility that overproduction of the v-SNAREs could cause a decrease in the Arf-GAP available for membrane transport. However, an increase in *GCS1* expression did not alleviate the effects of *SNC* overexpression in the COPI B mutants (Figure 4, D and E).

The Deletion of GCS1 Alters GFP-Snc1 Recycling

Because of a functional overlap with Age2, Gcs1 was proposed to play a role in post-Golgi protein sorting (Poon *et al.*, 2001). In addition, the present study shows the colocalization of Gcs1 with Golgi and endosomal markers, as well as physical and genetic interactions with v-SNAREs that facilitate post-Golgi transport. Because GFP-Snc1 recycles through early

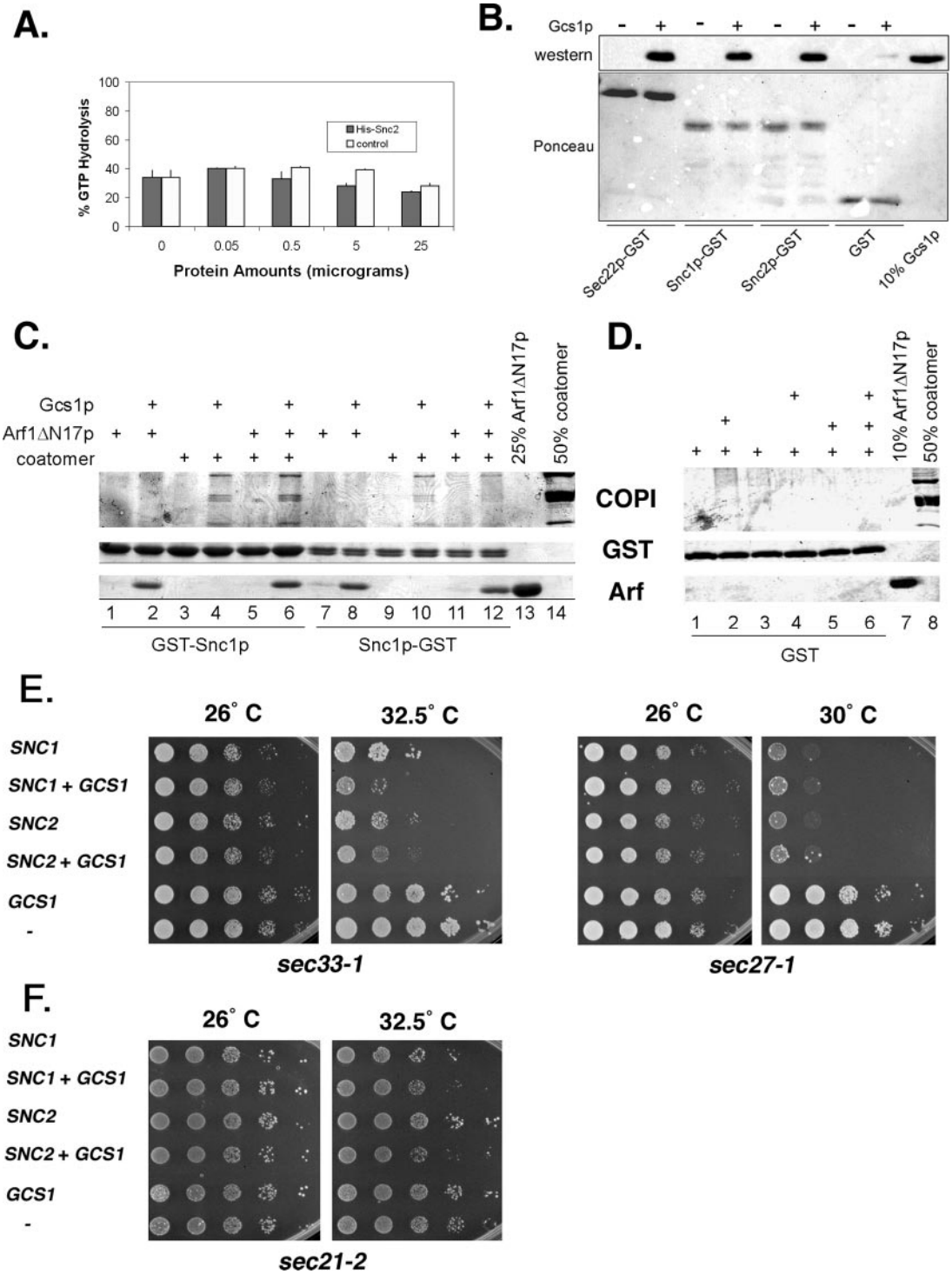

Figure 4. Snc v-SNAREs bind to Arf1ΔN17-Q71L and coatomer in a Gcs1-dependent manner in vitro and interact genetically with specific COPI subunits. (A) Recombinant Snc2 does not alter Gcs1 Arf-GAP activity in vitro. Recombinant His_6-tagged Gcs1 and His_6-tagged Snc2 (His-Snc2) or His_6-tagged Gcs1 alone (control) were mixed with GTP-bound myristoylated Arf1, and Arf-GAP activity was measured in vitro as described (see *Materials and Methods*). (B) Recombinant Gcs1 binds to the Snc v-SNAREs in vitro. Purified recombinant Snc1-GST, Snc2-GST, Sec22-GST, or GST alone (5 μg) were incubated with or without recombinant Gcs1 (20 nM) and a GST-pull down assay was performed. Samples subjected to SDS-PAGE and analyzed in immunoblots with anti-Gcs1 antibodies. Ponceau staining of the nitrocellulose filter after gel transfer is shown as a control for the amounts of GST fusion proteins added. Ten percent of added Gcs1 is shown as a control for loading. (C) Recombinant Snc1 binds to Arf1ΔN17-Q71L and purified coatomer in a Gcs1-dependent manner in vitro. Purified recombinant GST-Snc1 or Snc1-GST (5 μg) were incubated with recombinant Gcs1 (20 nM), recombinant Arf1ΔN17-Q71L (7.3 nM), and purified coatomer (40 nM), alone or in combination (see *Materials and Methods*). Binding was carried out for 1 h at 4°C. Samples were resolved on SDS-PAGE gels and visualized by Fairbanks (Coomassie R) staining. Loadings corresponding to 25% of added Arf1ΔN17-Q71L and 50% of added coatomer are shown in lanes 13 and 14, respectively. (D) GST alone does not bind recombinant Arf1ΔN17-Q71L or purified coatomer. Purified GST was incubated with recombinant Gcs1,

Table 3. Genes that are synthetic lethal with *gcs1*Δ

ORF	Gene name	Role
YIL044c	*AGE2*	Membrane trafficking
YDL192w	*ARF1*	Membrane trafficking
YAL026c	*DRS2*	Membrane trafficking
YKL204w	*EAP1*	Translation inhibition
YER122c	*GLO3*	Membrane trafficking
YOR070c	*GYP1*	Membrane trafficking
YMR224c	*MRE11*	DNA nuclease
YJL117w	*PHO86*	Phosphate transport
YGL167c	*PMR1*	Ion transport
YIL067w	*SEC28*	Membrane trafficking
YDR320c	*SWA2*	Membrane trafficking
YOL018c	*TLG2*	Membrane trafficking
YOR115c	*TRS33*	Membrane trafficking
YER151c	*UBP3*	Ubiquitin protease
YKL080w	*VMA5*	Membrane trafficking
YLR447c	*VMA6*	Membrane trafficking
YKR001c	*VPS1*	Membrane trafficking
YKR020w	*VPS51*	Membrane trafficking
YER031c	*YPT31*	Membrane trafficking
YBR111c	*YSA1*	Nuc. diphos. sugar hydrolase
YEL048c		Unknown
YGL081w		Unknown

endosomes back to the Golgi (Lewis *et al.*, 2000), we examined the trafficking of this v-SNARE in cells lacking the *GCS1* gene (Figure 5A). In addition, we also examined GFP-Snc1 localization in *rcy1*Δ cells, which are defective in early endosome–Golgi sorting (Galan *et al.*, 2001), and *end4-1* cells, which are defective in the endocytosis of endocytic markers such as GFP-Snc1 (Lewis *et al.*, 2000). Unlike in wild-type cells, we found that GFP-Snc1 accumulated in intracellular compartments in both *rcy1*Δ and *gcs1*Δ cells, while being restricted to the plasma membrane in *end4-1* cells (Figure 5A). Thus, proper GFP-Snc1 recycling is largely inhibited in the absence of Gcs1. This result is identical to that shown earlier for *rcy1*Δ and other mutations in proteins that facilitate early endosome–Golgi transport (Galan *et al.*, 2001).

To examine whether GFP-Snc1 reaches the late Golgi in *gcs1*Δ cells we performed a colocalization study with GFP-Snc1 and Sec7-RFP in both wild-type and *gcs1*Δ cells (Figure 5B). We found that Snc1 could not colocalize effectively with Sec7 in the absence of *GCS1*. In contrast, these proteins readily colocalize in wild-type cells (Figures 3B and 5B). Similar results were obtained using another Golgi marker, Yif1 (Matern *et al.*, 2000), that colocalizes in part with Gcs1 (our unpublished observations). We found that GFP-Yif1 could not colocalize with mRFP-Snc1 in the absence of *GCS1* (Figure 5C). In contrast, partial colocalization is observed between mRFP-Snc1 and GFP-Yif1 in wild-type cells (Figure 5C). Thus, the ability of Snc1 to recycle to the *trans*-Golgi (e.g., Sec7 compartment) is dependent on the Gcs1 Arf-GAP. We note that both Golgi markers (Sec7, Yif1) appeared to be more widely distributed and less punctate in *gcs1*Δ cells, indicating a possible alteration in Golgi morphology in the absence of Gcs1 function.

Figure 4 (cont). recombinant Arf1ΔN17-Q71L, and purified coatomer, alone or in combination, as described above. Binding and detection were performed as under *Materials and Methods*. (E) *SNC1* and *SNC2* overexpression inhibits the growth of COPI B subcomplex mutants. *sec27-1* and *sec33-1* cells were transformed with multicopy plasmids expressing *SNC1* (pAD54-cSNC1) or *SNC2* (pAD54-SNC2) and either a control vector (pAD54 or pRS426) or a multicopy plasmid expressing *GCS1* (pPP329). Cells were grown to midlog phase on selective medium, diluted serially, and plated on solid medium at different temperatures. (F) *SNC1* or *SNC2* overexpression do not inhibit the growth of a mutant in the COPI F subcomplex. *sec21-2* cells were transformed with plasmids expressing *SNC1* (pAD54-cSNC1) or *SNC2* (pAD54-SNC2) and either a control vector (pAD54) or a multicopy plasmid expressing *GCS1* (pPP329). Cells were grown to midlog phase on selective medium, diluted serially, plated on solid medium and incubated at different temperatures for 48 h.

GFP-Snc1 Localization Is Altered in COPI B and ESCRT Mutants

As GFP-Snc1 retrieval to the Golgi is blocked in *gcs1*Δ cells (Figure 5) and the *SNC* genes interact genetically with mutations in COPI B (Figure 4E), we tested whether mutants in the COPI B subcomplex play a role in Snc1 recycling. We followed the localization of GFP-Snc1 in COPI B mutants and a variety of other cell types (Figure 6). In wild-type cells, we found that GFP-Snc1 gave typical plasma membrane staining that was slightly bud-enriched (Lewis *et al.*, 2000), a process requiring endocytosis (Valdez-Taubas and Pelham, 2003). Similar results were observed in COPI B mutants (e.g., *sec27-1*, *sec28*Δ) and an ESCRT-I mutant (*vps23*Δ at 26°C; however, the extent of plasma membrane labeling seen on the buds of these mutants was considerably stronger than that observed on the buds of wild-type cells. This bud-enriched pattern of labeling differed greatly from GFP-Snc1 labeling of the entire plasma membrane in endocytosis-deficient *end4-1* cells. In addition, it differed from the extensive pattern of internal GFP-Snc1 labeling seen in a COPI F mutant (*sec21-2*), as well as *sec33-1* cells. This is probably because both *sec21-2* and *sec33-1* mutants are impaired in transport through the early secretory pathway at 26°C (Wuestehube *et al.*, 1996), unlike *sec27-1* and *sec28*Δ cells. The internal pattern of GFP-Snc1 labeling was reminiscent of that seen in *rcy1*Δ mutants, which are defective in Snc1 recycling from early endosomes to the Golgi (Galan *et al.*, 2001). Our results imply that Snc1 v-SNARE retrieval and recycling through early endosomes to the Golgi is also impaired in *sec27-1* and *sec28*Δ cells, resulting in their retargeting to the bud plasma membrane.

*gcs1*Δ *Cells Are Calcofluor-sensitive*

Mutations in both the *GCS1* and *AGE2* Arf-GAP genes lead to defects in post-Golgi transport. In particular, combined *gcs1* and *age2* mutations led to the impaired delivery of internalized Ste3 mating-factor receptor and the vital dye, FM4-64, to the vacuole (Poon *et al.*, 2001). This result suggests that Gcs1 might facilitate protein retrieval from endosomes to other organelles.

Here we show that the deletion of *GCS1* strongly affects the recycling of GFP-Snc1 from early endosomes to the Golgi (Figure 5). Yet, cells lacking *GCS1* tend to grow normally on synthetic medium at temperatures above 15°C (Figure 2A) and have only minor defects in protein trafficking to the cell surface (Poon *et al.*, 1996). This suggests that protein export pathways are not markedly affected by loss of the Gcs1 Arf-GAP, as long as the Age2 Arf-GAP is present. To verify that export from the Snc1 recycling compartment is not abolished, we examined whether *gcs1*Δ cells are resistant to calcofluor, a molecule that binds to chitin and inhibits cell growth. Cells that are resistant to calcofluor have either a mutation in chitin synthase III (Chs3) or are defective in Chs3 export from the chitosome, which is analogous to the early endosome (Valdivia *et al.*, 2002). However, we found that *gcs1*Δ cells are generally sensitive to calcofluor, unlike control *chs6*Δ cells (our unpublished observations). This im-

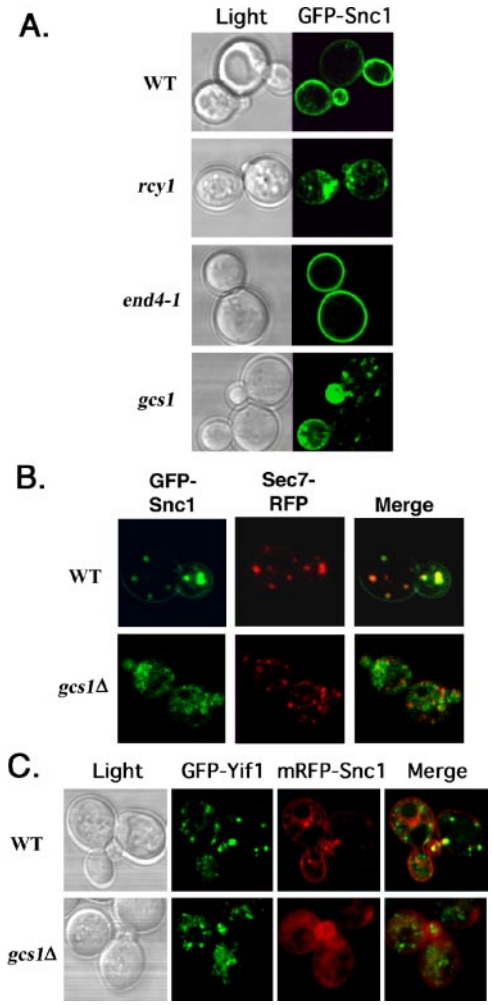

Figure 5. Snc v-SNARE retrieval to the *trans*-Golgi is defective in *gcs1*Δ cells. (A) GFP-Snc1 recycling is defective in *gcs1*Δ cells. Wild-type (SP1), *rcy1*Δ, *gcs1*Δ (MRY2), and *end4-1* cells expressing GFP-Snc1 from a single-copy plasmid (pRS315-GFP-cSNC1) were grown to midlog phase and processed for confocal fluorescence microscopy. (B) GFP-Snc1 is unable to access the Sec7 compartment (e.g., *trans*-Golgi) in the absence of Gcs1. Wild-type (SP1) and *gcs1*Δ (MRY4) cells were transformed with a linearized *SEC7-RFP* integrating plasmid (YIplac204-T/C-SEC7-dsRED.T4) and correctly integrated *SEC7-RFP*–expressing cells were transformed with a single copy plasmid expressing GFP-Snc1 (pRS315-GFP-cSNC1), and examined for fluorescence using confocal microscopy. (C) GFP-Snc1 is unable to access the Yif1 compartment (e.g., Golgi) in the absence of Gcs1. Wild-type (SP1) and *gcs1*Δ (MRY3) cells were transformed with single copy plasmids expressing mRFP-Snc1 (pRS316-mRFP-cSNC1) and GFP-Yif1 (pRS313-GFP-YIF1), and examined for fluorescence using confocal microscopy.

plies that the loss of Gcs1 function does not alter export of Chs3 to the cell surface.

Gcs1 Coimmunoprecipitates with Snx4

Normal cycling of the Snc v-SNAREs requires additional proteins, including the sorting nexins that play a role in retrieval, often as part of multiprotein complexes (Carlton *et al.*, 2005). In particular, the Snx4 sorting nexin is required for the retrieval of GFP-Snc1 from post-Golgi endosomes (Hettema *et al.*, 2003). Because Gcs1 and Snx4 both mediate

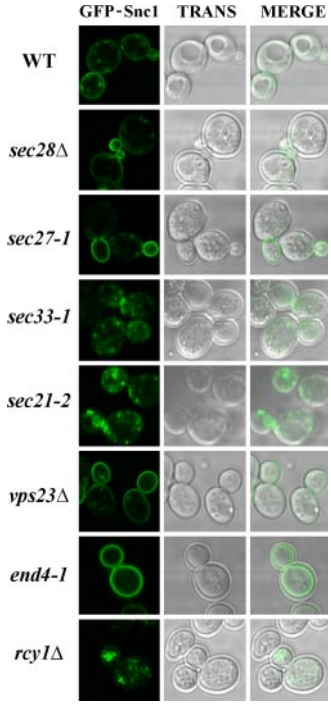

Figure 6. GFP-Snc1 is enriched on the bud plasma membrane in certain COPI mutants and in an ESCRT mutant. Wild-type yeast (W303-1a; WT) and mutants in COPI F (*sec21-2*), COPI B (*sec27-1*, *sec28*Δ, and *sec33-1*), ESCRT (*vps23*Δ), *RCY1* (*rcy1*Δ), and *END4* (*end4-1*) were transformed with a single-copy plasmid expressing GFP-Snc1 (pRS315-GFP-cSNC1) and examined by confocal microscopy.

GFP-Snc1 recycling and physically interact with Snc1 (Hettema *et al.*, 2003 and this study), we determined whether Gcs1 and Snx4 form a complex. We expressed both myc-tagged Snx4 and HA-tagged Gcs1 in wild-type cells and examined whether they can coimmunoprecipitate from cell lysates (Figure 7). We found that Gcs1 coprecipitates with Snx4 in a specific manner. No band corresponding to Gcs1 was detected in precipitates formed in the absence of either myc-Snx4 or HA-Gcs1. In contrast, a control reaction employing the anti-myc antibody to bring down myc-Snc2 demonstrated that it specifically precipitated an HA-tagged protein, in this case the Sso1 t-SNARE (Figure 7). Thus, Gcs1 and Snx4 interact in a specific manner.

DISCUSSION

Coatomer recruitment to Golgi membranes is necessary for formation of the COPI vesicles involved in intra-Golgi and Golgi–ER retrograde transport in yeast and mammals (Kirchhausen, 2000; Spang, 2002; McMahon and Mills, 2004). Despite the established role for coatomer, studies in mammalian cells also describe a post-Golgi role for coatomer and COPI in the transport of proteins to endosomes and multivesicular bodies (Whitney *et al.*, 1995; Aniento *et al.*, 1996; Gu and Gruenberg, 2000; Faure *et al.*, 2004). Coatomer binding in all systems is Arf-dependent and, thus, a mechanism for the recruitment of Arf and Arf-like proteins to different membranes also must depend on specific recruiting factors.

Here we demonstrate that the yeast exo- and endocytic v-SNAREs, Snc1 and Snc2, interact genetically and physically with the Gcs1 Arf-GAP (Figures 1 and 2) and colocalize with Gcs1 to late Golgi and endosomal compartments (Fig-

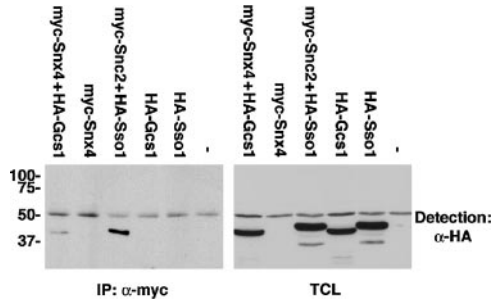

Figure 7. Gcs1 coimmunoprecipitates with Snx4. Wild-type (W303) cells were transformed with plasmids expressing both myc-Snx4 and HA-Gcs1 (pRS426-HA-GCS1 and pAD6-SNX4, respectively), or either myc-Snx4 or HA-Gcs1 alone. Empty vectors (pRS426 or pAD6) were also used as controls. In addition, cells used for a control immunoprecipitation were transformed with plasmids expressing myc-Snc2 and HA-Sso1 (pADH-myc-SNC2 and pRS426-HA-SSO1, respectively) or HA-Sso1 alone. Cells were grown to midlog phase and were processed for immunoprecipitation (see *Materials and Methods*). Immunoprecipitation (IP) was performed with anti-myc antibodies, whereas Western blotting was performed with both anti-myc and anti-HA antibodies. Blots for both the IP reactions and samples (50 μg) of the total cell lysates (TCL) were probed in parallel.

ure 3, A and B). This Arf-GAP has been previously shown to facilitate ER–Golgi and post-Golgi transport (Poon *et al.*, 1999, 2001). Thus, we hypothesize that the Snc v-SNAREs are actively involved in recruiting Gcs1 and, subsequently, Arf1 to these membranes. This idea is supported by in vitro binding data demonstrating the recruitment of coatomer to the Arf-GAP-v-SNARE complex (Figure 4C). One functional consequence of this interaction is retrieval of the Snc v-SNAREs and, perhaps, other cargo proteins to the *trans*-Golgi. Indeed, Snc1 does not reach the *trans*-Golgi, as visualized by Sec7-RFP or GFP-Yif1, in cells lacking *GCS1* (Figure 5, B and C). This role for Gcs1 is similar to that described for Rcy1 and Snx4, which also interact with Snc1 and mediate its retrieval to the *trans*-Golgi (Galan *et al.*, 2001; Hettema *et al.*, 2003; Chen *et al.*, 2005). Consistent with a role for Gcs1 in Snc1,2 recycling, we demonstrate that Gcs1 binds to the Snx4 sorting nexin (Figure 7). This suggests that the v-SNARE recruits a complex involving a sorting nexin, an Arf-GAP, Arf, and a coat for retrieval to the Golgi from endosomal compartments (see model, Figure 8). Thus, in the absence of any of these factors (i.e., Gcs1, Snx4, COPI B, etc.), Snc v-SNARE retrieval to the Golgi is altered.

Other components are also involved in GFP-Snc1 recycling to the Golgi (Lafourcade *et al.*, 2004), including the Ypt31,32 GTPases that facilitate Golgi export (Jedd *et al.*, 1997) and act upstream of Rcy1 (Chen *et al.*, 2005). In earlier work, a correlation between GFP-Snc1 phosphorylation and its presence at the cell surface was demonstrated (Galan *et al.*, 2001). Recently Chen *et al.* (2005) suggested that Ypt31,32 regulate the phosphorylation state of the Snc v-SNAREs, implying that phosphorylation targets these v-SNAREs for recycling (Chen *et al.*, 2005). This method of targeting would seem to be an important mechanism for controlling SNARE recycling and a potential means for facilitating interactions with either Gcs1 or Snx4, for example. However, we have been unable to demonstrate the phosphorylation of either endogenous or epitope-tagged Snc proteins expressed in yeast, either by in vivo labeling or by mobility shift analysis employing alkaline phosphatase treatment using wild-type, *rcy1*Δ, or *end4-1* cells (Couve *et al.*, 1995 and our unpublished

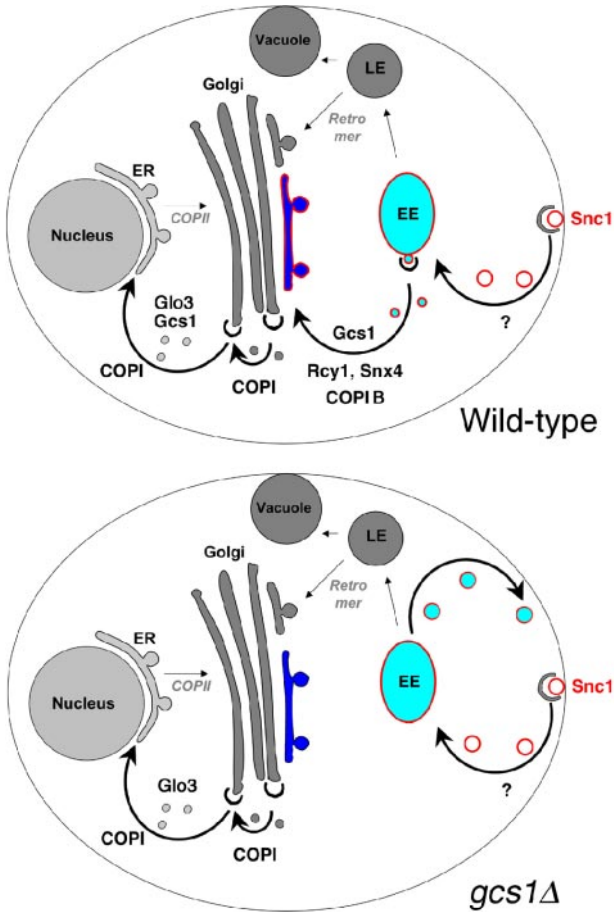

Figure 8. A model for Snc v-SNARE recycling involving Gcs1, Snx4, Rcy1, and COPI. After exocytosis, the Snc1 v-SNARE (designated with red line) undergoes retrieval from the cell surface to the early endosome (EE; light blue fill) and then to the *trans*-Golgi (blue fill). We propose that in wild-type cells, Gcs1 acts at the level of early endosomes to retrieve Snc1 back to the Golgi. This requires other proteins known to mediate Snc1 recycling to the Golgi, including Snx4 and Rcy1. Thus, Snc1 accumulates at the level of early endosomes in the absence of Gcs1 (*gcs1*Δ cells). We also propose that a subset of COPI subunits is involved in this step, either directly or indirectly. COPI is known to mediate retrograde Golgi–ER and intra-Golgi transport in yeast and mammals, as well as late endosome (LE)–multivesicular body transport in mammals. Thus COPI, like clathrin, acts as a coat for multiple trafficking pathways. In the absence of certain COPI B subunits (e.g., sec27-1 cells after temperature-shifting or *sec28*Δ cells), Snc1 is recycled back to the plasma membrane presumably by secretory vesicles derived from endosomal compartments.

observations). In contrast, GFP-Snc1 can clearly be modified into a form whose mobility is altered by phosphatase treatment, as demonstrated by several studies (Galan *et al.*, 2001; Hettema *et al.*, 2003; Chen *et al.*, 2005) as well as by us (our unpublished observations). This finding is consistent with the idea that GFP, and not the v-SNARE, is the phosphorylated substrate observed under these conditions. Thus, the role of phosphorylation as a recycling signal for v-SNAREs warrants further study.

In addition to its well-described role in Golgi–ER transport, the involvement of coatomer has also been demonstrated at the level of protein sorting to endosomes and multivesicular bodies in mammals (Whitney *et al.*, 1995; Aniento *et al.*, 1996; Gu and Gruenberg, 2000; Faure *et al.*,

2004). We show here that certain COPI subunits in yeast may assume this additional role by mediating protein retrieval from endosomes to the Golgi. For example, recombinant Snc v-SNAREs recruit purified coatomer in an in vitro binding assay (Figure 4C). Also, the *SNC* genes interact genetically with those encoding COPI B subunits, but not a COPI F subunit (Figure 4, E and F). Finally, GFP-Snc1 labeling of the plasma membrane of the growing bud is noticeably heightened in certain COPI B mutants, but not in a COPI F mutant (Figure 6). As a similar result was obtained in the ESCRT I mutant, *vps23*Δ (Figure 6), which is likely to be defective in its ability to target GFP-Snc1 for vacuolar degradation, it suggests that Snc1 recycles to the plasma membrane under conditions where trafficking to endosomal compartments is affected (see model, Figure 8). Unlike the *gcs1*Δ mutant, wherein recycling GFP-Snc1 accumulates in early endosomes (Figure 5), the enhanced bud localization observed with *sec27-1* and *sec28*Δ cells suggests an additional role for COPI (but not Gcs1) in sorting to multivesicular bodies (G. Gabriely and J. E. Gerst, our unpublished observations). These results suggest a broad role for COPI in endosome–Golgi transport and warrant further investigation.

Our findings support earlier studies that demonstrate a connection between Arf1 GTPases and v-SNAREs in conferring coat recruitment (Gommel *et al.*, 2001; Rein *et al.*, 2002; Lee *et al.*, 2004). This connection may guarantee incorporation of a v-SNARE into nascent vesicles in order to make them fusion-competent at the appropriate acceptor compartment. The classical view for Arf-GAP function has been that GAP-mediated hydrolysis of GTP on Arf1 leads to vesicle uncoating to allow for subsequent fusion (Tanigawa *et al.*, 1993; Bigay *et al.*, 2003; Reinhard *et al.*, 2003). Yet, other studies support the idea that Arf-GAP activity is required for the packaging of cargo into COPI vesicles (Nickel *et al.*, 1998; Pepperkok *et al.*, 2000; Lanoix *et al.*, 2001; Rein *et al.*, 2002; Lee *et al.*, 2004) and for COPI vesicle biogenesis (Yang *et al.*, 2002; Lee *et al.*, 2004). Our findings neither contradict nor reconcile these differing views, but may suggest additional functions for Gcs1 that are independent of its Arf-GAP activity. This is based on the fact that Snc binding does not involve the Arf-GAP domain of Gcs1 (Figure 1D) nor alters Gcs1 Arf-GAP activity (Figure 4A).

Finally, it has been suggested that Arf-GAPs themselves are coat components (Yang *et al.*, 2002; Lewis *et al.*, 2004). For example, interactions between Arf-GAPs and coat proteins that function in post-Golgi transport, including the GGA and AP3 clathrin adaptor proteins, have been demonstrated (Randazzo and Hirsch, 2004). Arf-GAPs have been shown to interact physically with coat proteins by two-hybrid analysis and coimmunoprecipitation. In particular, Glo3, a yeast Arf-GAP that acts with Gcs1 upon Golgi–ER transport (Poon *et al.*, 1999) binds to coatomer in both in vitro and two-hybrid assays (Eugster *et al.*, 2000; Lewis *et al.*, 2004). Although Gcs1 is not readily detected in COPI vesicles (Lewis *et al.*, 2004), this Arf-GAP may play a role in post-Golgi transport along with members of the COPI coat, as suggested here for the COPI B subcomplex. The possibility exists that multiple subpopulations of COPI occur in yeast, as was recently shown for mammalian cells (Malsam *et al.*, 2005). These different coats may define distinct sorting routes (i.e., Golgi–ER, intra-Golgi, early endosome–Golgi, and late endosome-MVB).

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5.3 Arf1p, Chs5p and the ChAPs are required for export of specialized cargo from the Golgi

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The *trans*-Golgi network (TGN) is the main sorting station for anterograde cargo in a cell. Most of the major routes from the TGN have been described in detail. However, there are minor routes that are not fully understood yet or even await discovery. The enzyme chitin synthase III (Chs3p), which is involved in the synthesis of the cell wall of budding yeast, might be a model cargo for such a trafficking route. Chs3p is secreted at the mother-bud junction during the G1 phase of the cell cycle, while for the rest of the cell cycle it is stored in internal stores termed chitosomes. Defects in Chs3p trafficking can be easily monitored by testing the sensitivity of yeast cells to the chitin-binding dye calcofluor white. Calcofluor is toxic for cells containing chitin in their cell wall. Thus, cells in which Chs3p transport is impaired are resistant to calcofluor white. Several proteins like Arf1p, Chs5p and Chs6p have been implicated in the traffic of Chs3p. Yeast strains in which either *CHS5* or *CHS6* is deleted are resistant to calcofluor. These strains become sensitive again when *ARF1* is deleted on top. These data reported by Valdivia *et al.* (2003) establish a genetic interaction between *CHS5*, *CHS6* and *ARF1*.

In the attempt to identify new interactors of Arf1p we used differential affinity chromatography with Arf1p-GTP. Two proteins specifically interacting with Arf1p-GTP were of special interest, Chs5p and an unknown ORF Ymr237w. Database research showed that Ymr237w and Chs6p belong to a fungi-specific protein family, which we termed the ChAP family (for Chs5 and Arf1p interacting proteins). This family has two additional members: Bud7p, which has been implicated in bud-site selection in diploid yeast, and one more unknown ORF Ykr027w. We termed Ymr237w *BCH1* and Ykr027w *BCH2* (for Bud7p, Chs6p homolog). Deletion of single members of the ChAPs family revealed individual phenotypes: calcofluor-resistance and temperature-sensitive growth for *Δchs6*, bud-site-selection defects and fast growth on high pH media for *Δbud7*, and impaired growth on plates containing high levels of ammonium for *Δbch1*. Interestingly,

a *Δchs5* strain displayed all the phenotypes of the single deletions of the ChAPs. This indicates that Chs5p either acts upstream of the ChAPs or functions as a downstream converging point of ChAP function. Immunoprecipitation experiments demonstrated that all ChAP proteins are found in complexes that also contain Arf1p. To probe a direct interaction between the ChAPs and Arf1p we used a liposome flotation assay. Using this assay we established Bch2p and Chs6p as direct binding partners for Arf1p. Next, we wanted to test whether other ChAP proteins besides Chs6p were also involved in the trafficking of Chs3p. We determined the calcofluor resistance of double deletions of ChAP genes in various combinations. A double deletion of *BCH1* and *BUD7* was also calcofluor-resistant. When *ARF1* was additionally deleted in the *Δbch1Δbud7* strain, the resulting strain became calcofluor-sensitive again. This result indicated that Bch1p and Bud7p are also involved in Chs3p transport. As three of the four ChAP proteins were implicated in Chs3p transport, we hypothesized that the ChAPs might be able to form complexes in order to achieve Chs3p transport. We generated a strain in which all four ChAP proteins were differently tagged and used lysates of this strain in co-immunoprecipitation experiments. Together with results from native PAGE gel electrophoresis, the co-immunoprecipitation experiments allowed us to establish that the ChAP proteins were able to form multimeric complexes with variable stoichiometries. These complexes might form at the TGN because all ChAP proteins at least partially overlapped with the TGN marker Sec7p. Brefeldin A (BFA) is a fungal metabolite, which inhibits the activation and thus stable membrane-localization of Arf1p. Upon BFA treatment, the TGN-localization of not only Arf1p, but also of Chs5p and the members of the ChAP family was lost. From this finding we concluded that Chs5p and the ChAP localization to the TGN is dependent on Arf1p.

To gain further insight in ChAPs function, we tested whether small deletions within the ChAP genes phenocopied the entire deletion of the corresponding gene. Interestingly, we found that the deletion of the 13-14 most C-terminal residues fulfilled this requirement. Further tests showed that the C-terminus is not only required for the interaction with Chs5p but is also essential to localize the ChAPs to the TGN. These truncated proteins allowed us to demonstrate that the ChAPs interact with Chs5p independently from Arf1p binding and that the interaction of the ChAPs with Chs5p requires the C-terminus of the ChAP proteins. We next wanted to assess the requirements of complex formation and tested whether the TGN localization of one ChAP protein was altered when either the three remaining ChAPs or Chs5p were absent. Localization of the ChAPs at the TGN

was severely affected in a *Δchs5* strain. Moreover, the expression levels of the ChAPs changed dramatically indicating a regulatory function for Chs5p. In contrast to that, the localization of a single ChAP protein was only slightly dependent on the other three family members. The localization of Chs5p at the TGN was not changed when all four ChAP proteins were absent. These findings indicate that Chs5p localized to the TGN is independent of the ChAP proteins. The formation of ChAP complexes takes place on membranes and requires Chs5p. To test whether the ChAP proteins interact with the cargo protein Chs3p, we used crosslinking in combination with co-immunoprecipitation. All ChAPs interacted with Chs3p and this interaction was dependent on presence of Chs5p.

Based on our findings, we suggest that the ChAP proteins are necessary for the transport of certain cargo in specialized transport vesicles. The ChAP proteins might function as cargo receptors, coat adaptors or even as novel coat.

Two reports recently published by the Schekman lab corroborate our findings (Sanchatjate and Schekman, 2006; Wang *et al.*, 2006). Using the TAP purification method, they were also able to establish the interaction between the ChAP proteins. Furthermore, they confirmed the existence of high molecular weight complexes containing the ChAP proteins and the cargo Chs3p. Similar to our results, they found that Chs5p is crucial for the formation of these complexes. Furthermore, they were able to purify a recombinant ChAP complex from insect cells and showed that this complex bound synthetic liposomes dependent on activated Arf1p.

Own contribution

My task was to prove the direct interaction between the ChAP family members and Arf1p. Therefore, I expressed the proteins in *E. coli* and established a purification protocol. Furthermore, I established the liposome flotation experiment which enabled us to show a direct interaction for Bch2p and Chs6p with activated Arf1p.

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Arf1p, Chs5p and the ChAPs are required for export of specialized cargo from the Golgi

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In *Saccharomyces cerevisiae,* the synthesis of chitin is temporally and spatially regulated through the transport of Chs3p (chitin synthase III) to the plasma membrane in the bud neck region. Traffic of Chs3p from the *trans*-Golgi network (TGN)/early endosome to the plasma membrane requires the function of Chs5p and Chs6p. Chs6p belongs to a family of four proteins that we have named ChAPs for Chs5p-Arf1p-binding Proteins. Here, we show that all ChAPs physically interact not only with Chs5p but also with the small GTPase Arf1p. A short sequence at the C-terminus of the ChAPs is required for protein function and the ability to bind to Chs5p. Simultaneous disruption of two members, Δ*bud7* and Δ*bch1*, phenocopies a Δ*chs6* or Δ*chs5* deletion with respect to Chs3p transport. Moreover, the ChAPs interact with each other and can form complexes. In addition, they are all at least partially localized to the TGN in a Chs5p-dependent manner. Most importantly, several ChAPs can interact physically with Chs3p. We propose that the ChAPs facilitate export of cargo out of the Golgi.

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Introduction

The *trans*-Golgi network (TGN) is the central sorting station in the cell (Gu *et al*, 2001). From the TGN, proteins are distributed to different compartments such as the lysosomes, endosomes or plasma membrane. To reach their specific destinations, cargo proteins interact with receptors that facilitate their inclusion into the appropriate transport vesicle. After cargo delivery, these cargo receptors are retrieved from the acceptor compartment to allow for another round of transport. The cargo receptors recognize distinct coat proteins, and might even help to recruit them or stabilize them on the membrane (Le Borgne *et al*, 1996). Multiple types of vesicles have been identified that transport cargo from the TGN to different organelles. The AP-1 complex and clathrin facilitate transport between the TGN and the early endosomes. AP-3 recruits clathrin to the TGN to produce vesicles targeted to the lysosome (or the vacuole in *Saccharomyces cerevisiae*). Finally, the clathrin-binding GGAs (Golgi-localized, Gamma-ear-containing, Arf-binding) are responsible for the exchange of proteins and lipids between the TGN and the late endosomes. Although these different pathways have been studied extensively, it is likely that there are other, as yet undefined vesicles transporting cargo to other specialized regions of the cell. For example, the identity of the coat protein essential for transport from the late endosome or from the TGN directly to the plasma membrane remains elusive. Although the FAPPs (4-phosphate adaptor proteins) have been recently implicated in post-Golgi carrier formation (Godi *et al*, 2004), they were not found on transport intermediates after fission. In addition, at least in *S. cerevisiae*, the major location of secretion changes during the cell cycle: normally, secretion occurs at the bud tip, which is the region where the bud grows; however, at the end of mitosis, secretion switches to the mother-bud junction. Although the exocyst and other factors necessary for consumption of vesicles at the plasma membrane also relocate from the bud tip to the mother-bud junction, it remains unclear whether the vesicles directed to the bud tip and into the bud neck carry the same coat. Furthermore, the number of cargo receptors identified at the TGN is clearly insufficient to distribute all the different cargo proteins to their various locations. In recent years, it has become clear that the vesicles directed to the mother-bud junction late in the cell cycle must carry specific cargoes, whereas soluble cargo like the yeast pheromone α-factor might be excluded from these vesicles.

One of the best-defined cargoes that cycle between the plasma membrane and internal stores/endosomes in a cell cycle-dependent manner is chitin synthase III, Chs3p. This enzyme serves as a valuable model to investigate regulated vesicular traffic in yeast (Valdivia and Schekman, 2003). Chs3p is polarized at the incipient bud site and the neck region between the mother and the small daughter. When the daughter reaches approximately the same size as the mother, Chs3p is internalized and sequestered into specialized endosomes called chitosomes (Ziman *et al*, 1998). Transport of Chs3p to the plasma membrane requires the function of two proteins called Chs5p and Chs6p. Chs5p is a peripheral late Golgi protein that colocalizes with the *trans*-Golgi marker Kex2p (Santos and Snyder, 1997). Chs6p is a cytosolic protein of unknown function (Ziman *et al*, 1998) in the transport of Chs3p to the plasma membrane. Mutants in *CHS6* are resistant to calcofluor, which is characteristic of factors involved in chitin biosynthesis (Ziman *et al*, 1998). Furthermore, Chs3p is trapped in internal compartments in *chs6* mutants. AP-1- and clathrin-coated vesicles (CCVs) have been impli-

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cated in retrieval of Chs3p from the early endosomes to the TGN (Valdivia *et al*, 2002). In yeast and mammals, the formation of CCVs that contain AP-1 is dependent on the small GTPase Arf1p (Le Borgne *et al*, 1996; Chen and Graham, 1998; Gaynor *et al*, 1998; Kirchhausen, 2000; Yahara *et al*, 2001). The interaction between AP-1 and the activated form of Arf1p is stabilized by cargo proteins that retain AP-1 at the membrane, leading to the assembly of the clathrin lattice and the formation of CCVs. Valdivia *et al* (2002) reported that *ARF1* interacts genetically with *CHS5* and *CHS6*, suggesting a link between ARF1 function and Chs3p traffic. However, Arf1p function was assumed to be connected to the formation of CCVs at the early endosomes. We wanted to explore the possibility that Arf1p and Chs5p and Chs6p might be more closely linked than was apparent from these data and to ask whether Chs5p and Chs6p might play a direct role in the formation of specialized vesicles at the TGN, which contain Chs3p as cargo.

In this report, we show that indeed Chs5p and Chs6p interact biochemically with Arf1p. Furthermore, we discovered that Chs6p is part of a family with three additional members: Bud7p, Ymr237p and Ykr027p, and we named this family ChAPs (Chs5p-Arf1p-binding Proteins). Although mutants in *BUD7* display a bud-site selection phenotype, for none of these proteins had a cellular role been established (Zahner *et al*, 1996; Ni and Snyder, 2001). Surprisingly, all ChAPs bind to Arf1p. Furthermore, double deletion of *BUD7* and *BCH1* results in the same phenotype as deletion of *CHS6* with respect to calcofluor resistance and Chs3p trafficking. The different ChAPs interact with each other and partially localize to the TGN, dependent on the presence of Chs5p. Moreover, the ChAPs interact directly with Arf1p, and most importantly, the ChAPs can be crosslinked to Chs3p, indicating a direct requirement of the ChAPs in export of certain cargo from the Golgi.

Results

Chs6p is part of an ancient eukaryotic family

Chs5p and Chs6p are involved in the traffic of Chs3p to the plasma membrane (Ziman *et al*, 1998; Valdivia *et al*, 2002). Yet, the role of Chs5p and Chs6p in this process is ill defined. We were wondering whether homologs of Chs5p or Chs6p with similar function might exist, which would give us a hint about the function of these proteins. We therefore searched the database for homologs of Chs5p and Chs6p in *S. cerevisiae*. Although there were no clear homologs of Chs5p, we found three homologs for Chs6p: Bud7p, and the uncharacterized proteins Ymr237w and Ykr027w. We named Ymr237w and Ykr027w, Bch1p and Bch2p, respectively, for Bud7p and Chs6p homologs 1 and 2. The entire family was referred to as ChAPs.

Bud7p was identified as a mutant in the bud-site selection process (Zahner *et al*, 1996). Homozygous diploid deletions in *BUD7* result in a random budding pattern, but the role of Bud7p in this process is unknown. ChAPs were found to be present also in other fungi, like zygomycetes, basidiomycetes and nonbudding ascomycetes, and bioinformatic analysis revealed that Bch1p is most likely the most ancient member of this protein family (Supplementary Figure S1). A gene duplication event founded the Chs6p subbranch of the family. The duplication of the genome during development of the genus *Saccharomyces* resulted finally in Bud7p and Bch2p as closest homologs of Bch1p and Chs6p, respectively (Supplementary Figure S3; Wolfe and Shields, 1997). Therefore, it is not surprising that all members of this family are located on different chromosomes. *BCH1*, *CHS6*, *BUD7* and *BCH2* are located on chromosomes XIII, X, XV and XI, respectively. Outside of fungi, Bch1p was also identified in mycetozoa, red algae and ciliates, but not in the various completely sequenced genomes of metazoa and viridiplanta. Thus, the ChAPs represent an ancient eukaryotic protein family that ramified during development of ascomycotic yeasts. But what is the role of these proteins?

The ChAPs are involved in different processes

To study the ChAPs in a systematic manner, we created single deletions. The single deletion strains obtained were assayed for growth at various temperatures and on different nutrient sources. From all the ChAP deletion strains tested, only the Δ*chs6* strain was temperature-sensitive at 37°C and resistant to calcofluor (Figures 1A and 3A; Valdivia *et al*, 2002). Calcofluor is a toxic dye, which binds to chitin and thereby poisons yeasts containing chitin in their cell walls. Resistance to calcofluor is used as a diagnostic tool for Chs3p mislocalization. Remarkably, calcofluor-sensitivity was restored upon deletion of *ARF1* in a Δ*chs6* strain (Figure 3A; Valdivia *et al*, 2002).

In contrast to the deletion phenotype of *CHS6*, a Δ*bch1* strain grew slowly at 23°C and was highly sensitive to growth on YMP+ plates (Figure 1A and B). YMP+ is a rich medium containing elevated levels of ammonium. Deletion of any of the other three members of the family was no more sensitive towards YMP+ than the wild type. For a Δ*bch2* strain, we did not observe any obvious defect under any of a large number of different growth conditions. As reported before, a homozygous diploid Δ*bud7* strain displayed a random budding pattern (Figure 1C). The bipolar budding pattern in Δ*bch1* and Δ*bch2* homozygous diploid deletions was indistinguishable from wild type. To assess the budding pattern of Δ*chs5* and Δ*chs6* homozygous diploid strains, which do not stain with calcofluor, we used concanavalin A coupled to FITC for the visualization of bud scars in Δ*chs5* and Δ*chs6* cells (Figure 1D). As reported before, deletion of *CHS5* resulted in a random budding pattern (Santos *et al*, 1997). In contrast, no defect in bud-site selection was observed in Δ*chs6* cells. We also observed another previously undescribed phenotype for a Δ*bud7* deletion, which was the fast growth on plates buffered at pH 7.5, demonstrating also a role for Bud7p in haploid cells (Figure 1E). The fact that phenotypes observed for specific mutants were only associated with one member of the ChAPs indicates that the individual family members serve different functions in the cell and are not entirely redundant. However, they might still act in the same process.

Strikingly, however, the Δ*chs5* deletion strain exhibited all phenotypes observed for the deletion of different members of the ChAPs (Figures 1 and 3A), namely resistance to calcofluor, temperature- and cold-sensitivity, YMP+ sensitive growth, fast growth on plates at pH 7.5 and a random budding pattern in diploid strains. These data suggest that Chs5p acts in the same pathway either as an upstream regulator or a downstream convergence point in ChAPs family-related functions.

Chs5p and Bch1p interact with the activated form of Arf1p

Given the genetic interaction between *CHS5*, *CHS6* and *ARF1* (Figure 3A; Valdivia *et al*, 2002), we asked whether they also interact biochemically. Cytosolic extracts from a wild-type yeast strain were passed over Arf1p affinity columns preloaded with GTP or GDP, respectively (Trautwein *et al*, 2004). A conformational change was provoked on the Arf1 proteins by spontaneous nucleotide exchange to elute specific interactors. To test for the specificity of the affinity matrix, we assayed for the binding of known interactors of Arf1p. As expected from their biological functions, the coatomer complex and the ARF-GAP Glo3p bound preferentially to Arf1p-GTP, whereas the ARF-GEF Gea2p bound only to Arf1p-GDP (Figure 2A). This approach has been also used successfully to identify Pab1p as an Arf1p-binding protein, which uses COPI-coated vesicles for mRNA transport to the ER (Trautwein *et al*, 2004). We found that Chs5p bound specifically to the activated form of Arf1p (Figure 2B). We also identified Arf1p-GTP-associated proteins by mass spectrometry. Out of 10 protein bands analyzed, seven corresponded to already known interactors of Arf1p, thus again validating the approach and an eighth protein band corresponded to Chs5p, confirming the immunoblot assay. Interestingly, another band was identified as Bch1p, demonstrating that at least one of the ChAPs can associate, directly or indirectly, with Arf1p.

To test whether the other ChAPs can also interact with Arf1p, we performed co-immunoprecipitations with α-Arf1p antibodies on lysates from strains where the ChAPs were appended chromosomally with a 9myc-tag. The proteins were expressed from their endogenous promotor and were fully functional. A signal for Bch1p-9myc, Bud7p-9myc, Chs6p-9myc and Bch2p-9myc was obtained in the precipitate (Figures 2C and 7A), indicating that all ChAPs can associate with Arf1p. This interaction seemed to be specific because another small GTPase, Sar1p, was unable to precipitate any of the ChAPs (Figure 2C and data not shown). However, the interaction between Arf1p and the ChAPs might be indirect. To clarify this point, we performed a liposome binding experiment. One of the ChAPs, Bch2p, was incubated with activated Arf1p and liposomes. The association of Bch2p with Arf1p and the liposomes was assessed by flotation. Bch2p binding to liposomes was increased in the presence of Arf1p (Figure 2D). The increase in binding occurred to a similar extent as in the positive control, when coatomer was present, indicating a specific Arf1p-dependent recruitment of Bch2p to liposomes. A similar result was obtained for Chs6p (data not shown). These data demonstrate that at least Bch2p and Chs6p can interact directly with Arf1p.

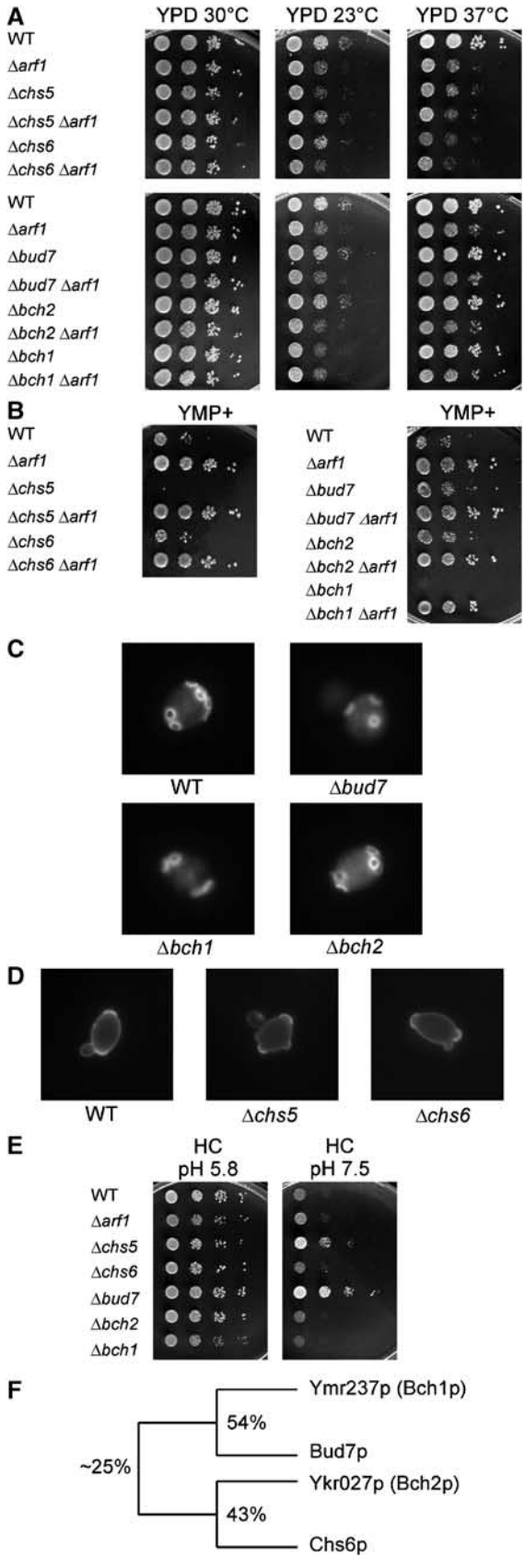

Figure 1 Analysis of single deletion mutants of the ChAPs together with mutants in *CHS5* and *ARF1*. For the drop assays, haploid strains were grown overnight to logarithmic phase in rich medium. Serial dilutions (1:10) were dropped onto plates and incubated for 2 days at 30°C, unless indicated otherwise. (**A**) Growth at different temperatures. (**B**) Growth on YMP+ plates, which contain an elevated level of ammonium sulfate. (**C**) Analysis of the budding pattern. Diploid yeast strains were grown to logarithmic phase and stained with calcofluor. (**D**) Analysis of the budding pattern of mutants in *CHS5* and *CHS6*. Diploid yeast strains were grown to logarithmic phase in YPD + 0.7 M sorbitol. After fixation, cells were stained with FITC–concanavalin A. (**E**) Growth on minimal medium plates buffered at pH 5.8 and 7.5. (**F**) Scheme depicting the relationship of the members of the ChAP family. The percentage of protein sequence identity is given.

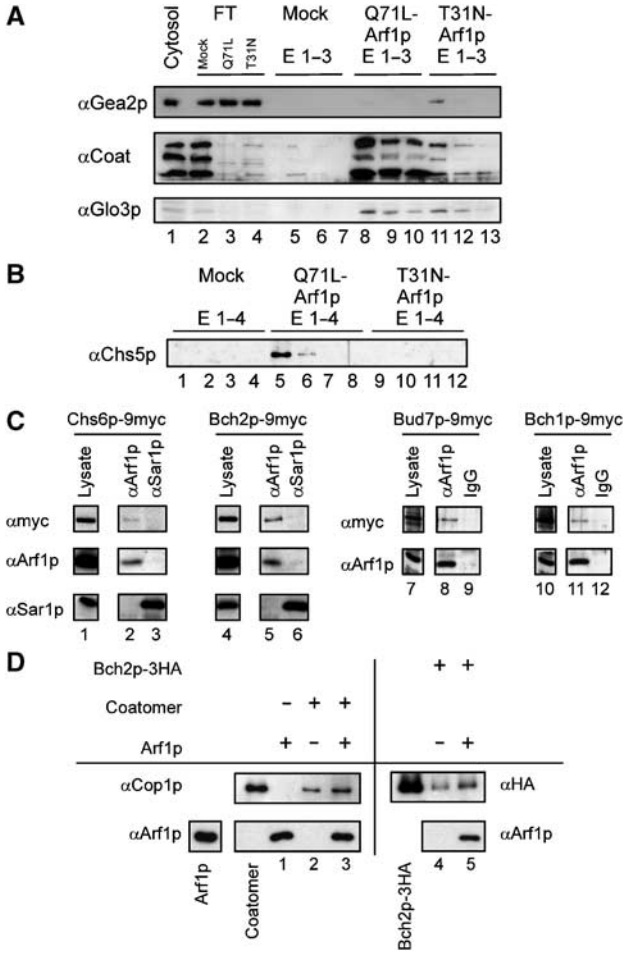

Figure 2 Chs5p binds to activated Arf1p. (**A**) Differential Arf1p affinity chromatography to evaluate binding to Arf1p. Yeast cytosol was incubated with either Arf1p-Q71L (Arf1p-GTP) or Arf1p-T31N (Arf1p-GDP) column material. After washing, spontaneous nucleotide exchange was elicited resulting in a conformational change on Arf1p and the release of conformation-specific bound proteins. The eluates (E1–E3) from an affinity chromatography experiment were analyzed by immunoblot. FT is the flow-through of unbound protein. Beads without Arf1p were mock-treated and served as negative control. The Arf1p-GEF Gea2p is enriched in the Arf1p-GDP column, whereas both the coatomer complex (three subunits are shown) and the Arf1p-GAP Glo3p bind predominately to Arf1p-GTP. (**B**) Chs5p binds to activated Arf1p. Eluates (E1–E4) from the Arf1p affinity chromatography were analyzed for the presence of Chs5p by immunoblot. (**C**) The ChAPs interact with Arf1p. Co-immunoprecipitation experiments were performed using strains in which the ChAPs were chromosomally tagged with 9myc. The lysates were treated with affinity-purified α-Arf1p-IgGs or α-Arf1p and α-Sar1p sera and Protein A–Sepharose. The precipitate was analyzed by immunoblot with antibodies directed against the myc epitope, Sar1p and Arf1p. Lanes 1 and 4 and lanes 7 and 10 represent 1.3 and 1.7% of the lysate, respectively. The lysates and corresponding precipitates were from the same immunoblot. Image processing was identical for lysate and corresponding precipitates. (**D**) Bch2p interacts directly with Arf1p. Liposomes were incubated with Bch2p in the presence or absence of Arf1p-GTP. The liposomes were floated through a sucrose cushion and the top layer was analyzed by immunoblot against Arf1p and Bch2p. The flotation of coatomer served as control and Cop1p was detected by immunoblot. In all, 10% of the input of Arf1p, coatomer and Bch2p were loaded for comparison.

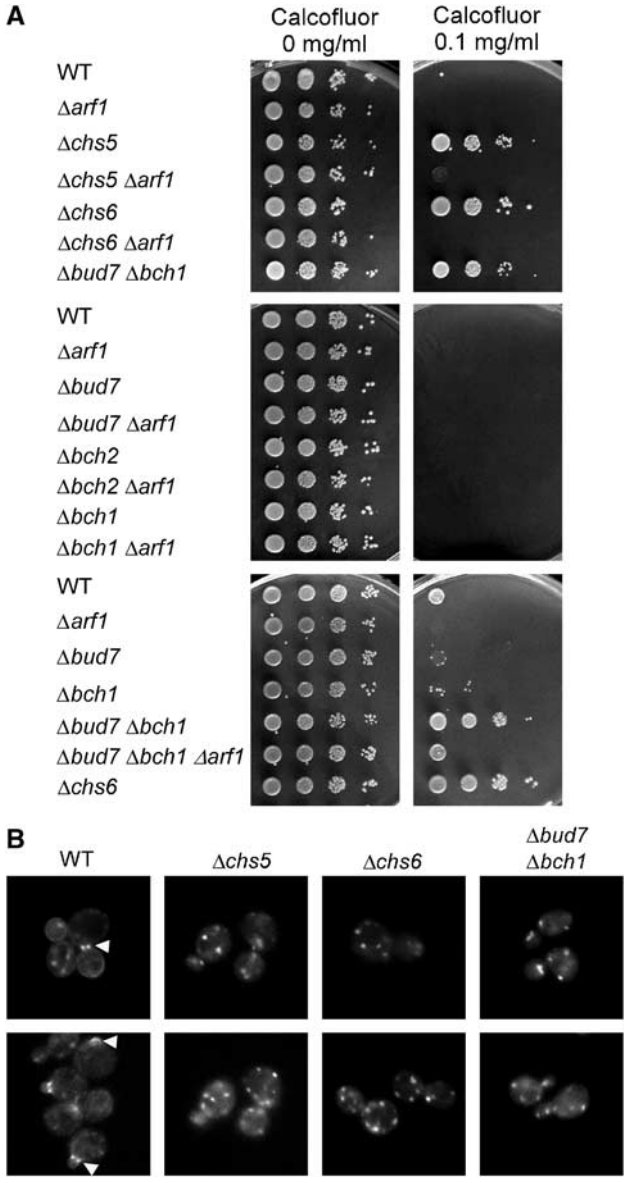

Figure 3 Chs5p and members of the ChAPs are involved in transport of chitin synthase Chs3p to the plasma membrane. (**A**) Haploid strains were grown to logarithmic phase in rich medium. Serial dilutions (1:10) were dropped onto plates containing the toxic dye calcofluor and incubated for 2 days. (**B**) Haploid yeast cells bearing a chromosomal *CHS3-GFP* were grown to logarithmic phase. Arrowheads point to the signal at the bud neck and the incipient bud site. Pictures were taken from freshly mounted cells and two images per strain are shown.

Δbud7Δbch1 display the same phenotype as Δchs6

The ChAPs seemed to be linked to both Chs5p and Arf1p. The only established role for a ChAP in conjunction with Chs5p and Arf1p is the transport of Chs3p, which requires Chs6p. Our results raise the possibility that Chs5p acts not only together with Chs6p but also together with the other three family members to export Chs3p from the TGN. Alternatively, Chs5p may play different roles in conjunction with various members of the ChAPs.

To test the possibility that ChAPs interact with Chs5p to export Chs3p from the Golgi, we generated deletions of the ChAPs in various combinations. These deletion strains were tested on plates containing calcofluor. As described above, Δ*chs5* and Δ*chs6* strains were resistant to calcofluor and became sensitive again when *ARF1* was disrupted (Figure 3A; Valdivia *et al*, 2002). However, more importantly,

a double deletion of *BUD7* and *BCH1* resulted also in calcofluor resistance (Figure 3A). To test whether the calcofluor resistance of a Δ*bud7*Δ*bch1* strain was owing to a defect in transport of Chs3p to the plasma membrane, we created a functional Chs3p-GFP, which is expressed from the *CHS3* chromosomal location under the endogenous promotor. In WT cells, we observed a GFP signal primarily at the bud neck and the incipient bud site and also sometimes in structures resembling the TGN as well as a more dispersed signal, which probably corresponds to chitosomes (Figure 3B). This localization is somewhat different from the localization for Chs3p-GFP described by Valdivia *et al* (2002). However, they coexpressed Chs7p, an ER-resident export factor for Chs3p and Chs3p-GFP from plasmids, whereas we are observing the endogenous protein only. As expected, in Δ*chs5* and in Δ*chs6* cells, the GFP signal was exclusively restricted to structures corresponding to the TGN/chitosomes and almost never (<1%) observed in the bud neck (Figure 3B; Valdivia *et al*, 2002). Similarly, in a Δ*bud7*Δ*bch1* strain Chs3p-GFP was present only in TGN/chitosomal structures (Figure 3B).

To confirm that the calcofluor resistance of a Δ*bud7*Δ*bch1* strain was owing to reduced chitin levels at the plasma membrane, we stained the deletion strains with calcofluor. In both Δ*chs5* and Δ*chs6* cells, the cell wall was stained very poorly. This poor staining was rescued by an additional *ARF1* deletion, because Chs3p reaches the plasma membrane by an alternative pathway (Valdivia *et al*, 2002, and data not shown). In case of the Δ*bud7*Δ*bch1* double deletion, only weak staining of the cell wall was visible (data not shown). Taken together, these observations indicate that in the Δ*bud7*Δ*bch1* strain, Chs3p transport to the plasma membrane was disturbed. The calcofluor-sensitivity was restored in the Δ*bud7*Δ*bch1*Δ*arf1* strain, indicating that Bud7p and Bch1p might be involved in the same pathway as Chs6p (Figure 3A). Moreover, the results strongly suggest that not only Chs6p but also other members of the ChAPs are required for efficient Chs3p transport from the TGN to the plasma membrane.

The ChAPs interact with each other

As more than one ChAP member is necessary for Chs3p transport to the plasma membrane, this raised the possibility that the ChAPs interact with each other. We constructed a quadruply tagged strain to be able to detect all ChAPs in the same strain and performed co-immunoprecipitation experiments.

Chs6p was fused to GFP, Bud7p was appended with 9myc, Bch1p carried a double AU5 epitope and Bch2p was tagged with 3HA. The resulting strain behaved like the corresponding wild-type strain, indicating that all chromosomal fusions were functional and expressed at endogenous levels. When polyclonal AU5 antibodies were used for precipitation, all the three other ChAPs were detected (Figure 4). Co-precipitation was specific, because no ChAPs were detected from control strains lacking the AU5 tag. A similar result was obtained for Bud7p-9myc that was precipitated with monoclonal myc antibodies. Again, Bch2p, Chs6p and Bch1p were detected when Bud7p-9myc was precipitated and were absent when control lysates lacking the myc epitope were used. Similar results were obtained for co-immunoprecipitations with monoclonal HA antibodies and polyclonal GFP antibodies (data not shown).

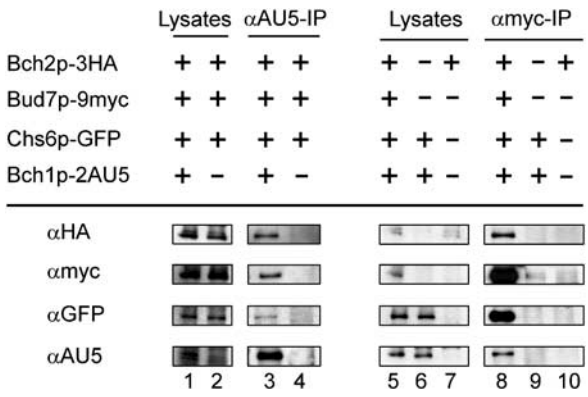

Figure 4 The ChAPs interact with each other. A quadruply chromosomally tagged strain along with control strains were subjected to immunoprecipitation. The precipitates were analyzed by SDS–PAGE and immunoblot with antibodies directed against all four different epitope tags. Precipitations with α-AU5 and with α-myc-IgGs are shown. In all, 1.7% of the lysates were loaded in lanes 1, 2 and 5–7. The lysates and corresponding precipitates were from the same immunoblots. Image processing was identical for lysates and corresponding precipitates.

These experiments demonstrate that the four ChAPs are able to interact with each other, either directly or indirectly. However, the intensities of the signals of the precipitations suggest that ChAPs form complexes with variable stoichiometries. We verified the presence of multiple complexes by blue native gel electrophoresis (data not shown). This variability suggests that a single ChAP might assemble individually into distinct complexes with other ChAPs.

The ChAPs localize at least partially to the TGN

Our results suggest that the ChAPs collaborate to transport Chs3p to the plasma membrane. But where does this transport step occur? We have shown that the ChAPs interact with Arf1p and it has been reported that activated Arf1p resides on Golgi membranes (Stearns *et al*, 1990). Furthermore, Chs5p, which might be implicated in the same trafficking step, is associated with the TGN (Santos and Snyder, 1997). Importantly, Δ*chs5*, Δ*chs6* and Δ*bud7*Δ*bch1* all accumulate Chs3p in the TGN. To investigate the localization of the members of the ChAPs, we performed immunofluorescence on strains in which the ChAPs were chromosomally tagged with 9myc. These strains also contained a chromosomal *SEC7-GFP*, which was used as a marker of the TGN. Bch2p was localized exclusively to the TGN (colocalization with Sec7p-GFP), whereas Chs6p and Bud7p each localized to the TGN and to other punctate structures in the cytoplasm (Figure 5). Bch1p exhibited a very diffuse staining throughout the cytoplasm, which overlapped partially with the TGN. Whereas Chs6p, Bud7p and Bch2p appeared to be expressed to about the same level, Bch1p was expressed at a noticeably higher level. This was evident from immunofluorescence (the exposure time was much shorter for Bch1p) as well as from immunoblots of cell lysates (Figure 9B). Taken together, all members of the ChAPs were at least partially localized to the TGN.

As activated Arf1p, Chs5p and the ChAPs all localize to the TGN, we were wondering whether the localization of Chs5p and the ChAPs might be dependent on the presence of activated Arf1p. Therefore, we treated yeast cells with the

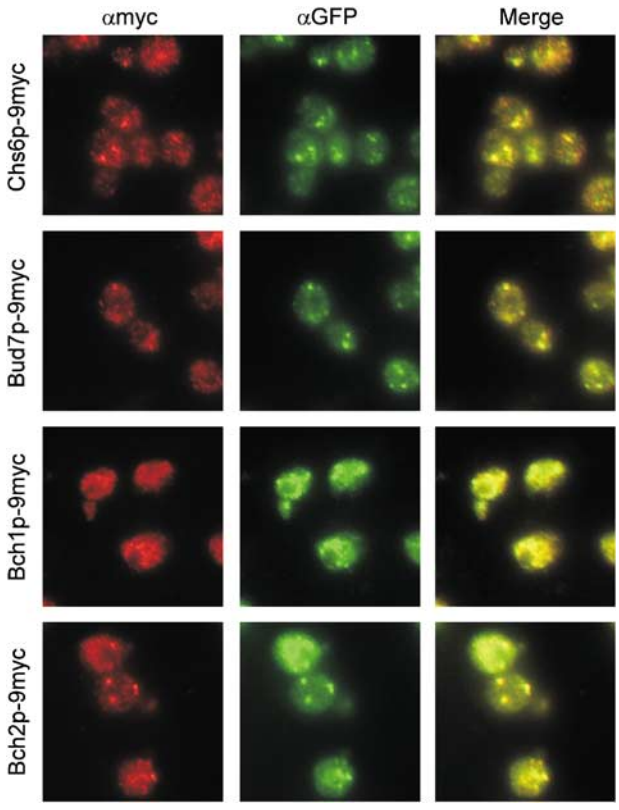

Figure 5 The ChAPs are at least partially localized to the TGN. The ChAPs were chromosomally tagged with 9myc and Sec7p with GFP. Strains were grown to logarithmic phase, fixed and stained with α-myc and α-GFP antibodies. α-Myc and α-GFP were detected with Cy3 and FITC, respectively.

fungal metabolite brefeldin A (BFA), which is an inhibitor of ARF-GEF function. Already after 15 min of BFA treatment, Golgi localization of Chs5p-6HA and Bch2p-9myc was lost (Supplementary Figure S2), indicating that Chs5p and the ChAPs depend on Arf1p-GTP for their Golgi localization.

The C-terminus of the ChAPs is essential for function

None of the ChAPs is essential and even the quadruple knockout did not show a detrimental phenotype under normal growth conditions (data not shown). Therefore, to study further the molecular role of the different ChAPs, we generated small deletions in the different genes. Remarkably, when we removed the C-terminal 13–14 amino acids in Chs6p, Bud7p and Bch1p, the strains phenocopied the gene disruption (Figure 6). Similar to a Δ*chs6* strain, a Chs6pΔc-9myc strain was temperature-sensitive and resistant to calcofluor (Figure 6B). Furthermore, Chs3p-GFP was trapped in the TGN (data not shown). Likewise, Bud7pΔc-9myc homozygous diploid cells displayed a random budding pattern, indistinguishable from Δ*bud7* cells (Figure 6C) and Bch1pΔc-3HA was sensitive to YMP+ (Figure 6D). The observed phenotypes for these ChAPs were not owing to decreased stability or absence of the proteins in the cell, because we could detect similar levels by immunoblot (Figure 7). However, Bch2pΔc-9myc was degraded rapidly, so the role of the C-terminus could not be ascertained (Figure 6A). Nonetheless, because the other nonfunctional proteins were still expressed, the C-terminal-deleted strains served as valuable tools in the course of further experiments.

The C-terminus of the ChAPs is not required for the interaction with Arf1p

We have shown that the ChAPs are able to bind to Arf1p (Figure 2C and D). To test if the C-terminus of the ChAPs is required for this interaction, we performed co-immunoprecipitation experiments with lysates from strains in which the C-terminus of individual ChAPs was replaced by 9myc using affinity-purified α-Arf1p IgGs. In addition, the ChAPs were tagged individually in either wild type, Δ*chs5* or in a background where the other three ChAPs had been deleted and tested for the ability to associate with Arf1p under the different conditions. As shown in Figure 7A (lane 5), Chs6p-9myc, Bud7p-9myc, Bch1p-9myc and Bch2p-9myc were all co-precipitated with affinity-purified α-Arf1p-IgGs. Neither the deletion of *CHS5* (Figure 7A, lane 7) nor the deletion of the three other ChAPs (Figure 7A, lane 9) altered the precipitation efficiencies. However, deletion of the C-terminus increased the amount of protein precipitated in the case of Chs6p, Bud7p and Bch1p (Figure 7A, lane 8). As mentioned above, Bch2pΔc-9myc was too unstable to be evaluated. In conclusion, all ChAPs interact independently with Arf1p and neither their complete C-termini nor Chs5p is required for this interaction.

The C-terminus of the ChAPs is required for the interaction with Chs5p

Mutations in *CHS5* and *CHS6* result in a similar phenotype and both proteins interact with Arf1p, raising the possibility that Chs6p might bind to Chs5p. To test if the other ChAPs would also bind to Chs5p, we performed precipitations with Chs5p antiserum on the same lysates that were used to detect the Arf1p interaction (Figure 7). All ChAPs co-immunoprecipitated with Chs5p (Figure 7B, lane 5). The deletion of three ChAPs did not alter significantly the precipitation efficiency of the remaining one (Figure 7B, lane 8). However, Chs6pΔc-9myc and Bch1pΔc-9myc were not co-precipitated with Chs5p (Figure 7B, lane 7). The amount of precipitated Bud7pΔc-9myc was slightly reduced. Most importantly, however, these experiments provide evidence that the ChAPs bind independently from each other to Chs5p and that their C-termini facilitate this interaction. Furthermore, the requirement for an intact C-terminus argues that ChAP functions are mediated through Chs5p.

Chs5p is essential for the TGN localization of the ChAPs

As the interaction of the members of the ChAP family with Chs5p appeared to be essential for protein function, we asked whether this function is related to the localization of the ChAPs. Chs5p localizes to the TGN (Santos and Snyder, 1997, and Figure 8). This localization is independent of the ChAPs, because we observed the same TGN staining in the absence of the entire ChAP family (Figure 8A). This result indicated that Chs5p might either recruit the ChAPs or stabilize the ChAPs at the TGN. If this were the case, one would expect that the TGN localization of the ChAPs would require Chs5p.

The deletion of *CHS5* had a dramatic impact on both expression levels as well as localization of the ChAPs. In Δ*chs5* strains, Bud7p is up- and Bch1p downregulated, both evident in immunofluorescence (Figure 8B) and immuno-

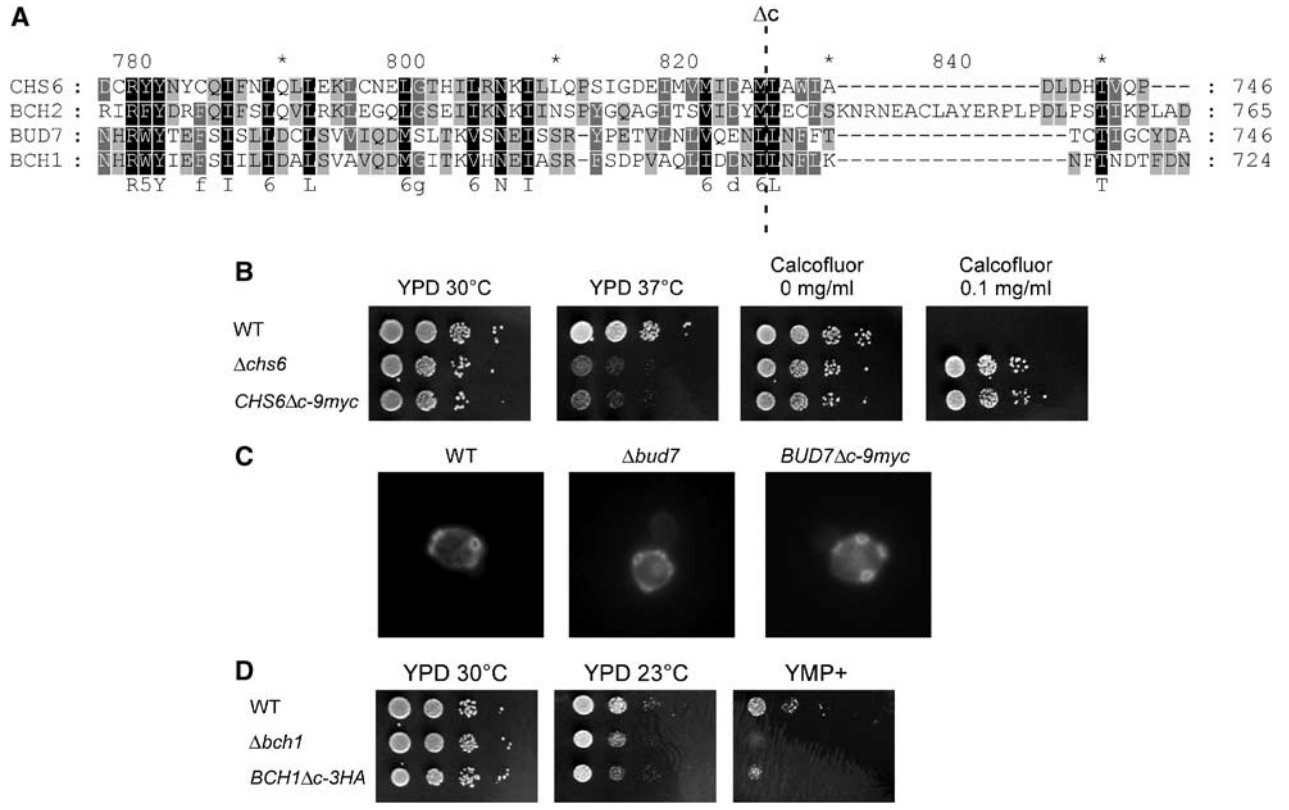

Figure 6 The C-terminus of the ChAPs is essential for their function. (**A**) Sequence alignment of the C-terminus of the ChAPs. The last 13 amino acids of Chs6p were deleted and replaced by 9myc. The C-termini of the other three family members were deleted and replaced by epitope tags according to this alignment. A dashed line indicates the site of deletion. (**B**) Growth of *CHS6*Δ*c-9myc* as compared to WT and Δ*chs6* at different temperatures and on calcofluor plates. (**C**) Budding pattern of a homozygous diploid *BUD7*Δ*c-9myc* strain. Diploid yeast strains were grown to logarithmic phase and stained with calcofluor. (**D**) Growth of *BCH1*Δ*c-3HA* as compared to WT and Δ*bch1* at different temperatures and on YMP + plates.

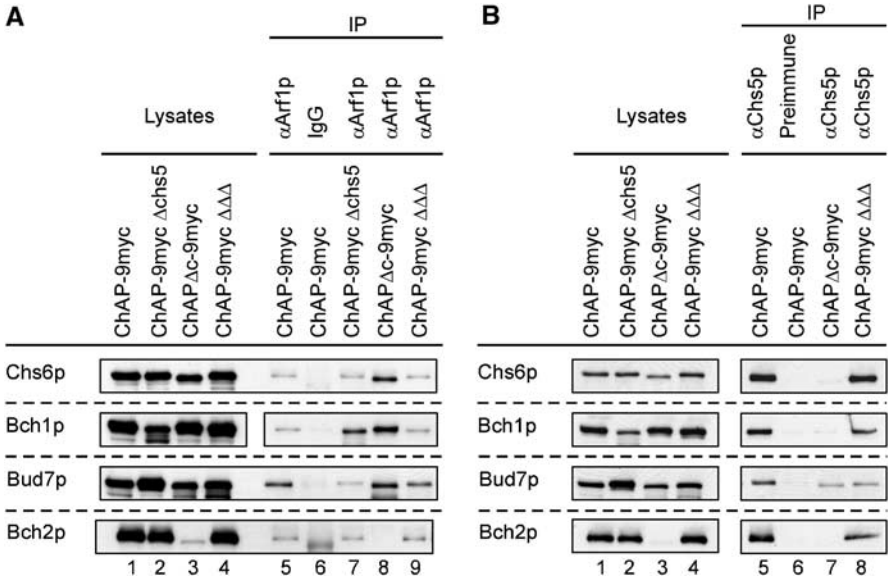

Figure 7 The ChAPs interact with Arf1p and Chs5p. Co-immunoprecipitation experiments were performed using strains in which one ChAP was individually chromosomally tagged with 9myc in either WT, in Δ*chs5* or in a strain in which the three remaining ChAPs were deleted. Furthermore, the experiment was also performed with lysates from ChAPΔc-9myc strain. The lysates were treated with either affinity-purified α-Arf1p (**A**) or α-Chs5p serum (**B**) and Protein A–Sepharose. The precipitate was analyzed with α-myc. In all, 1.7% of the lysates was loaded in lanes 1–4. The lysates in (A) and (B) are from the same experiment. The lysates and corresponding precipitates were from the same immunoblot.

blots (Figure 7B, lanes 1 and 2). The same exposure time as in Figure 5 was used to allow a direct comparison. In addition, Sec7p-GFP staining in Δ*chs5* and in C-terminally deleted ChAP strains indicated a normal Golgi morphology (data not shown). Chs6p, Bud7p and Bch2p lost their TGN localization in a Δ*chs5* background and showed a diffuse

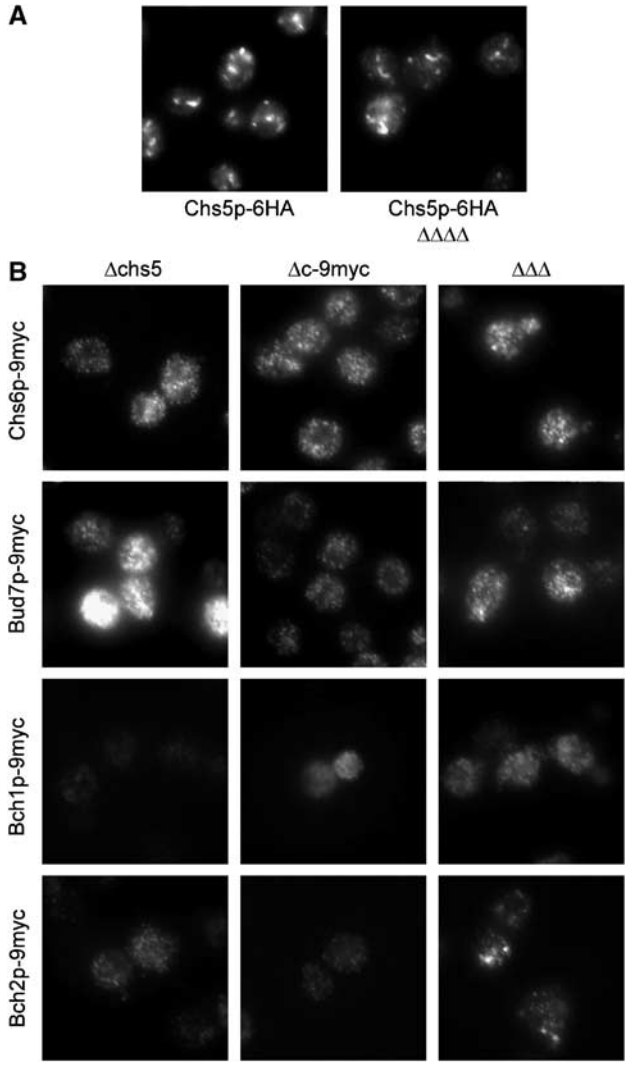

Figure 8 Chs5p is essential for the TGN localization of the ChAPs. (**A**) Chs5p-6HA staining in WT cells and in cells in which the whole ChAP family is deleted (ΔΔΔΔ). (**B**) Staining of ChAP-9myc in Δ*chs5* strains or strains in which the other three ChAPs were deleted (ΔΔΔ) or the ChAPΔc-9myc in a WT background. The same exposure time as in Figure 5 was used for each protein within one row.

staining (Figure 8B). Furthermore, the deletion of the C-terminus of the ChAPs had a similar effect on their TGN association. The impact on the localization of Bch1p was generally hard to score because of the already very diffuse staining of this protein in WT cells. These experiments provide further evidence that Chs5p as well as the C-terminus of the ChAPs is required for either their recruitment to or their stabilization at the TGN. Taken together, these results indicate that localization of the ChAPs to the TGN is a prerequisite for protein function. Because Chs5p and Chs6p have been shown to be required for export of Chs3p out of the TGN, the ChAPs might act together with Chs5p as cargo receptors/coat for specialized transport vesicles that bud from the TGN.

Next, we investigated the influence of the ChAPs on the localization of each other by performing immunofluorescence in triple deletion strains (Figure 8B). The most dramatic effect was observed for Chs6p and Bud7p, which

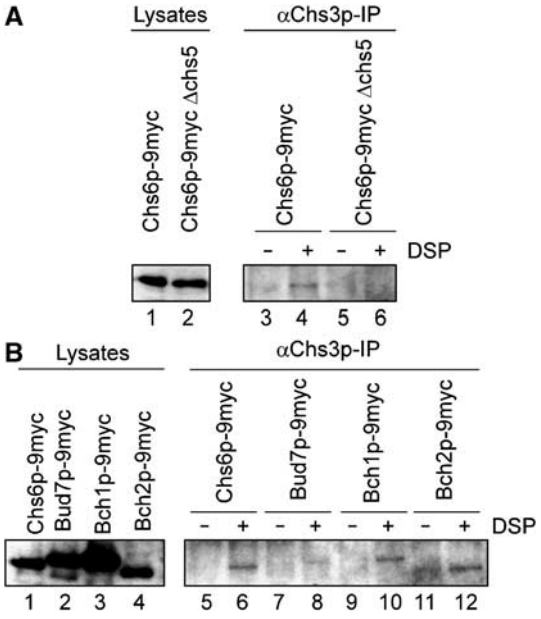

Figure 9 Chs3p interacts with the ChAPs. (**A**) Crosslinking experiments were performed using either WT or Δ*chs5* strains carrying Chs6p-9myc. The lysates were treated with either DMSO or DSP before Chs3p immunoprecipitation. The precipitate was analyzed by immunoblot with α-myc. In all, 8% of the lysates was loaded in lanes 1 and 2. (**B**) Crosslinking experiments were performed using strains in which the ChAPs were chromosomally tagged with 9myc as in (A). The lysates and corresponding precipitates were from the same immunoblot, and image processing was identical.

showed a dispersed staining in the absence of the other three ChAPs, similar to what was observed in the Δ*chs5* strain. Bch2p retained TGN staining. Thus, the localization of Bch2p was less dependent on the other ChAPs than on Chs5p or its C-terminus. These results are corroborated by the finding that a Δ*chs5* strain displays all the phenotypes of the different ChAPs deletions. The data are in agreement with ChAPs assembly probably on membranes rather than the presence of a preformed complex.

Chs3p interacts with the ChAPs

If the interpretation of our data were correct, one would expect an interaction between Chs3p and at least a subset of the ChAPs. Because this interaction might be only transient, we chose a crosslinking approach to address this point. Cell lysates from different strains were treated with the cleavable crosslinker DSP, Chs3p was immunoprecipitated and the co-precipitating ChAPs were visualized by immunoblot. Chs6p-9myc was crosslinked to Chs3p (Figure 9A). This interaction was dependent on the presence of Chs5p. We could also detect an interaction of Chs3p with Bch1p-9myc and Bch2p-9myc (Figure 9B), indicating that they might also be involved in export of Chs3p from the TGN. Taken together, our results are consistent with a role of the ChAPs as cargo receptor for Chs3p or even a coat for Chs3p-containing vesicles.

Discussion

In this paper, we characterized and analyzed a new family of proteins, the ChAPs, which is involved in transport of chitin

synthase Chs3p to the plasma membrane. We found that they interact with Chs5p, with the small GTPase Arf1p and with the cargo Chs3p. These interactions most likely take place at the *trans*-Golgi. The C-terminus of the ChAPs is necessary for the interaction with Chs5p and for localization to the TGN. We and others have established scoreable phenotypes for the individual deletion for three of the four ChAPs (Bulawa, 1992; Zahner *et al*, 1996). The deletion of *CHS5* displayed all these phenotypes, indicating that Chs5p acts in the same pathway, either upstream of the ChAPs or as a convergence point. What could be the role of the ChAPs in Chs3p transport?

It is tempting to speculate that the ChAPs could form together with Chs5p a novel coat. This conclusion is supported by (1) their direct binding to Arf1p-GTP; (2) their ability to interact with each other; (3) their recruitment to the *trans*-Golgi in a Chs5p-dependent manner and (4) probably most importantly by their ability to bind to cargo. This idea is supported by the results from Santos and Snyder (1997), who suggested that Chs5p is present on transport vesicles. In addition, we could not detect any clathrin or coatomer in co-immunoprecipitates with the ChAPs (MT and AS, unpublished results), indicating that the ChAPs and Chs5p may act separately from the major coats.

Alternatively, the ChAPs could act as chaperones like the ER-resident Shr3p and Chs7p, which are required for inclusion of Gap1p and Chs3p, respectively, into COPII-coated vesicles, but do not enter the vesicle themselves (Kuehn *et al*, 1996, 1998; Trilla *et al*, 1999). However, it seems unlikely that ChAPs are chaperones, because they are soluble proteins that have to be recruited to the membrane in order to fulfill their function. This recruitment to membranes requires the presence of Chs5p on the late Golgi, because deletion of *CHS5* abolished ChAPs' TGN localization. Yet, another possibility would be that Chs5p and the ChAPs control the generation of specialized transport carriers at the *trans*-Golgi, but are not part of a coat, similarly to the FAPPs, which are phosphoinositide-binding proteins on nascent carriers and which can also interact with Arf1 to control budding at the TGN (Godi *et al*, 2004). Although neither ChAPs nor Chs5p contain a PH domain, a different anchoring mechanism possibly through Chs5p could restrain the ChAPs at the late Golgi. However, Chs6p is not exclusively localized to the TGN. A significant portion is also found on chitosomes, which are specialized endosomes (Ziman *et al*, 1996). In addition, Bch1p was mostly cytoplasmic. This indicates that the association of the ChAPs with Golgi membranes might be more dynamic than that of the FAPPs.

In the case of Chs3p, Chs6p would recognize the cargo and therefore allow the inclusion into the polymerizing coat. It is tempting to speculate that each of the ChAPs recognizes a specific subset of cargo. Bud7p might be able to interact specifically with proteins involved in bud-site selection and polarity establishment, whereas Bch1p could be required to deliver amino-acid permeases or specific amino-acid transporters to the membrane, because of the YMP+ phenotype. What all these cargoes would have in common is their tightly controlled temporal and spatial localization to a subdomain of the plasma membrane. It is unlikely that Chs5p is a specific cargo receptor, because deletion of *CHS5* accumulates not only all phenotypes of single ChAPs deletions but also additional phenotypes. Therefore, it is likely that Chs5p may play a much more general role in the vesicle formation at the TGN.

One specific member of the ChAPs is necessary but not sufficient for transport of a particular cargo. Chs3p requires the presence of Chs6p for export from the *trans*-Golgi; however, simultaneous deletion of *BCH1* and *BUD7* trapped Chs3p in the Golgi, despite the presence of Chs6p, clearly indicating a role for the other members of the ChAPs. Furthermore, in addition to Chs6p, Bch1p and Bch2p were also crosslinked to Chs3p. One possibility might be that Bch1p, Bch2p and Bud7p could help to form primers that would represent a starting point for vesicle formation (Springer *et al*, 1999). Alternatively, Bch1p, Bch2p and Bud7p could help to stabilize a Chs6p–Chs3p interaction until a transport vesicle could be generated. Both of these explanations would not require the presence of all ChAPs for a specific transport event, which is what we observe for Chs3p transport, bud-site selection and sensitivity to YMP+. However, more than one ChAP might be necessary for efficient cargo transport and thus the ChAPs might form facultative oligomers. This conclusion is supported by the apparent different stoichiometries in the co-immunoprecipitation experiments (Figure 4) and the various complexes detected by blue native gel electrophoresis (MT and AS, unpublished results).

Whatever the involvement of the ChAPs in the generation of vesicles at the *trans*-Golgi is, these vesicles are most likely not default secretory vesicles, rather than special ones, because they carry cargoes that are only required at a certain time in the cell cycle. These specialized vesicles are most likely targeted to the bud neck and the incipient bud site to deliver Chs3p and at least a polarity cue, which might help the cell to find the correct next budding site. However, we did not observe any change in localization of Chs5p and the ChAPs over the cell cycle, indicating that there might be an additional factor, which controls the vesicle generation temporally. The identification of this elusive factor might be difficult because neither the ChAPs nor Chs5p are essential under standard laboratory growth conditions. Therefore, generation of these specialized transport vesicles might not be essential either. In addition, multiple pathways lead out of the Golgi. Valdivia *et al* (2002) have provided evidence that there is an alternative trafficking route for Chs3p to the plasma membrane when *ARF1* and *CHS6* or *CHS5* are deleted. Although the cells of these double deletions were rendered calcofluor-sensitive again, a similar combination of deletions with *ARF1* and *BUD7* did not rescue the bud-site selection defect of the Δ*bud7* strain (MT and AS, unpublished results), suggesting that the cargo for Bud7p might still reach the plasma membrane, but as the temporal and spatial control of the delivery of the polarity cue was lost, the random budding pattern could not be rescued.

Materials and methods

Yeast media

Standard yeast media were prepared as described (Sherman, 1991). Calcofluor plates were based on minimal medium containing additionally 0.1% yeast extract, 1% MES (pH 6.0) and 0.1 mg/ml calcofluor (Sigma). YMP+ plates contained 6.3 g/l yeast nitrogen base without amino acids and ammonium sulfate (Difco), 4.5 g/l yeast extract, 9 g/l peptone, 9 g/l succinic acid, 4.5 g/l NaOH and 25 g/l ammonium sulfate (Lillie and Pringle, 1980).

Yeast genetic methods

Standard genetic techniques were used throughout (Sherman, 1991). Chromosomal tagging and deletions were performed as described (Knop *et al*, 1999; Gueldener *et al*, 2002). For *CHS6*, *BCH1* and *BCH2*, the whole ORF was deleted, whereas *BUD7* was deleted only until 100 bp upstream the Stop codon leaving the overlapping ORF *YOR300w* intact. Strains and primers used are given in Table I and Supplementary Table S1. All PCR-based chromosomal manipulations were confirmed by analytical colony PCR.

Protein purifications

BCH2-3HA and *CHS6-GFP* were cloned into pTYB12 (NEB) and purified from *Escherichia coli* according to the manufacturer's instructions. Proteins were buffer exchanged to B88 (20 mM HEPES, pH 6.8, 5 mM $Mg(Ac)_2$, 150 mM KAc, 250 mM sorbitol) using PD10 columns (Amersham). *CHS5* was cloned into pET100/D-TOPO (Invitrogen) and purified from *E. coli* using standard procedures. Coatomer, Arf1p and Sar1p were purified as described (Spang and Schekman, 1998).

Table I Yeast strains used in this study

Designation	Genotype	Reference
YPH499	*MAT a ade2 his3 leu2 lys2 trp1 ura3*	Sikorski and Hieter (1989)
YPH500	*MAT α ade2 his3 leu2 lys2 trp1 ura3*	Sikorski and Hieter (1989)
YPH501	*MAT a/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	Sikorski and Hieter (1989)
YAS431	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS5::LEU2 (Kluveromyces lactis)*	This study
YAS571	*MAT a ade2 his3 leu2 lys2 trp1 ura3 ARF1::HIS3MX6*	This study
YAS525	*MAT α ade2 his3 leu2 lys2 trp1 ura3 CHS6::URA3 (K. lactis) BCH2::KAN (Tn903)*	This study
YAS430	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7::LEU2 (K. lactis) BCH1::HIS5 (Schizosaccharomyces pombe)*	This study
YAS563-2a	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6::URA3 (K. lactis)*	This study
YAS563-3a	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7::LEU2 (K. lactis)*	This study
YAS563-4a	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH2::KAN (Tn903)*	This study
YAS563-5a	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH1::HIS5 (S. pombe)*	This study
YAS563-10a	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7::LEU2 (K. lactis) BCH1::HIS5 (S. pombe)*	This study
YAS988	*MAT a/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 CHS5::LEU2 (K. lactis)/CHS5::TRP1 (K. lactis)*	This study
YAS778	*MAT a/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 CHS6::URA3 (K. lactis)/CHS6::URA3 (K. lactis)*	This study
YAS779	*MAT a/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 BUD7::LEU2 (K. lactis)/BUD7::URA3 (K. lactis)*	This study
YAS780	*MAT a/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 BCH2::KAN (Tn903)/BCH2::KAN (Tn903)*	This study
YAS781	*MAT a/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 BCH1::HIS5 (S. pombe)/BCH1::HIS5 (S. pombe)*	This study
YAS793	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS5::LEU2 (K. lactis) ARF1::HIS3MX6*	This study
YAS794	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6::URA3 (K. lactis) ARF1::HIS3MX6*	This study
YAS795	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7::LEU2 (K. lactis) ARF1::HIS3MX6*	This study
YAS796	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH2::KAN (Tn903) ARF1::HIS3MX6*	This study
YAS797	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH1::HIS5 (S. pombe) ARF1::KANMX6*	This study
GPY60	*MAT α ura3–52 leu2,3–112 his4–579 pep4::URA3 prb1 trp1 gal2*	R Schekman
YAS603	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH2::BCH2-3HA-His3MX6*	This study
YAS839	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS5::CHS5-6HA-TRP1 (K. lactis) CHS6::URA3 (K. lactis) BUD7::LEU2 (K. lactis) BCH2::KAN (Tn903) BCH1::HIS5 (S. pombe)*	This study
YAS328	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6::CHS6-9myc-TRP1 (K. lactis)*	This study
YAS335	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7::BUD7-9myc-TRP1 (K. lactis)*	This study
YAS576	*MAT α ade2 his3 leu2 lys2 trp1 ura3 BUD7::BUD7-9myc-TRP1 (K. lactis)*	This study
YAS697	*MAT a/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 BUD7::BUD7-9myc-TRP1 (K. lactis)/BUD7::BUD7-9myc-TRP1 (K. lactis)*	This study
YAS339	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH1::BCH1-9myc-TRP1 (K. lactis)*	This study
YAS589	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH2::BCH2-9myc-TRP1 (K lactis)*	This study
YAS333	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6::CHS6-9myc-TRP1 (K. lactis) CHS5::LEU2 (K. lactis)*	This study
YAS379	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7::BUD7-9myc-TRP1 (K. lactis) CHS5::LEU2 (K. lactis)*	This study
YAS380	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH1::BCH1-9myc-TRP1 (K. lactis) CHS5::LEU2 (K. lactis)*	This study
YAS582	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH2::BCH2-9myc-TRP1 (K lactis) CHS5::LEU2 (K. lactis)*	This study
YAS653	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6::CHS6-9myc-TRP1 (K. lactis) BUD7::LEU2 (K. lactis) BCH2::KAN (Tn903) BCH1::HIS5 (S. pombe)*	This study
YAS615	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7::BUD7-9myc-TRP1 (K. lactis) ura3 CHS6::URA3 (K. lactis) BCH2::KAN (Tn903) BCH1::HIS5 (S. pombe)*	This study
YAS614	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH1::BCH1-9myc-TRP1 (K. lactis) CHS6::URA3 (K. lactis) BUD7::LEU2 (K. lactis) BCH2::KAN (Tn903)*	This study
YAS654	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH2::BCH2-9myc-TRP1 (K lactis) CHS6::URA3 (K. lactis) BUD7::LEU2 (K. lactis) BCH1::HIS5 (S. pombe)*	This study
YAS684	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6::CHS6Δc-9myc-TRP1 (K. lactis)*	This study
YAS798	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7::BUD7Δc-9myc-TRP1 (K. lactis)*	This study
YAS799	*MAT α ade2 his3 leu2 lys2 trp1 ura3 BUD7::BUD7Δc-9myc-TRP1 (K. lactis)*	This study
YAS802	*MAT a/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 BUD7::BUD7Δc-9myc-TRP1 (K. lactis)/BUD7::BUD7Δc-9myc-TRP1 (K. lactis)*	This study
YAS698	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH1::BCH1Δc-9myc-TRP1 (K. lactis)*	This study
YAS800	*MAT α ade2 his3 leu2 lys2 trp1 ura3 BCH1::BCH1Δc-3HA-His3MX6*	This study
YAS801	*MAT α ade2 his3 leu2 lys2 trp1 ura3 BCH2::BCH2Δc-9myc-TRP1 (K lactis)*	This study
YAS792	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7::BUD7-9myc-TRP1 (K. lactis) CHS6::CHS6-yEGFP-KanMX6 BCH2::BCH2-3HA-HIS3MX6 BCH1::BCH1-2AU5-LEU2 (K. lactis)*	This study

Table I continued

Designation	Genotype	Reference
YAS699	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7*::*BUD7-9myc-TRP1 (K. lactis) CHS6*::*CHS6-yEGFP-KanMX6 BCH2*::*BCH2-3HA-HIS3MX6*	This study
YAS700	*MAT* α *ade2 his3 leu2 lys2 trp1 ura3 CHS6*::*CHS6-yEGFP-KanMX6 BCH1*::*BCH1-2AU5-LEU2 (K. lactis)*	This study
YAS604	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7*::*BUD7-9myc-TRP1 (K. lactis) BCH2*::*BCH2-3HA-His3MX6*	This study
YAS381	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS5*::*CHS5-6HA-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS382	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6*::*CHS6-9myc-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS385	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7*::*BUD7-9myc-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS386	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH1*::*BCH1-9myc-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS655	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH2*::*BCH2-9myc-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS384	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6*::*CHS6-9myc-TRP1 (K. lactis) CHS5*::*LEU2 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS387	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7*::*BUD7-9myc-TRP1 (K. lactis) CHS5*::*LEU2 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS388	*MAT a ade2 his3 leu2 lys2 trp1ura3 BCH1*::*BCH1-9myc-TRP1 (K. lactis) CHS5*::*LEU2 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS656	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH2*::*BCH2-9myc-TRP1 (K. lactis) CHS5*::*LEU2 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS835	*MAT a ade2 his3 leu2 lys2 trp1 ura3CHS6*::*CHS6c-9myc-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS836	*MAT a ade2 his3 leu2 lys2 trp1 ura3BUD7*::*BUD7c-9myc-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS837	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH1*::*BCH1c-9myc-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS838	*MAT* α *ade2 his3 leu2 lys2 trp1 ura3 BCH2*::*BCH2c-9myc-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3*	This study
YAS906	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7*::*LEU2 (K. lactis) BCH1*::*HIS5 (S. pombe) ARF1*::*KAN (Tn903)*	This study
YAS947	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS3*::*CHS3-yEGFP-KanMX6*	This study
YAS941	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS5*::*LEU2 (K. lactis) CHS3*::*CHS3-yEGFP-KanMX6*	This study
YAS942	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6*::*URA3 (K. lactis) CHS3*::*CHS3-yEGFP-KanMX6*	This study
YAS943	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BUD7*::*LEU2 (K. lactis) BCH1*::*HIS5 (S. pombe) CHS3*::*CHS3-yEGFP-KanMX6*	This study
YAS947	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS6*::*CHS6*Δ*c-9myc-TRP1 (K. lactis) CHS3*::*CHS3-yEGFP-KanMX6*	This study
YAS1057	*MAT a ade2 his3 leu2 lys2 trp1 ura3 CHS5*::*CHS5-6HA-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3 ERG6*::*LEU2 (K. lactis)*	This study
YAS1055	*MAT a ade2 his3 leu2 lys2 trp1 ura3 BCH2*::*BCH2-9myc-TRP1 (K. lactis) SEC7*::*SEC7-GFP-URA3 ERG6*::*LEU2 (K. lactis)*	This study

Differential affinity chromatography

The affinity chromatography was performed as described by Trautwein *et al* (2004). In brief, equal amounts of recombinant ΔN17-Arf1Q71Lp and ΔN17-Arf1T31Np were coupled to sepharose–resin and a nucleotide exchange was performed in NE buffer (25 mM HEPES (pH 7.5), 100 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT) for 1 h at 37°C (GTP for Arf1pQ71Lp, GDP for Arf1T31Np). Yeast cytosol was allowed to bind for 1 h at 4°C. Proteins were eluted with exchange buffer containing 500 μM GX′P (GDP for Arf1pQ71Lp, GTP for Arf1T31Np). The samples were separated on SDS–PAGE and immunoblotted.

Antibodies

Antisera against Gea2p (Spang *et al*, 2001), coatomer (Rexach *et al*, 1994), Glo3p (Poon *et al*, 1999) and Arf1p (Spang and Schekman, 1998) have been described. Polyclonal antibodies against Sar1p, Chs5p and the N-terminal fragments 1–171 of Chs3p were raised in rabbits.

Co-immunoprecipitation

Yeast lysates from equal amounts of cells were prepared by spheroplasting as described (Rexach *et al*, 1994). Spheroplasts were sedimented (2 min, 1000 *g*), lysed in 20 mM HEPES (pH 6.8), 150 mM KAc, 5 mM $Mg(Ac)_2$, 1% Tween-20 with protease inhibitors and cleared by centrifugation (10 min, 16 000 *g*).

Immunoprecipitations were performed with 10 μg affinity-purified α-Arf1p antibodies or affinity-purified rabbit IgGs, 10 μl α-Arf1p, α-Sar1p, α-Chs5p or preimmune serum, 5 μl α-HA-11 (Eurogentec), 5 μl α-Myc (9E10; Sigma), 5 μl α-AU5 (Abcam) or 5 μl α-GFP (Torrey Pines) and 100 μl 20% Protein A–Sepharose per 1 ml cleared lysate for 1 h at 4°C. The beads were washed, resuspended in sample buffer and analyzed by immunoblot.

Liposome-binding experiments

Azolectin (20 mg; Sigma) was mixed with 6 ml diethylether and 1 ml 20 mM HEPES (pH 7.4). After sonication, the ether was removed by evaporation. Liposomes were extruded 12 times through 0.6 μm membranes (Avanti) and then incubated with 1.35 μM Arf1p and 625 μM GTPγS in NE buffer for 1 h at 30°C. Bch2p (110 nM) or coatomer (40 nM) were added to the reaction and incubated for 10 min at 4°C. The samples were adjusted to 1 M sucrose in B88, overlaid with 75 μl 0.75 M sucrose in B88 and 20 μl B88. The liposomes were floated for 3 h at 100 000 *g* (TLA100). The top 30 μl were analyzed by immunoblot.

Immunofluorescence and fluorescence microscopy

Immunofluorescence was performed as described (Pringle *et al*, 1991). Cells expressing chromosomally epitope-tagged proteins were grown to log phase. After fixation and spheroplasting, the cells were stained with α-HA-7 (Sigma) or α-myc (9E10; Sigma) antibodies. Cy3-coupled secondary antibodies were used for visualization (Jackson ImmunoResearch Laboratories). For comparison, all strains except the triple and quadruple deletion of the ChAPs also contained a chromosomal copy of *SEC7-GFP* to assess colocalization with the TGN. *SEC7-GFP* was integrated by transformation of a *Spe*I-linearized pUSE-URA3 integration plasmid containing *SEC7-GFP* (Seron *et al*, 1998). For the visualization of GFP after fixation, α-GFP and secondary antibodies coupled to FITC were used.

Analysis of the budding pattern was carried out as described (Lord *et al*, 2002). Briefly, cells grown for at least 16 h to log phase were stained after fixation in either 1 mg/ml calcofluor or 1 mg/ml FITC-ConA (Molecular probes). To determine the budding pattern, only cells were scored with no more than four bud scars.

Pictures were acquired with an Axiocam mounted on an Axioplan 2 fluorescence microscope (Zeiss) using Axiovision

software. Image processing was performed using Adobe Photoshop 7.0.

Crosslinking experiments

For each sample, yeast lysate from six OD_{600} was prepared by glass bead lysis in 200 µl B88 buffer with protease inhibitors. The lysate was cleared by centrifugation at 25 000 *g* for 15 min at 4°C. A measure of 20 µl DMSO or 20 µl DSP (Pierce) dissolved in DMSO were added to 140 µl lysate. The crosslinking reaction took place 30 min at RT and was stopped with 7 µl 1 M Tris (pH 7.5) for 15 min. Then, 8 µl of 20% SDS were added and heated to 65°C for 15 min. A measure of 900 µl IP buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS) were added and the sample was centrifuged for 10 min at 20 000 *g*. The supernatant was subjected to immunoprecipitation for 1 h at RT using 5 µg affinity-purified α-Chs3p antibodies crosslinked to Protein A–Speharose with DMP (Pierce). The precipitates were analyzed by immunoblot.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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7 Abbreviations

AAA-ATPase	ATPases Associated with diverse cellular Activities
Ac	Acetate
AP	Adaptor protein
ADP	Adenosine-5´-diphosphate
Bch	Bud7p Chs6p homolog
ATP	Adenosine-5´-triphosphate
BFA	Brefeldin A
CCV	Clathrin-coated vesicle
ChAP	Chs5-Arf1p-binding protein
COP	Coat protein
d	day
DMP	Dimethylpimelimidate
DSP	Dithiobis[succinimidylpropionate]
DTT	DL-Dithiothreitol
E. coli	*Eschericia coli*
ECL	Enhanced chemoluminiscence
EDTA	Ethylenediaminetetraacetic acid
EE	Early endosome
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ER	endoplasmic Reticulum
ERGIC	ER-Golgi-intermediate compartment
ESCRT	Escort complex
FAPP	4-phosphate adaptor protein
FITC	Fluorescine-isothiocyanate
GAP	GTPase-activating protein
GDI	Guanine-nucleotide-dissociation inhibitor
GDP	Guanosine-5´-diphosphate
GFP	Green fluorescent protein
GGA	Golgi-localized gamma-ear containing
GPI	Glycosylphosphatidylinositol
GST	Gluthathione-S-transferase
GTP	Guanosine-5´-triphosphate
GTPase	GTP hydrolase
GTPγS	Guanosine-5´-O.(3-thiophosphate)
HEPES	N-[2-Hydroxyethyl]piperazine-N´-[2-ethanesolfonic acid]
IP	immunoprecipitation
K. lactis	*Kluyveromyces lactis*
LE	Late endosome
LB	Luria-Bertani
LMA1	Low-molecular weight activity 1
MALDI	Matrix-associated Laserionization/Desorption
MS	Mass spectometry
MVB	Multi-vesicular body
NEM	N-ethylmaleimide
NSF	NEM-sensitive factor
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide-Gel-Electrophoresis

PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PH	Pleckstrin homology
PMSF	Phenylmethanesulfonyl fluoride
RFP	Red fluorescent protein
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecylsulfate
SNAP	Soluble NSF attachment protein
SNARE	Soluble NSF attachment protein receptor
S. pombe	*Saccharomyces pombe*
TAP	Tandem-affinity purification
TBS	Tris-buffered saline
TCA	Trichloro acetic acid
TLC	Total cell lysate
TGN	Trans-Golgi-network
Tris	Tris(hydoxymethylaminomethane)
t-SNARE	Target membrane SNARE
TX-100	Triton-X100
v-SNARE	Vesicle-SNARE
WT	Wild type

8 Curriculum Vitae

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