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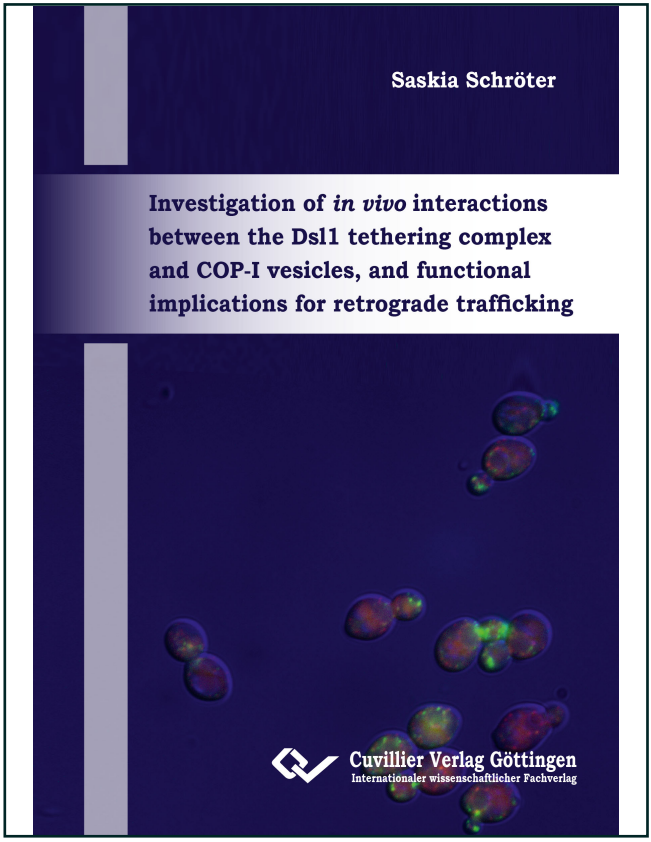
Investigation of in vivo interactions between the Dsl1 tethering complex and COP-I vesicles, and functional implications for retrograde trafficking



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1 Introduction

A unique feature of eukaryotic cells is their compartmentalization into organelles with specialized structure, function and composition. This ensures the execution of many distinct cellular processes within different micro-environments. However, with this separation, certain complications arise. Cells have had to develop mechanisms by which organelles exchange material (trafficking). Additionally, cells need to ensure that during growth and division, organelles are passed on to their progeny (inheritance). In the interplay between endoplasmic reticulum (ER) and Golgi apparatus, diverse processes are involved in trafficking and inheritance. The budding yeast *Saccharomyces cerevisiae* is a versatile model organism to study these intracellular processes.

1.1 ER and Golgi in yeast

The ER in *S. cerevisiae* exhibits a comparably simple subcellular distribution. ER membranes form the nuclear envelope (perinuclear ER), and are found at the cell cortex (cortical ER). These membrane systems are connected by ER tubules (tubER) (Preuss *et al.*, 1991).

The Golgi is built up of cisternal substructures with distinct protein compositions. The cisternae are transient structures, exchanging their early resident proteins for late resident proteins within minutes (Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006). The Golgi cisternae in *S. cerevisiae* do not form stack structures (Novick *et al.*, 1981; Svoboda and Necas, 1987), but have distinct punctate distributions (Antebi and Fink, 1992; Chapman and Munro, 1994; Lussier *et al.*, 1995). This seems to be a unique feature of *S. cerevisiae*, as budding yeast *Pichia pastoris*, for instance, exhibits a more conventional stacked structure of ER exit sites and Golgi (Rossanese *et al.*, 2001).

Interestingly, there are several reports of protein mutants which lead to multilamellar Golgi structures (Novick *et al.*, 1981; Franzusoff *et al.*, 1991; Jedd *et al.*, 1997), or colocalization of formerly separate entities of different Golgi compartments (Weinberger *et al.*, 2005). However, these structures are probably exaggerated late Golgi compartments rather than ordered stacks of cis, medial and trans cisternae (Rossanese *et al.*, 1999).

1.2 Organelle inheritance during budding

Vegetative reproduction in budding yeast requires a targeted asymmetric distribution of organelles, such as Golgi and ER, between the mother and the daughter cell. Generally, bud outgrowth is facilitated by fusion of secretory vesicles with the plasma membrane at the bud site (Pruyne *et al.*, 1998), resulting in local cell surface expansion. This process is executed by means of actin structures and myosin motors.

In yeast, actin appears as filaments, patches, and rings, which follow a dynamic equilibrium of polymerization and depolymerization. Actin filaments, with the barbed ends pointed towards the bud, form the tracks of material transport. They mediate most, if not all polarized organelle transport in yeast (Hammer and Sellers, 2012). The actin localization is coordinated with the stages of the cell cycle, exhibiting a very distinct localization pattern throughout budding (Figure 1-1) (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Pruyne *et al.*, 2004a).

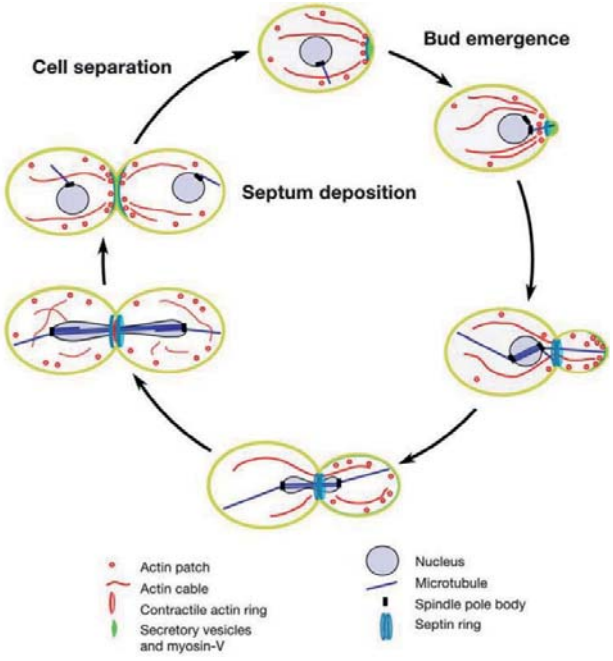

Figure 1-1: Localization of actin during budding in yeast. As the cells select a bud site during G1 phase, actin patches form a ring at the site of future bud outgrowth. During apical growth, the polarized cortical actin initially localizes to the tip of small buds (S, G2 phase), and actin cables are oriented along the mother-bud axis toward the actin patches. Meanwhile, a subset of actin is anchored at the mother cell–bud neck region in order to mediate secretory vesicle entry into the bud. During isotropic growth (M phase), actin patches distribute uniformly over mother and bud cortex, relocate at the neck during cytokinesis, and new growth is directed to this region during septum formation. Image from (Pruyne *et al.*, 2004b).

Secretory vesicles are transported along the actin cytoskeleton to distinct sites in the emerging bud, where they fuse with the plasma membrane and thus mediate bud outgrowth. However, not only secretory vesicles are transported along the actin cytoskeleton. Actin has been implicated in bud-directed movement of Golgi, mitochondria, ER, peroxisomes, and vacuoles (Pruyne *et al.*, 2004b), while organelle transport seems to be completely microtubule-independent (Fagarasanu *et al.*, 2010).

The motors responsible for organelle translocation along actin structures are *S. cerevisiae's* two class V myosins, Myo2p and Myo4p.

The inheritance of Golgi has to be differentiated into early and late Golgi inheritance. Late Golgi is localized to the bud in a Myo2p- dependent manner (Rossanese and Glick, 2001; Losev *et al.*, 2008). During budding, it is found at sites of secretory activity (Preuss *et al.*, 1992; Rossanese *et al.*, 2001). The RAB GTPase Ypt11p, which interacts with the globular tail domain of Myo2p, may link Myo2p to Golgi elements via the Golgi-located δ-COP (Arai *et al.*, 2008). Meanwhile, early Golgi inheritance appears to occur independently of Myo2p (Rossanese *et al.*, 2001; Fagarasanu *et al.*, 2010).

ER inheritance, on the other hand, largely does not require microtubule or actin filaments. This is due to the fact that yeast does not break down its nuclear envelope during mitosis (closed mitosis), which results in perinuclear ER being partitioned together with the DNA by astral microtubules (Huffaker *et al.*, 1988; Jacobs *et al.*, 1988).

However, certain dynamic aspects of ER behavior have been related to actin and myosin, even though the successful execution of these processes does not seem to be essential for the cells. A connection of ER dynamics to the actin cytoskeleton was shown by the finding that ER tubules and actin filaments sometimes align (Prinz *et al.*, 2000), and the number of ER tubules arising from the perinuclear ER is decreased in latrunculin A[1]–treated cells (Estrada *et al.*, 2003). A Myo4p-dependent inheritance mechanism of cortical ER (cortER) into small buds was also identified (Estrada *et al.*, 2003; Schmid *et al.*, 2006), but perturbations again only affect a subpopulation of the cells.

Finally, inheritance of cortical ER has been related to exocyst function. The exocyst tethering complex localizes to the sites of bud outgrowth and is implicated in secretory vesicle tethering. The exocyst subunit Sec3p, which acts as a spatial landmark for polarized secretion during

[1] Prevents F-actin polymerization (Spector *et al.*, 1989)

budding (Finger *et al.*, 1998; Wiederkehr *et al.*, 2003), anchors cortER tubules to the bud tip. In addition, exocyst subunit Sec6p plays a role in ER regulation. It interacts with Rtn1p (De Craene *et al.*, 2006), which in turn is responsible for the shaping of ER into tubules (Voeltz *et al.*, 2006).

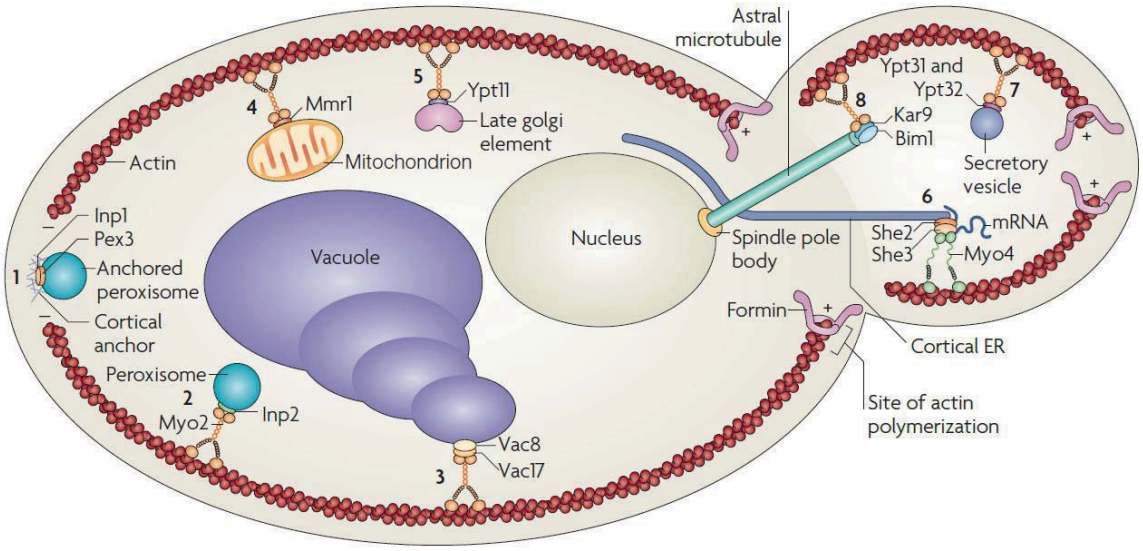

Figure 1-2: Bud-directed organelle transport in yeast. Myo2p is associated with bud-directed transport of peroxisomes (1 and 2), the vacuole (3), mitochondria (4), late Golgi (5), secretory vesicles (7) and assists in the initial orientation of the nucleus by directing microtubule plus ends to the bud (8). Myo4p on the other hand mediates the co-migration of cortER, and specific mRNAs (6). Image from Fagarasanu *et al.*, 2010.

1.3 Dynamics of ER-Golgi interactions

ER and Golgi show extensive interplay, as nascent proteins from the ER are relayed to the Golgi for further processing, and Golgi seems to function as a steady-state structure undergoing continuous outgrowth from and re-consumption by the ER through bidirectional anterograde and retrograde trafficking (Altan-Bonnet *et al.*, 2004). Rossanese and Glick, 2001 suggest that Golgi is deconstructed and reconstructed in every round of cell division. In fact, in vertebrates, during mitosis the majority of Golgi material transits through the ER (Zaal *et al.*, 1999). This may be similar in yeast. The presence of early Golgi structures in small buds correlates strongly with the localization of ER membranes, and small buds with ER inheritance defects also show no early Golgi, which supports the hypothesis that early Golgi is produced *de novo* from bud-localized ER membranes (Reinke *et al.*, 2004). Rossanese *et al.*, 1999 proposed that Golgi

cisternae always form by the coalescence of tER[2]-derived membranes. This relationship is particularly evident in cells that contain Golgi stacks immediately adjacent to tER sites (Bevis *et al.*, 2002; Glick, 2002). Interestingly, a colocalization of tER and early Golgi marked by γ-COP^{YFP} has been reported in glucose-starved cells (Levi *et al.*, 2010).

1.4 Vesicle-mediated material transport between ER and Golgi

ER and Golgi exchange material by means of vesicular trafficking. Vesicles are small membrane enclosed transport entities, often covered by a coat of proteins, which carry out specific transport steps. Accordingly, they differ in protein composition of cargo, adapters, SNAREs[3], and coat proteins. These protein subsets confer vesicle identity, function and destination. The best-studied transport systems are those of clathrin-coated vesicles, COP-II and COP-I coated vesicles.

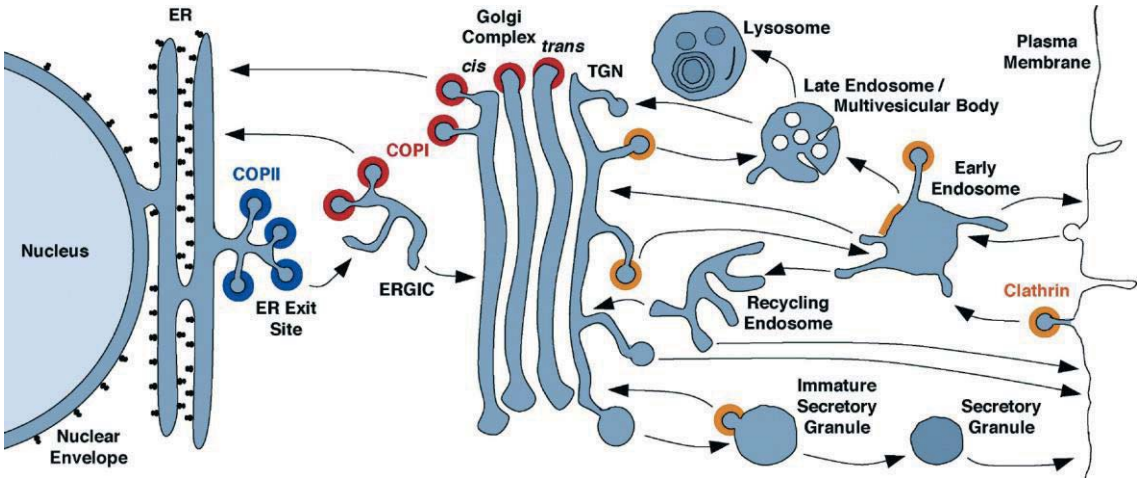

Figure 1-3: Vesicle transport between organelles of eukaryotic cells. COP-II vesicles mediate the anterograde transport of ER material to the Golgi, while COP-I vesicles assume the function of material retrieval to the ER, as well as mediating intra-Golgi transport. Clathrin-coated vesicles take on the task of relaying endocytic material, and facilitate material transport beyond the trans Golgi network (TGN). Image from Bonifacino and Glick, 2004.

While clathrin-coated vesicles are largely associated with endocytosis and transport between endosomes, and the trans Golgi network (TGN) in higher eukaryotes, COP-II and COP-I coated

[2] Transitional ER: specialized ER subdomain at which proteins destined for the Golgi are packaged into transport vesicles (Palade 1975)

3 SNARE: soluble (N-ethylmaleimide-sensitive factor) attachment protein receptor, mediates membrane fusion

vesicles carry out the material exchange between endoplasmic reticulum (ER) and Golgi apparatus and within the Golgi apparatus (Wooding and Pelham, 1998) (Figure 1-3). These organelles require vigorous transport of newly synthesized proteins, which undergo maturation as they pass from ER through the Golgi subcompartments. This anterograde movement of material from ER to Golgi has to be balanced by a retrograde transport system, which retrieves membranes, transport machinery components (e.g. cargo receptors, coat and adaptor proteins, SNAREs) and escaped resident proteins back to the ER. This retrograde trafficking step is carried out by COP-I coated vesicles (Letourneur *et al.*, 1994; Spang and Schekman, 1998; Shima *et al.*, 1999).

1.4.1 COP-I mediated transport

1.4.1.1 COP-I coat structure

The COP-I coat is composed of seven subunits, termed α-, β-, β′, γ, δ, ε- and ζ-COP[4]. They exist in assembled heptameric complexes abundantly present in the cytosol (Ghaemmaghami *et al.*, 2003). The complexes of COP-I coat subunits were also termed coatomer (Waters *et al.*, 1991). They form an adaptin-like, globular βδ/γζ subcomplex and a cage-forming αβ′ε subcomplex (Eugster *et al.*, 2000; Yip and Walz, 2011).

To date, the precise structure of the COP-I coat is still unknown. Partial crystal structures of the αβ′ε subcomplex suggest that it forms a triskelion, in which three copies of the β′-COP β-propeller domains converge by their axial ends, constituting the vertex of the COP-I cage. Meanwhile, the solenoid structures of α-COP and β′-COP curve around one another, perhaps forming the ‘legs’ of a cage. The C-terminal domain of α-COP interacts with ε-COP’s tetratricopeptide repeat (Hsia and Hoelz, 2010; Lee and Goldberg, 2010). Faini *et al.*, 2012 were able to obtain electron tomographic evidence for the surface structure of *in vitro* reconstituted COP-I vesicles (Figure 1-4).

[4] COP stands for *coat protein*

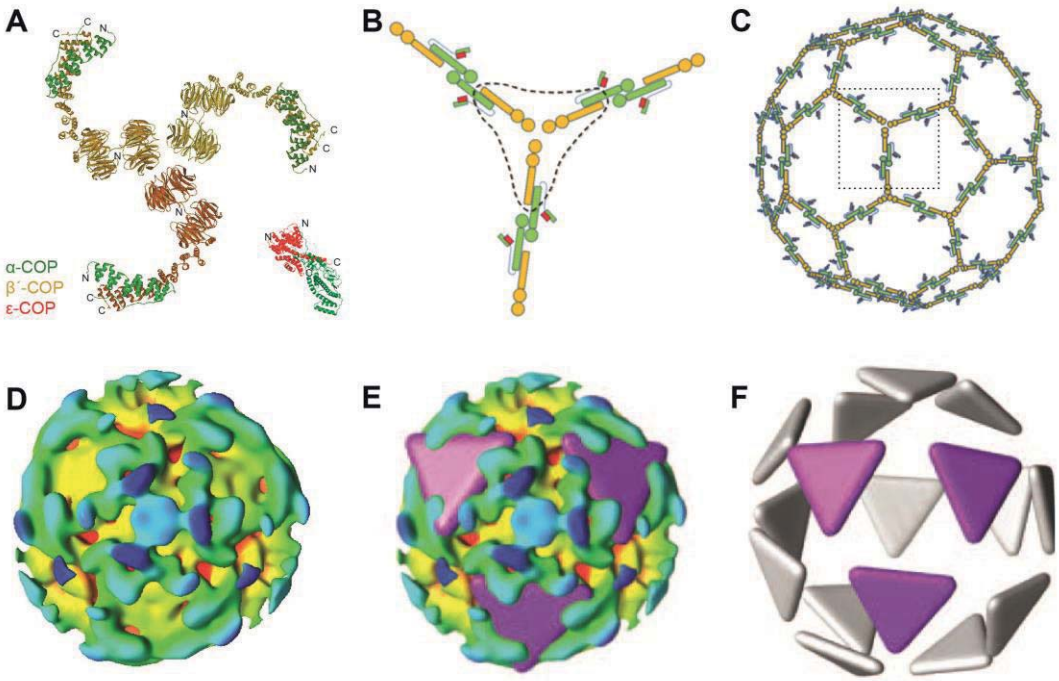

Figure 1-4: Insights into the structure of the COP-I coat. A. Crystal structure the αβ′ subcomplex (residues 1–814 of β′-COP and residues 642–818 of α-COP, PDB ID 3MKQ), and the αε subcomplex (residues 899-1224 of α-COP and residues 10-308 of ε-COP, PDB IDs 3MKR, 3MV2). **B**. schematic depiction of the proposed structure of the αβ′ε subcomplex of the COP-I coat. Area marked by dotted line represents the vertex crystal structure. **C**. Possible cage structure of the COP-I αβ′ε subcomplex. Area marked by dotted line marks one vertex repeat as in **B**. **D**. Depiction of the COP-I coat of *in vitro* reconstituted COP-I vesicles, as visualized by cryoelectron tomography. **E**. Isosurface representations of the COP-I coat structure, superimposed on lattice map (**F**). **F**. Lattice map of the COP-I structure. Triangles are believed to represent the αβ′-COP vertices as depicted in **A** and **B**. A-C modified from Lee and Goldberg, 2010; D-F modified from Faini *et al.*, 2012.

1.4.1.2 Cargo recognition by coatomer

Several mechanisms of cargo recognition ensure specific packaging of cargo in nascent COPI vesicles. The N-terminal WD40 β-propeller domains of α-COP and β′-COP are able to bind di-lysine K(x)Kxx motifs (Cosson and Letourneur, 1994; Letourneur *et al.*, 1994; Lowe and Kreis, 1995; Bremser *et al.*, 1999; Eugster *et al.*, 2004.) These are sorting signals at the C-terminus of proteins, which promote packaging into COP-I vesicles (Nilsson *et al.*, 1989; Jackson *et al.*, 1990). These protein motifs were found to bind at a site close to the vertex of the triskelion formed by three αβ′ε-COP assembly units (Lee and Goldberg, 2010; Jackson *et al.*, 2012; Ma

and Goldberg, 2013). This suggests that COPI coat nucleation may be coupled to recruiting cargo molecules. In fact, dilysine-tagged cargo molecules were reported to be required for efficient COPI coat assembly and vesicle formation (Bremser *et al.*, 1999; Aguilera-Romero *et al.*, 2008).

Another sorting motif is a group of arginine- based signals, which are recognized by subunits of the adaptor-like subcomplex, β- and δ-COP (Michelsen *et al.*, 2007).

Additionally, a di-phenylalanine motif with the consensus sequence FFXX(KR)(KR)X_n, $n \geq 2$ was discovered in p24 proteins (Fiedler *et al.*, 1996; Sohn *et al.*, 1996). The signal mediates binding to γ-COP (Harter and Wieland, 1998).

1.4.1.3 The p24 cargo receptor family

An example of COP-I cargo proteins relevant for this work is the p24 protein family (Lanoix *et al.*, 2001; Malsam *et al.*, 2005). This is a family of type I transmembrane proteins with eight members in yeast: Emp24p, Erv25p, and Erp1p to Erp6p. The members of the p24 family members are localized to COP-I and COP-II vesicles, as well as ER and cis-Golgi, and constantly shuttle between these compartments. p24 proteins are thought to primarily function as cargo receptors, but are also involved in COPI vesicle biogenesis. They act as receptors for the small GTPase Arf1p-GDP, thereby binding and inducing oligomerization of the coatomer complex (Strating and Martens, 2009). Additionally, a regulatory role in uncoating was proposed (Goldberg, 2000; Lanoix *et al.*, 2001).

All proteins have similar sequences (Stamnes *et al.*, 1995; Dominguez *et al.*, 1998; Marzioch *et al.*, 1999) and can be divided into the four subfamilies p24α, β, γ and δ (Dominguez *et al.*, 1998). They are thought to form heterooligomeric complexes with one member of each subfamily present, as was shown for the yeast p24 protein complex of Emp24p, Erv25p, Erp1p and Erp2p by Belden and Barlowe, 1996; Marzioch *et al.*, 1999; and Muniz *et al.*, 2000, and for mammalian p24 complexes by Lanoix *et al.*, 2001.

As mentioned in the previous section, the cytosolic tails of p24 proteins carry signal sequences for the packaging into COP-I vesicles (Belden and Barlowe, 1996; Marzioch *et al.*, 1999; Muniz *et al.*, 2000).

Intriguingly, besides their diverse functions, p24 proteins do not appear to be essential *in vivo*, as simultaneous deletion of all eight p24 proteins in yeast did not lead to major adverse effects (Springer *et al.*, 2000).

1.4.1.4 The COP-I transport cycle: a brief overview

Budding of COP-I vesicles if initiated the activation of a membrane-anchored ARF[5] GTPase through ARF-GEFs[6]. This recruits coatomer to the Golgi membrane (Palmer *et al.*, 1993; Hara-Kuge *et al.*, 1994; Yu *et al.*, 2012). The coating process is regulated by ARF GAPs[7], which bind not only ARF, but also the COP-I subunits γ-COP and β′-COP. This way it is ensured that only vesicles containing cargo are produced (Goldberg, 2000; Luo *et al.*, 2009). Subsequently, transmembrane cargo receptors, adapter proteins and SNAREs concentrate at the assembling coat, and Arf1 curves the membrane (Beck *et al.*, 2008; Krauss *et al.*, 2008; Lundmark *et al.*, 2008) (Figure 1-5). The forming vesicle neck is severed either by direct action of the coat or by accessory proteins (as in the case of clathrin), and set free from its donor organelle (Brandizzi and Barlowe, 2013).

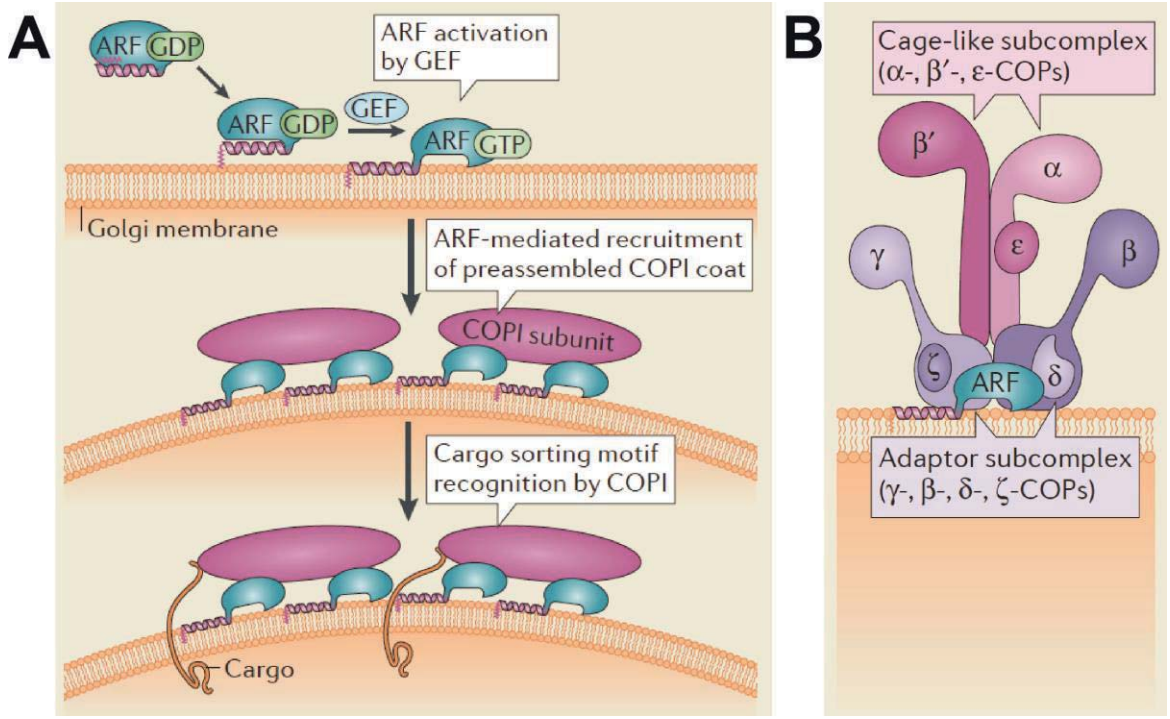

Figure 1-5: COP-I coat formation. A. COP-I coat formation is initiated through ARF recruitment to the membrane and activation through ARF-GEF. This leads to the assembly of COP-I complexes onto the membrane, as well as the recognition of COP-I cargo proteins. **B**. Schematic structure of the COP-I coat, including the cage-like αβ′ε- subcomplex and the adaptor-like γβ/δζ- subcomplex. Images from Brandizzi and Barlowe, 2013.

[5] ARF: ADP-ribosylation factor

[6] GEF: guanine nucleotide exchange factor

[7] GAP: GTPase activating protein

In contrast to mammalian cells, the subsequent transport between Golgi and ER does not rely on cytoskeletal components in yeast. Here, the short distances between ER and Golgi may be the reason that no ER intermediate compartment or cytoskeletal elements to restrict diffusion of transport intermediates, as found in higher eukaryotes, are needed (Presley *et al.*, 1997).
During the course of the COP-I transport cycle, uncoating of the vesicle must occur. This is due to the fact that for fusion, the vesicle must expose its membrane at least at the site of the future fusion pore. Stimulation of the GTPase activity of Arf1p by ARF GAPs leads to the release of Arf1p from the complex, which is followed by coat dissociation (Szul and Sztul, 2011; Shiba and Randazzo, 2012). It remains unclear whether ARF GAPs remain components of mature COPI vesicles, but it is understood that GTP hydrolysis through ARF is insufficient to cause coat dissociation (Szul and Sztul, 2011). An open question is the precise moment when fusion occurs. It was long assumed that vesicles undergo uncoating before they arrive at the target membrane, but recently the Dsl1 tethering complex at the ER membrane was proposed to be associated with the uncoating process (Zink *et al.*, 2009). Tethering complexes facilitate the initial attachment of arriving vesicles at the target membranes (Barlowe, 1997; Cao *et al.*, 1998). They are not required for *in vitro* fusion (Weber *et al.*, 1998; McNew *et al.*, 2000; Paumet *et al.*, 2004), but essential *in vivo*.

1.5 The Dsl1 tethering complex

The yeast Dsl1 complex tethers Golgi-derived COP-I vesicles at the ER (Andag *et al.*, 2001; Vanrheenen *et al.*, 2001). It belongs to the CATCHR[8] family, and with only three subunits is the simplest known multi-subunit tethering complex (MTC).
Its crystal structure was recently solved by Ren *et al.*, 2009 from a set of four overlapping structures (Figure 1-6). The three complex subunits Dsl1p, Dsl3p (also termed Sec39p) and Tip20p form a 30 nm tower which is anchored at the ER through a stable association with the ER SNAREs Ufe1p, Use1p, and Sec20p (Sweet and Pelham, 1993; Lewis *et al.*, 1997; Kraynack *et al.*, 2005). Dsl3p and Tip20p form the base of the complex, while Dsl1p at the

[8] CATCHR stands for *Complex Associated with Tethering Containing Helical Rods*. All proteins in this family share sequence homology of subunits and play a role at compartments along the exocytic and endocytic pathways. Other members are the COG complex at the Golgi, the GARP complex at endosomes and the exocyst at the plasma membrane (Whyte and Munro, 2001; Whyte and Munro 2002; Koumandou *et al.* 2007; Yu and Hughson 2010).