Grh1-dependent Unconventional Protein Secretion

Caroline Bruns

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THESIS DIRECTOR

Dr. Vivek Malhotra

Intracellular Compartmentation Laboratory

Cell and Developmental Biology Programm

Center for Genomic Regulation (CRG)

UNIVERSITAT POMPEU FABRA

Für meine Familie,

die immer zu mir stand,
und die mir all das ermöglicht hat
ohne genau zu verstehen, was ich mache;
aber immer an mich und meine Schritte geglaubt hat.

I Summary

Besides conventional secretion, in which proteins are transported through the endoplasmic reticulum (ER) and the Golgi complex, unconventional secretion routes bypassing the Golgi complex have been described for different proteins in several organisms. However, the mechanisms of their release remain poorly understood. It was reported that the unconventional secretion of the acyl-CoA binding protein Acb1 from *Saccharomyces cerevisiae* requires a diverse group of proteins including the GRASP ortholog Grh1, autophagy-related proteins, proteins involved in fusion of membranes with endosomes, members of the ESCRT-machinery, and the plasma membrane t-SNARE Sso1. How these proteins work together for Acb1 secretion remains elusive.

Our findings indicate that upon nutrient starvation, the condition known to induce unconventional secretion of Acb1, Grh1 is concentrated in a phosphatidylinositol 3-kinase-dependent manner to unique membrane structures near the ER exit sites. These membranes –shaped like cups– are enriched in PI(3)P and contain the ESCRT-I protein Vps23 as well as the autophagy-related proteins Atg8 and Atg9 thereby bringing together different proteins required for Acb1 secretion. We have named these structures CUPS (Compartment for Unconventional Protein Secretion), based on their shape and content. CUPS, we propose, are the starting point for the formation of Acb1-containing vesicular intermediates dedicated for unconventional secretion.

II Resum

A part de la secreció convencional, en la qual les proteïnes són transportades a través del reticle endoplasmàtic (RE) i de l'aparell de Golgi, s'han descrit, per a diferents proteïnes i en organismes diversos, rutes de secreció no-convencional que circumval·len l'aparell de Golgi. No obstant, aquests mecanismes de secreció no estan ben entesos. Se sap que per a la secreció no-convencional de la proteïna d'unió a acil-CoA Acb1 a *Saccharomyces cerevisiae* són necessàries diverses proteïnes, com ara l'ortòleg de GRASP Grh1, proteïnes relacionades amb l'autofàgia, proteïnes involucrades en la fusió de membranes amb els endosomes, membres de la maquinària ESCRT, i la t-SNARE de la membrana plasmàtica Sso1. La manera per la qual totes aquestes proteïnes cooperen per a dur a terme la secreció d'Acb1 és encara una incògnita.

Els resultats de les nostres investigacions indiquen que sota inanició de nutrients, condició que indueix la secreció no-convencional d'Acb1, Grh1 es concentra en estructures membranoses prop dels llocs de sortida del RE, de manera dependent de fosfatidilinositol-3-cinasa. Aquestes membranes, en forma de tassa, estan enriquides en PI(3)P i contenen la proteïna d'ESCRT-I Vps23 així com les proteïnes d'autofàgia Atg8 i Atg9, reunint així diverses proteïnes necessàries per a la secreció d'Acb1. Hem batejat aquestes estructures CUPS (per les sigles en anglès de compartiment per a la secreció no-convencional de proteïnes), basant-nos en la seva forma i el seu contingut. Proposem que els CUPS són l'estació de sortida per a la formació d'intermediaris vesiculars dedicats a la secreció no-convencionals que contenen Acb1.

III Preface

The work presented in this doctoral thesis was carried out in the Cell and Developmental Biology Program at the Centre for Genomic Regulation (CRG) in Barcelona, Spain under the supervision of Dr. Vivek Malhotra (ICREA, CRG).

The content of this thesis provides novel insights in the mechanisms of unconventional protein secretion and part of this thesis was published in the Journal of Cell Biology (2011), 195:979-992.

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1 Introduction

1.1 Conventional Secretory Pathway

The endoplasmic reticulum (ER) and the Golgi apparatus are the central organelles of the classical secretory pathway in eukaryotic cells, through which the vast majority of proteins are transported to their final destinations. Soluble secretory proteins contain a signal peptide (SP) at their N terminus, typically consisting of 16-30 amino acids with a positively charged region at the N-terminal end, followed by a central hydrophobic stretch and a cleavage site for the signal peptidase (von Heijne, 1985). In a nascent polypeptide, this SP will be recognized by the signal recognition particle, thereby allowing co-translational transport into the lumen of the ER, where the SP will be immediately cleaved after translocation (von Heijne, 1985; Walter et al., 1984). In the ER, the newly synthesized polypeptide chain is properly folded and core glycosylated. In most of the cases, proteins, that do not contain an ER retention signal, are exported via coat protein complex II (COPII)-coated vesicles. The formation of COPII vesicles occurs in most eukaryotes at specific ER domains known as ER exit sites. However, in the budding yeast *Saccharomyces cerevisiae,* it is thought to occur throughout the entire ER (Orci et al., 1991a; Rossanese et al., 1999; Schekman and Novick, 2004). The formation of this small vesicular transport carrier (typically 60-90nm in diameter) is initiated by the activity of the GTP exchange factor (GEF) Sec12 (Nakano et al., 1988). Sec12 catalyzes the exchange of GDP for GTP on the small

GTPase Sar1 and thereby induces the insertion of the N-terminal amphipathic helix of Sar1 into the ER membrane (Bielli et al., 2005). Sar1-GTP then binds to Sec23 of the Sec23/Sec24 heterodimer and recruits it to the membrane (Barlowe et al., 1994; Hicke et al., 1992). In yeast, the heterodimer can also consist of Sec23 and one of the Sec24 homologues, Sfb2 or Sfb3. Sec24 (and Sfb2/Sfb3) is involved in recognition and clustering of transmembrane domain containing cargo molecules into the so-called prebudding complex (Aridor et al., 1998; Miller et al., 2002; Miller et al., 2003). Then, the outer layer of the coat, consisting of Sec13/Sec31, is recruited to the prebudding complex by direct binding of Sec31 to Sec23 (Bi et al., 2007). Sec23 itself is a GTPase activating protein (GAP) for Sar1, thus stimulating the hydrolysis of Sar1-GTP to Sar1-GDP. The interaction of Sec31 with Sec23 further enhances this GAP activity. The activation of the GTPase activity and the conversion of GTP to GDP lead to the release of the components from the membrane (Barlowe and Schekman, 1993; Hughes and Stephens, 2008; Yoshihisa et al., 1993). The activation and regulation of Sar1 GTPase activity and the recruitment of the outer coat components are thought to time the formation and completion of the COPII vesicle. Its scission occurs independently of a dynamin-like GTPase (Lee et al., 2005a; Matsuoka et al., 1998). The six proteins mentioned above are sufficient for COPII vesicle biogenesis; however, additional factors like Sec16 are involved in this process putatively by stabilizing the prebudding complex (Supek et al., 2002). COPII vesicles are essential for the export of proteins from the ER and they are transported along microtubules towards to the *cis*-face of the Golgi stack (Barlowe et al., 1994; Ellgaard and Helenius, 2003; Jensen and Schekman, 2011; Kirchhausen, 2000; Murshid and Presley, 2004).

Proteins reaching the Golgi apparatus are either recycled back to the ER in coat protein complex I (COPI)-coated vesicles by binding to the KDEL receptor (HDEL receptor in yeast) or transported through the cisternae of the Golgi stack (Bonifacino and Glick, 2004; Lewis and Pelham, 1996).

Similarly to COPII vesicle biogenesis, the formation of COPI-coated vesicles also depends on a small GTPase, the ADP-ribosylation factor 1 (ARF1) (Donaldson et al., 1992). Soluble ER resident proteins contain an HDEL/KDEL motif at their C terminus, which is recognized by a specific receptor (Erd2 in yeast and KDELR in mammals) (Lewis and Pelham, 1990; Semenza et al., 1990). The receptor itself interacts with the GEF of ARF1, GBF1 (Golgi-specific brefeldin A [BFA]-resistant guanine nucleotide exchange factor 1). GBF1 then activates ARF1 by exchanging GDP to GTP and thereby anchoring ARF1-GTP to the membrane (Kawamoto et al., 2002). The initial activation step also seems to facilitate interaction with the coatomer and additional cargo molecules. The COPI coatomer consist of seven subunits (α, β, β', γ, δ, ε, ζ) which form a heptameric complex (Malhotra et al., 1989; Serafini et al., 1991). The coatomer itself binds to transmembrane proteins containing a KKXX motif and allowing their return to the ER (Cosson and Letourneur, 1994; Letourneur et al., 1994). Both the coatomer and the GAP for Arf1, ARFGAP1 (ADP-ribosylation factor GTPase activating protein 1) are required for vesicle budding and cargo recruitment/sorting (Lee et al., 2005b) but unlike COPII vesicles, COPI vesicles need additional factors for their membrane fission. The BFA ADP-Ribosylated Substrate (BARS) together with phosphatidic acid were identified as factors involved in membrane

bending and are a prerequisite for the final fission step (Deng et al., 2009).

On their route through the Golgi apparatus, proteins can be further post-translationally modified (Bergmann and Singer, 1983; Dunphy et al., 1981) and at the level of the *trans*-Golgi network (TGN) they are sorted in specific carriers for transport to their final destination. Whereas proteins exiting the ER are transported to the Golgi apparatus, sorting of cargo at the level of the TGN does not only need to distinguish between resident proteins and those that are exported, the cargo molecules also need to be sorted into different classes of vesicles according to their destination, which can be an endosomal-lysosomal compartment, the cell surface or, in professional secretory cells, the secretory storage granules (Figure 1) (De Matteis and Luini, 2008; Farquhar, 1985; Griffiths and Simons, 1986; Gu et al., 2001). For instance, the sorting of lysosomal hydrolases is mediated by cargo binding to the mannose 6-phosphate receptor and subsequent export in clathrin-coated vesicles (Ghosh et al., 2003; Kornfeld and Mellman, 1989). Integral membrane proteins are thought to expose an export signal at their cytoplasmic tail, but no general export mechanism at the level of the TGN has been yet identified (Ang et al., 2003; Folsch et al., 1999; Salvarezza et al., 2009). It is even less clear how soluble proteins are sorted for transport to their final destination. However, if the secretory proteins do not contain a signal for trafficking to specific compartments inside the cell, they are transported to the plasma membrane (bulk secretion) (Pfeffer and Rothman, 1987). The fusion step of the transport carrier with the correct target membrane is controlled by specific target (t-) and vesicular (v-) SNAREs (soluble N-ethylmaleimide sensitive fusion protein [NSF] attachment

protein receptors), small Rab-GTPases and various tethering complexes (Barlowe, 2000; Rothman, 1994).

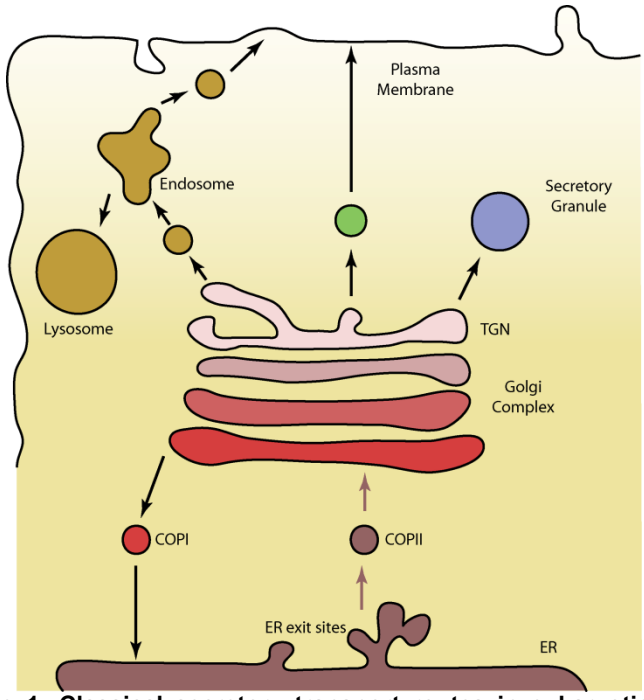

Figure 1: Classical secretory transport routes in eukaryotic cells (Malhotra and Campelo, 2011; modified). Proteins containing a SP are translocated into the ER and leave the ER at specific ER exit sites in COPII vesicles directed to the Golgi complex. In case they are resident proteins of the ER, they are transported back to the ER in COPI vesicles. Otherwise they cross the Golgi stack and at the level of the TGN they are sorted for transport to their final destination which can be the endolysosomal system, a secretory storage granule, or the cell surface/extracellular space.

The vast majority of proteins being secreted from the cell into the extracellular milieu use the organelles of the conventional pathway, for instance extracellular matrix components or hormones. Their secretion can be constitutive, meaning that newly synthesized proteins are secreted as soon as they are made and processed, or regulated (Burgess and Kelly, 1987). Protein that are secreted in a regulated manner are transported from the Golgi apparatus to secretory storage granules, which will eventually fuse with the plasma

membrane to release their content but only when the appropriate stimulus is detected, for example Ca^{2+}-influx for neurotransmitter release from the synapse (Sudhof, 2004).

The basic concept for this classical secretory pathway, namely the entering of secretory proteins into the ER prior to their transport to different organelles within the cells or beyond the cellular boundaries, was first postulated by Palade and co-workers (Palade, 1975). Since then, this pathway has been extensively investigated. For instance, studies in the budding yeast led to the identification of several genes required at different stages of the secretory pathway, the SEC (secretion) genes (Kaiser and Schekman, 1990; Novick et al., 1981) and also the COPII coat was first described in yeast (Barlowe et al., 1994). In many cases, orthologs were identified in mammalian cells so that a map describing the key events in this highly conserved conventional secretory route could be established, which is similar to the pathway suggested by Palade (Pfeffer, 2007).

1.2 Unconventional Protein Secretion

The conventional secretory pathway is a highly efficient transport route used by the vast majority of secretory proteins as mentioned above. However, for secretion of some proteins, eukaryotic cells use another pathway that does not require transport through the Golgi apparatus: the so-called unconventional protein secretion. Proteins following this pathway have crucial roles for cell survival, homeostasis, and tissue surveillance and their secretion is a regulated process stimulated by external triggers, mostly related to stress (Nickel, 2003; Nickel and Rabouille, 2009; Radisky et al.,

2009). In several cases, the specific functions of the unconventionally secreted proteins outside the cell are quite distinct from their intracellular functions (transglutaminase or thioredoxin) whereas other proteins seem to be solely functional outside the cell (Interleukin [IL]-1ß; galectin-1 and 3, fibroblast growth factor 2 [FGF2]) (Nickel and Seedorf, 2008; Radisky et al., 2009).

By far the best understood mechanism of unconventional secretion is the release of the yeast a-factor pheromone, which occurs through the cell surface ATP-binding cassette (ABC) transporter Ste6 (Kuchler et al., 1989; McGrath and Varshavsky, 1989). Although the number of proteins identified to be secreted unconventionally has increased over the years, the mechanism of their release into the extracellular space remains poorly understood in most cases (Nickel, 2010; Nickel and Rabouille, 2009; Nickel and Seedorf, 2008). Some unconventionally secreted proteins are translocated into the ER but leave the ER in a COPII-independent mechanism and, therefore, their secretion is independent of the Golgi apparatus. However, most unconventionally secreted proteins are cytosolic/nuclear proteins believed to be secreted independently of both the ER and the Golgi apparatus. Whether translocated into the ER or not, unconventionally secreted proteins lack the post-translational modifications usually obtained during transport through the Golgi stack and their secretion is resistant to treatment with the fungal metabolite BFA, which blocks vesicular transport along the classical secretory pathway (Lippincott-Schwartz et al., 1989; Misumi et al., 1986; Orci et al., 1991b). Examples of the first type of unconventional secretion, where proteins translocate into the ER, are the *Drosophila melanogaster* αPS1 integrin subunit, the yeast protein Ist2 or the cystic fibrosis

transmembrane conductance regulator (CFTR) (Juschke et al., 2005; Schotman et al., 2008; Yoo et al., 2002). By contrast, proteins like IL-1ß, galectin-1 and 3, transglutaminase, FGF2, or acyl-CoA binding proteins (ACBPs) are cytoplasmic proteins without an obvious ability to enter the ER –as they neither possess a SP nor a transmembrane domain. Their secretion is therefore independent of the ER and the Golgi apparatus (Cooper and Barondes, 1990; Duran et al., 2010; Florkiewicz et al., 1995; Kinseth et al., 2007; Loomis et al., 2010; Lorand and Graham, 2003; Manjithaya et al., 2010; Rubartelli et al., 1990).

Four different principal mechanisms have been postulated for the unconventional secretion of cytoplasmic/nuclear proteins: (1) secretion mediated by secretory lysosomes; (2) direct translocation across the plasma membrane; (3) secretion of exosomes; and (4) secretion by plasma membrane blebbing and vesicle shedding (Figure 2) (Nickel, 2005). Nevertheless, even if the secretion process is in the same category, different proteins might require several unique factors (Nickel and Seedorf, 2008). Also, as the secretion routes are quite distinct amongst the unconventionally secreted proteins, a common recognition motif similar to the SP for the conventional pathway has yet to be identified. However, crucial amino acids have been mapped for single proteins (insulin degrading enzyme (IDE), ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) (Glebov et al., 2011; Konya et al., 2011).

An example for direct translocation across the plasma membrane is the release of FGF2, which is thought to be mediated by binding to phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) at the plasma membrane and subsequent membrane translocation in a heparan sufate proteoglycan-dependent manner (Schafer et al., 2004;

Temmerman et al., 2008; Zehe et al., 2006). Galectin-1 and 3, which are known to bind to ABO blood group structures and matrix glycoproteins, are potentially released by plasma membrane blebbing and vesicle shedding (Cooper and Barondes, 1990; Mehul and Hughes, 1997; Sato and Hughes, 1992). Only about 20-30% of newly synthesized galectin-3 has been reported to be secreted within 24 hours, with the transport to the plasma membrane as a rate limiting step (Mehul and Hughes, 1997; Sato et al., 1993); and lectin-loaded structures blebbing from the cell surface have been detected suggesting a secretion by membrane evaginations (Harrison and Wilson, 1992; Sato et al., 1993). IL-1ß as well as high-mobility group box 1 (HMGB1) were reported to be sequestered into secretory lysosomes and released upon fusion with the plasma membrane (3) (Andrei et al., 1999; Andrei et al., 2004; Bonaldi et al., 2003; Gardella et al., 2002; Rubartelli et al., 1990) however, molecular factors or mechanisms involved in this process remain elusive.

The fourth proposed secretion mechanism involving exosomes most likely requires multivesicular bodies (MVB). MVBs are usually derived from endosomes by inward budding of its surrounding membrane to give rise to cargo-filled intralumenal vesicles (ILVs) (Piper and Katzmann, 2007). The targeting of transmembrane proteins to the MVBs relies on specific ubiquitination and the conserved endosomal sorting complexes requited for transport (ESCRTs) of which some are able to bind to mono-ubiquitinated and Lys-63-linked poly-ubiquitinated cargo molecules (Duncan et al., 2006; Hicke and Dunn, 2003).

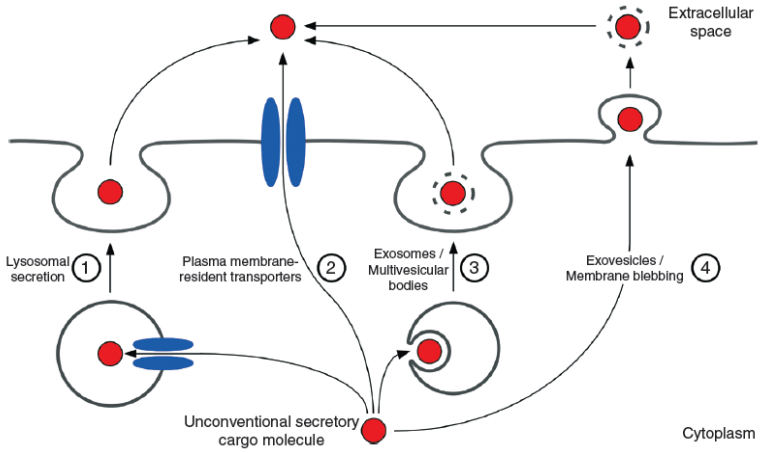

Figure 2: Four main routes for unconventional protein secretion (Nickel, 2005). Putative vesicular and non-vesicular pathway supposedly involved in unconventional protein secretion. (1) secretion by secretory lysosomes; (2) direct translocation across the plasma membrane; (3) secretion of exosomes; and (4) export of microvesicles through plasma membrane blebbing.

ESCRT components were first described in yeast by the accumulation of abnormal endocytic structures referred to as class E compartments (Banta et al., 1988; Raymond et al., 1992; Robinson et al., 1988). Four complexes were identified: ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, which are soluble complexes unless they interact with ubiquitinated transmembrane proteins, phosphatidylinositol 3-phosphate (PI(3)P) or other ESCRT complexes (Hurley and Emr, 2006; Piper and Katzmann, 2007; Saksena et al., 2009). ESCRT-0 consists of a Vps27/Hse1 heterodimer, of which Vps27 is able to bind to PI(3)P and both proteins bind to ubiquitinated cargo, thereby recruiting itself and downstream ESCRT complexes sequentially to endosomes (Henne et al., 2011). ESCRT-0 is required to recruit ESCRT-I (Vps23, Vps28, Vps37, Mvb12) by binding of Vps23 to Vps27. Vps23 is in addition able to bind to ubiquitin (Curtiss et al., 2007; Katzmann et al., 2001; Katzmann et al., 2003). ESCRT-I also interacts with ESCRT-II (Vps22, Vps25, Vps36), but

on its opposite end via binding of Vps28 to Vps36 (Babst et al., 2002b; Gill et al., 2007; Langelier et al., 2006; Teo et al., 2006). Vps36 is also able to bind to PI(3)P and to ubiquitinated cargo molecules (Slagsvold et al., 2005). ESCRT-III consists of Vps2, Vps20, Vps24, and Snf7 (Babst et al., 2002a). In contrast to ESCRT-0,-I and -II, ESCRT-III assembly occurs at the endosomes after Vps20 recruitment by its binding to ESCRT-II subunit Vps25 and its subsequent interaction with the membrane via an N-terminal myristoylation (Teo et al., 2004). Crucial for cargo sorting into the ILVs is the recruitment of the deubiquitinating enzyme Doa4 via interaction with Snf7 and subsequent cargo deubiquitination (Luhtala and Odorizzi, 2004; Odorizzi et al., 2003). Once it is assembled, ESCRT-III is thought to drive inward budding and vesicle maturation via controlled Snf7-homo-oligomerization and maybe via filaments of the Vps24/Vps2 subcomplex (Ghazi-Tabatabai et al., 2008; Hanson et al., 2008; Wollert et al., 2009). The recruitment of the ATPase Vps4 is believed to be involved in the disassembly of the mature ESCRT-III complex (Babst et al., 1998) and this disassembly seems to play a role in the final constriction of the bud neck required to release the ILVs into the lumen of the MVB (Saksena et al., 2009). Upon fusion of the MVB with the lysosome the ILVs are eventually degraded (Piper and Katzmann, 2007).

However, sorting of proteins into ILVs does not exclusively destine them for lysosomal degradation. For example, class II major histocompatibility complex and tetrapanins accumulate in specialized MVBs, which upon fusion with the plasma membrane release the ILVs as exosomes (Simons and Raposo, 2009; Thery et al., 2009; van Niel et al., 2006). Additionally, the unconventional secretion

process of some proteins seems to rely on exosome release upon fusion of a MVB with the cell surface. Secretion of IL-1ß has also been shown to depend on exosome release after inflammasome activation, indicating a potential role of MVB or MVB-like structures (Qu et al., 2007). HMGB1, which functions extracellularly as a pro-inflammatory cytokine, is secreted mainly from monocytes and macrophages and at least partially associated with exosomes (Bonaldi et al., 2003; Gardella et al., 2002; Liu et al., 2006). Furthermore, the ACBP orthologs in the social amoebae *Dictyostelium discoideum* (AcbA) and in the yeasts *S. cerevisiae* and *Pichia pastoris* (Acb1) were identified as unconventionally secreted proteins under nutrient-starvation conditions and the secretion of Acb1 was reported to require factors involved in MVB biogenesis (Duran et al., 2010; Kinseth et al., 2007; Manjithaya et al., 2010). Interestingly, Acb1 secretion not only requires MVB pathway, it additionally relies on proteins known to be necessary for autophagy, the Atg proteins (see chapter 1.3) (Duran et al., 2010; Manjithaya et al., 2010), Also, the secretion of IL-1ß has been recently linked to the autophagy machinery (Dupont et al., 2011) suggesting that cytosolic cargoes could be captured by an autophagosome and, at least in mammals, autophagosomes are known to be able to fuse with MVB to generate amphisomes (Berg et al., 1998). Not only is the requirement of autophagy-related proteins common for the secretion of Acb1 and IL-1ß, but they both also require a protein called GRASP (*G*olgi *R*e-*A*ssembly *S*tacking *P*rotein) (Dupont et al., 2011; Duran et al., 2010; Manjithaya et al., 2010), which has been localized to Golgi membranes in higher eukaryotes (Barr et al., 1997; Shorter et al., 1999). The proposed functions and the current knowledge of GRASP in unconventional protein secretion are discussed in more detail below (see chapters 1.4 and 1.4.1).

1.3 Autophagy

Autophagy is a highly conserved, stress-induced bulk degradation process by which cytoplasmic material like proteins and lipids or even entire organelles (mitochondria, peroxisomes, ER) are transported to the lysosome (vacuole in yeast) for degradation. Depending on the pathways by which the material is delivered to the lysosome, three types of autophagy are described: (1) chaperone-mediated autophagy, (2) microautophagy and (3) macroautophagy (Figure 3) (Lynch-Day and Klionsky, 2010). Chaperone-mediated autophagy occurs via direct translocation of a cytosolic protein harboring a specific signal sequence through the lysosomal membrane via LAMP-2A. It requires the molecular chaperone Hsp70 and has so far only been described in mammalian cells (Cuervo and Dice, 1996). Microautophagy is characterized by inward invaginations of the lysosomal membrane thereby sequestering a portion of the cytoplasm (Wang and Klionsky, 2003). The third mechanism, macroautophagy, hereafter referred to as autophagy, requires the formation of an autophagosome. In yeast, autophagosome formation initiates at the pre-autophagosomal assembly site (PAS), an organization centre close to the vacuole where several Atg proteins are sequestered upon induction of autophagy (Kim et al., 2002; Suzuki et al., 2001).

From the PAS, nucleation of a double-membrane structure called phagophore or isolation membrane occurs which engulfs cytoplasmic content and organelles. After closure of the phagophore, the vesicular intermediate termed autophagosome fuses with the vacuole (lysosome) and delivers its content for degradation (Rubinsztein et al., 2012). Degraded material is eventually transported back into the cytosol for reuse, which is especially important

when nutrients are limiting (for instance upon starvation) (Klionsky, 2007). However, besides this recycling process, autophagy is also important for clearance of old or misfolded proteins and damaged organelles. Recently, this process has also been linked to cancer, immunity, neurodegenerative disorders, aging, cell development, and differentiation (in yeast also for sporulation) (Cuervo and Macian, 2012; Ravikumar et al., 2010).

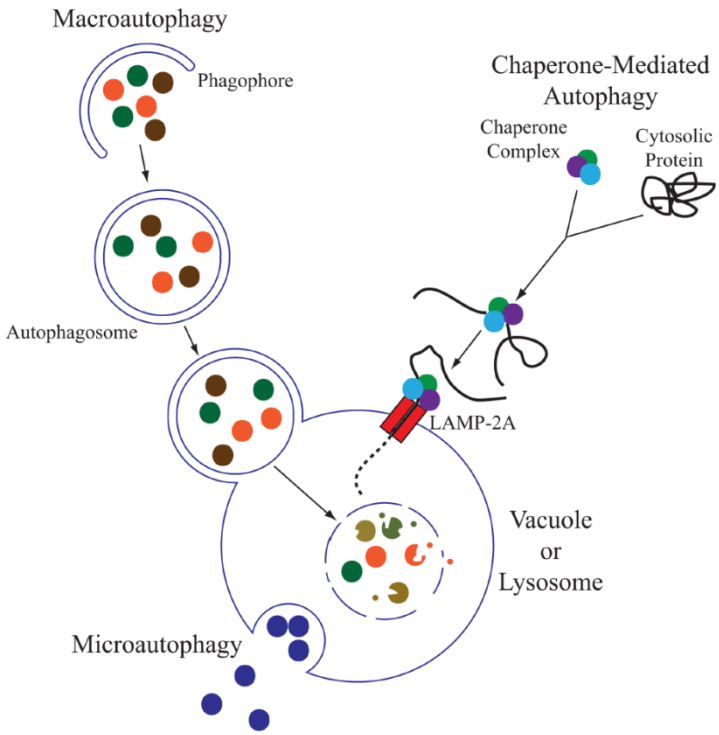

Figure 3: Three main types of autophagy (Lynch-Day and Klionsky, 2010). Three main degradative routes of autophagy have been described. For microautophagy, the lysosomal membrane invaginates and thereby sequesters cytoplasmic material. Chaperone-mediated autophagy is mammalian-specific and requires the molecular chaperone Hsp70. It recognizes proteins harboring a specific signal sequence and those will be transported through the lysosomal membrane via LAMP-2A. Macroautophagy involves the formation of a double-membrane vesicular structure, the autophagosome into which cytoplasmic material and organelles are captured and delivered to the vacuole/lysosome.

The term autophagy was first described by de Duve in 1963 as “self” (auto) “eating” (phagy) process (De Duve, 1963). However, only during the past two decades has the molecular machinery for the formation of autophagosomes started to be identified. Studies from the Ohsumi, Thumm and Klionsky lab used yeast genetics to identify mutants that were defective in autophagy, which led to the description of around thirty autophagy-related (Atg) genes (Harding et al., 1995; Klionsky et al., 2003; Thumm et al., 1994; Tsukada and Ohsumi, 1993).

Autophagy is induced in response to physiological stress, like nutrient starvation or oxidative stress. Two key signaling proteins for autophagy have been identified: target of rapamycin (TOR) and class III phosphatidylinositol 3-kinase (PI3K), amongst which TOR was described as a master regulator in response to amino acid starvation (Blommaart et al., 1997; Blommaart et al., 1995; Jung et al., 2010; Seglen and Gordon, 1982). TOR is present in two distinct complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2) of which only TORC1 is sensitive to rapamycin treatment and known to negatively regulate autophagy (Blommaart et al., 1995; Noda and Ohsumi, 1998; Ravikumar et al., 2004). Induction of autophagy requires the activity of the Atg1 complex at the PAS, which consists of the serine/threonine kinase Atg1, Atg13 and the Atg17/Atg31/Atg29 subcomplex (Mizushima, 2010). Under nutrient-rich conditions, Atg13 is hyperphosphorylated by TORC1 which abolishes the interaction with Atg1, thereby inhibiting Atg1 activity and autophagy (Kamada et al., 2010). TORC1 inhibition by nutrient starvation or treatment with rapamycin leads to dephosphorylation of Atg13, recruitment of Atg1 to the complex and subsequent Atg1 autophosphorylation, and finally induction of autophagy (Cebollero

and Reggiori, 2009; Kamada et al., 2010; Mizushima, 2010; Yeh et al., 2010). The active complex is crucial for the recruitment of downstream Atg proteins, however, this seems to be kinase-independent and so far, there are no known direct phosphorylation targets of Atg1 (Cheong et al., 2008; Kabeya et al., 2005; Kamada et al., 2000). It is worth mentioning that Atg1-complex is also the target of two other kinases, the cAMP dependent protein kinase A (PKA) and the AMP kinase (AMPK): PKA phosphorylates Atg1 and Atg13, thereby inhibiting autophagy; whereas AMPK phosphorylates Atg1, thereby inducing autophagy (Stephan et al., 2009; Wilson et al., 2002).

To initiate autophagosome nucleation, PI(3)P and therefore the activity of the PI3K Vps34 is essential. Vps34 is present in two distinct complexes: complex I is composed of Atg14, Atg6, Vps15, and Vps34, whereas complex II contains Vps38, Atg6, Vps15, and Vps34 (Kihara et al., 2001). Only complex I is implicated in autophagy and Atg14 is known to recruit the complex to the PAS (Figure 4) (Obara et al., 2006). However, exactly how complex I is regulated and its relationship to TORC1 and the Atg1-complex still need to be unraveled.

Three PI(3)P-binding proteins have been identified in yeast, which are linked to autophagy: Atg18, which has important roles for autophagy and the cytoplasm to vacuole transport (Cvt) pathway (chapter 1.2.1), Atg21, which is mainly required for the Cvt pathway; and Ygr223c, which is implicated in micronucleophagy (Krick et al., 2008). These three proteins contain WD-40 repeats and bind to PI(3)P (and also to $PI(3,5)P_2$) via a FRRG motif (Dove et al., 2004; Stromhaug et al., 2004). Atg18, together with its binding partner Atg2, act downstream of the Atg1- and PI3K-complexes,

and presumably function at late steps where they might facilitate the recruitment of Atg8 and/or Atg9 to the PAS (Nair et al., 2010).

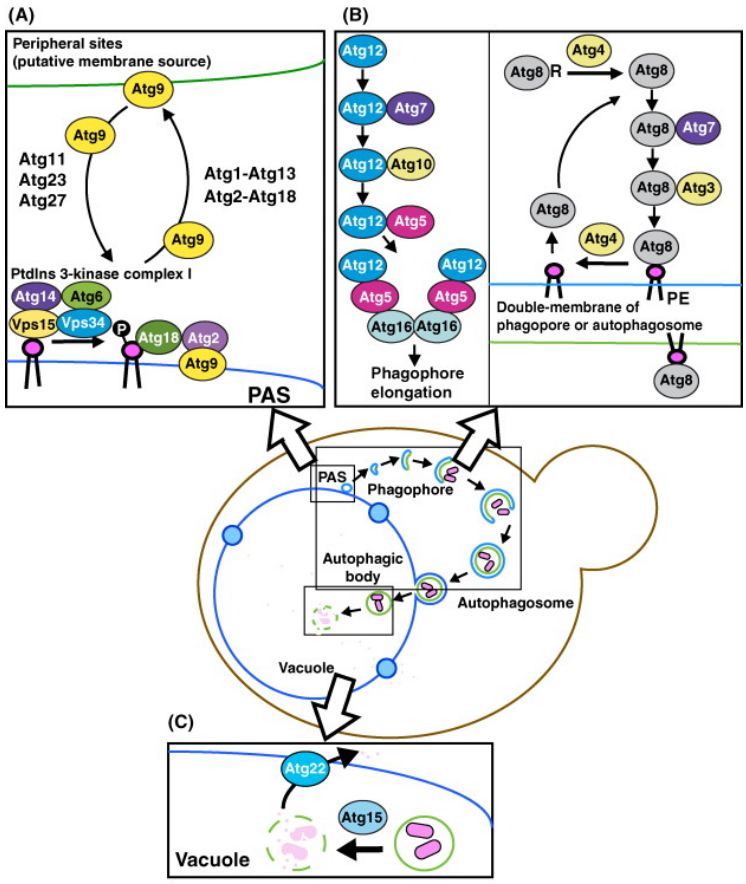

Figure 4: Molecular mechanism of autophagosome biogenesis (Inoue and Klionsky, 2010). (A) Trafficking of the integral membrane protein Atg9 between the PAS and a peripheral site depends on several Atg proteins which are either involved in anterograde transport to the PAS (Atg11/23/27) or in retrograde transport (Atg1/13 and Atg2/18). (B) Two ubiquitin-like conjugation systems are involved in expansion of the phagophore membrane. Atg12 is activated by Atg7 and conjugated to Atg5 by Atg10. The Atg5/Atg12 complex then binds to Atg16, allows the dimerization of the latter one and the recruitment of the entire complex to the phagophore. They will dissociate upon autophagosome completion. Atg8 as a ubiquitin-like protein is modified by Atg4, activated by Atg7 and conjugated to PE by Atg3. Atg8-PE is localized to inner and outer membrane of the expanding phagophore and removed from the outside upon autophagosome completion by Atg4. Atg8-PE on the inner surface will be delivered into the vacuole. (C) For the final degradation of the autophagic body within the vacuole, the putative lipase Atg15 is required. The breakdown products are transported back into the cytoplasm through permeases like Atg22.

Although a clear function has not yet been assigned to Atg9, it is believed to be involved in lipid-supply to the growing phagophore as it is the only multi transmembrane-spanning Atg protein (Noda et al., 2000). Under non autophagy-inducing conditions, Atg9 is present in vesicular intermediates close to the mitochondria, which origin and composition are just starting to be deeper elucidated (Kakuta et al., 2012; Mari et al., 2011; Yamamoto et al., 2012). Upon induction of autophagy, Atg9 cycles between these vesicles, also termed Atg9-reservoir, and the PAS. Atg11/23/27 are required to target Atg9 to the PAS and its transport back to the vesicular intermediates involves Atg1/13 and Atg2/18. By contrast, Atg9 localization at the PAS is necessary to efficiently recruit Atg8 and Atg12 (Suzuki et al., 2001).

Atg8 and Atg12 are part of two ubiquitin-like conjugation systems, which are required for expansion of the phagophore. Atg12 is activated by the E1-ubiquitin activation enzyme-like protein Atg7 and conjugated by Atg10 (E2-ubiquitin conjugation enzyme) to Atg5 (Mizushima et al., 1998). The Atg5/Atg12 complex binds to Atg16 thereby inducing the dimerization of Atg16, which leads to the formation of a dimeric complex (2x Atg5/Atg12/Atg16) and to the recruitment of this complex to the PAS and the phagophore where it might act as an E3 ligase for Atg8 (Hanada et al., 2007; Kuma et al., 2002). Atg8 is an ubiquitin-like molecule and its C-terminal arginine is cleaved by the cysteine protease Atg4 thereby exposing a C-terminal glycine (Kabeya et al., 2004; Kirisako et al., 2000). The processed Atg8 is activated by Atg7 and conjugated by Atg3, a second E2-ubiquitin conjugation enzyme, to an amino group of the lipid phosphatidylethanolamine (PE) (Ichimura et al., 2000). Atg8-PE localizes to both the growing phagophore and the

autophagosome, but how this recruitment occurs is still unknown. Atg4 also functions as a deconjugation enzyme, removing Atg8-PE from the outer membrane but not the Atg8-PE bound to the inner membrane, which is kept in the autophagosome and further transported to the vacuole (Kirisako et al., 1999; Kirisako et al., 2000). The outer membrane fuses with the vacuole and the inner membrane will be released as an autophagic body. This is degraded by the putative lipase Atg15 and the breakdown products are transported back into the cytosol through permeases like Atg22 (Epple et al., 2001). In the past twenty years, the key proteins involved in autophagosome formation have been identified and also upstream kinase complexes have been unraveled. Nevertheless, the interplay between these complexes to induce autophagy is still not fully understood and the source of the membrane still remains an unanswered question. Therefore a lot of effort still needs to be done to get a complete understanding of the mechanism of autophagosome formation.

1.3.1 Cytoplasm to vacuole transport (Cvt) pathway

Besides the non-selective bulk degradation process induced upon starvation, selective pathways of autophagy have been described, for example the specific degradation of mitochondria (mitophagy) or the ER (reticulophagy), which occur in both yeast and higher eukaryotes (Kim et al., 2007; Kraft et al., 2009). Additionally, in yeast, the selective Cvt pathway is used for the trafficking of vacuolar hydrolases (aminopeptidase 1, Ape1 or α-mannosidase, Ams1), which are synthesized as inactive precursors in the cytoplasm (Figure 5) (Harding et al., 1995; Yoshihisa and Anraku, 1990).

The Cvt pathway requires the core Atg proteins involved in autophagosome formation as mentioned above as well as additional Atg proteins, which are required for cargo selection. This selective autophagy transport pathway is present under nutrient-rich conditions, however, if cells are starved or if macroautophagy is induced by other stress factors, the Cvt pathway cargo will be incorporated into an autophagosome for its transport to the vacuole (Lynch-Day and Klionsky, 2010). The precursor of Ape1 (prApe1) rapidly oligomerizes into a homododecamer, of which several units assemble into the higher ordered Ape1 complex (Kim et al., 1997). This complex then binds to Atg19, a peripheral protein that serves as a receptor for the Cvt pathway cargoes. Other proteins, like Ams1, also bind to Atg19 but at different sites, allowing simultaneous transport of different cargoes. Atg11 binds to the C terminus of Atg19 and thereby tethers the complex to the PAS under nutrient-rich conditions (Scott et al., 2001; Shintani et al., 2002). Atg19 then interacts with Atg8-PE at the PAS and Atg11 is involved in the transport of Atg9 to the PAS. Atg11 is also postulated to be part of a putative Atg1 regulatory complex consisting of Atg1/Atg13/Atg17 (as for macroautophagy) and Atg20, Atg24, and Vac8, of which the latter three are Cvt pathway-specific (Scott et al., 2001; Shintani et al., 2002).

For the elongation and completion of the phagophore membrane to generate the Cvt vesicle, the two previously mentioned ubiquitin-like conjugation systems as well as the PI(3)P-binding proteins are required (Lynch-Day and Klionsky, 2010). The outer membrane of the Cvt vesicle eventually fuses with the vacuole and Atg15 is needed for the breakdown of the inner membrane and the release of the content into the vacuole. The protease Pep4 is then pro-

cessing the precursors of Ape1 and Ams1 into their active forms (Klionsky et al., 1992; Zubenko et al., 1983).

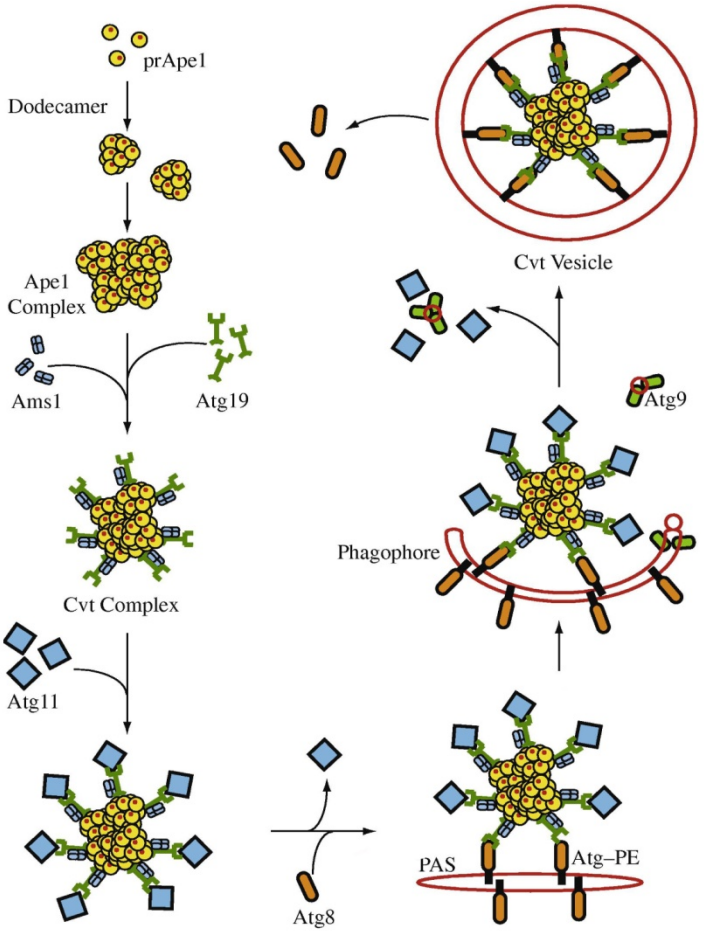

Figure 5: Cytoplasm to vacuole transport (Cvt) pathway (Lynch-Day and Klionsky, 2010; modified). The precursors of Ape1 (prApe1) form a dodecamer, of which several copies assemble into the Ape1 complex. This complex as well as other Cvt cargoes (like Ams1) are recognized by Atg19 and they assemble into the Cvt complex. Atg19 then binds the scaffold protein Atg11 and moves to the PAS, where Atg19 binds Atg8-PE which in turn drives the sequestration of the complex into the Cvt membrane/phagophore. After completion, the outer membrane fuses with the vacuole and the Cvt body is degraded similarly to autophagic bodies in an Atg15-dependend manner and the precursor of Ape1 is eventually cleaved into its active form.

1.4 Golgi Re-Assembly Stacking Protein (GRASP)

GRASP proteins are peripheral membrane proteins that are ubiquitously expressed in eukaryotes and only missing in the plant kingdom (Barr et al., 1997; Short et al., 2001; Shorter et al., 1999). They are attached to the cytoplasmic side of the Golgi apparatus via a highly conserved N-terminal myristoylation anchor, which is only absent in yeast and the malaria parasite *Plasmodium falciparum*, where the proteins are attached to membranes via an N-terminally acetylated amphipathic helix (Barr et al., 1997; Behnia et al., 2007; Struck et al., 2008). They contain a conserved N-terminal domain consisting of two PDZ domains, which are known protein-protein interaction motifs (Lee and Zheng, 2010), and a less conserved C terminus, which is enriched in serine and proline residues and, at least in mammals, known to be phosphorylated by cell cycle-specific kinases (Feinstein and Linstedt, 2008; Lin et al., 2000; Preisinger et al., 2005; Sengupta et al., 2009; Sutterlin et al., 2002; Truschel et al., 2011; Wang et al., 2005). Mammalian cells possess two GRASP orthologs (GRASP55 and 65) which were first identified in an *in vitro* assay as factors required for tethering of Golgi membranes to build the Golgi ribbon (Barr et al., 1997; Shorter et al., 1999). GRASP65 is localized to *cis*-Golgi membranes and interacts with GM130, the vesicle tethering factor p115, and the small GTPase Rab1 (Allan et al., 2000; Barr et al., 1998; Barr et al., 1997; Shorter and Warren, 1999; Weide et al., 2001); whereas GRASP55 is found on medial Golgi cisternae and interacts with the vesicle tethering factor golgin45 and the small GTPase Rab2 (Short et al., 2001; Shorter et al., 1999). Besides these interacting partners, GRASP proteins are also found to interact with other GRASP proteins on adjacent cisternae which is thought to be in-

volved in stacking the Golgi apparatus (Barr et al., 1998; Feinstein and Linstedt, 2008; Puthenveedu et al., 2006). GRASP oligomerisation depends on its PDZ domains and is inhibited by phosphorylation in the C-terminal half (Jesch et al., 2001; Sengupta et al., 2009; Truschel et al., 2011; Wang et al., 2003). However, the involvement of GRASP proteins in stacking of Golgi membranes remains controversial: as mentioned above, plants do not contain a GRASP ortholog, yet their Golgi membranes are perfectly stacked, and *S. cerevisiae and P. falciparum* contain a GRASP protein, yet their Golgi membranes are not stacked and are distributed throughout the cell. Additionally, RNAi-based knockdown of GRASP orthologs in mammals did not affect the overall Golgi architecture (Duran et al., 2008; Sutterlin et al., 2005) and deletion of the single GRASP gene in *D. discoideum* did not cause unstacking of Golgi membranes *in vivo* (Kinseth et al., 2007) thereby questionning the assigned role for GRASPs as stacking factors.

1.4.1 GRASPs in protein secretion

Even if GRASP proteins interact with vesicle tethering factors like p115 and GM130, they are not directly required for conventional protein transport (Behnia et al., 2007; Duran et al., 2008; Feinstein and Linstedt, 2008; Kondylis et al., 2005). However, via their PDZ domains they can, in addition to homo-oligomerization, interact with proteins containing C-terminal valine motifs (Barr et al., 2001; Kuo et al., 2000). Interestingly, this motif also serves as an export or transport signal for some proteins to leave the ER (D'Angelo et al., 2009; Nufer et al., 2002) and GRASP proteins have been implicated in the conventional transport of some specific C-terminal valine-

harboring proteins through the Golgi stack like transforming growth factor alpha (TGFα), CD8α, or Frizzled 4 (D'Angelo et al., 2009; Kuo et al., 2000).

Even more striking is the identification of GRASP proteins as key players in the unconventional secretion of several proteins (Dupont et al., 2011; Duran et al., 2010; Gee et al., 2011; Kinseth et al., 2007; Manjithaya et al., 2010; Schotman et al., 2008; Schotman et al., 2009). *Drosophila* αPS1 integrin is an adhesion factor, which is involved in establishing contacts between the extracellular matrix and the plasma membrane (Hynes, 1992). It usually enters the secretory pathway at the level of the ER and is secreted conventionally from the cell via the Golgi complex. However, at a specific developmental stage where morphological changes in the epithelia occur, allowing for the formation of new plasma membrane-extracellular matrix contact sites, αPS1 integrin was shown to reach these sites at the plasma membrane by a mechanism that appears to bypass the Golgi apparatus, since it was shown to be insensitive to BFA-treatment (Horne-Badovinac and Bilder, 2005; Schotman et al., 2008). Interestingly, the transport of αPS1 integrin at this specific stage to these new contact sites is dependent on the *Drosophila* GRASP ortholog *d*GRASP (Schotman et al., 2008). Moreover, *d*GRASP re-localizes from its standard ER-Golgi localization to the plasma membrane, where it is required for the unconventional secretion of αPS1 integrin (Kondylis et al., 2005; Schotman et al., 2008). In the absence of *d*GRASP, αPS1 integrin is retained within the cell causing deformation of focal adhesion sites and wing epithelia, indicating a novel developmentally regulated unconventional secretion mechanism (Schotman et al., 2008).

GRASP proteins have also been shown to be involved in the unconventional trafficking of CFTR from the ER to the plasma membrane (Gee et al., 2011). CFTR is an ion channel involved in chloride and bicarbonate transport in the airway, pancreas and intestine (Park et al., 2010). CFTR is usually transported to the cell surface via the conventional ER-Golgi apparatus dependent pathway. Mutations in CFTR can lead to severe diseases, for instance deletion of phenylalanine 508 (ΔF508) results in a misfolded, but still functional protein. However, $CFTR^{\Delta F508}$ is incapable of leaving the ER, causing cystic fibrosis (Denning et al., 1992; Ward et al., 1995). Interestingly, Gee et al. showed that, upon induction of the unfolded protein response caused by ER stress and the presence of GRASP55, transport of $CFTR^{\Delta F508}$ to the cell surface could be rescued and that the export occurs independently of the Golgi apparatus, as $CFTR^{\Delta F508}$ remains in its core-glycosylated, immature form (Gee et al., 2011).

In addition to the unconventional secretion of proteins entering into the ER, GRASPs have also been implicated in the secretion of cytosolic proteins. The first protein identified to require GRASPs for its unconventional secretion was the ACBP homolog in *D. discoideum*, AcbA (Kinseth et al., 2007). ACBPs are highly conserved proteins of around 10kDa found in all eukaryotes (Mandrup et al., 1993). They were identified as cytosolic proteins that specifically bind to medium to long-chain fatty acid acyl-CoA esters and they are thought to serve as acyl-CoA transporters *in vivo* (Mogensen et al., 1987; Rosendal et al., 1993). ACBPs have also been suggested to stimulate steroid hormone synthesis and to suppress glucose-induced insulin secretion from the pancreas (Ostenson et al., 1994; Papadopoulos et al., 1991). The yeast

ortholog Acb1 was shown to affect ceramide levels, vacuole structure, and life span regulation (Fabrizio et al., 2010; Faergeman et al., 2004). However, ACBPs were first identified in mammals as peptides which inhibit binding of diazepam to γ-aminobutyric acid (GABA) receptor and was therefore assigned as a diazepam binding inhibitor (DBI) (Guidotti et al., 1983). Proteolytic cleavage of ACBP/DBI results in the production of different fragments: ODN (octadecaneuropeptide) and TTN (triakontatetra-neuropeptide), which were shown to be active fragments since they were still able to bind to GABA receptors (Ferrero et al., 1986; Slobodyansky et al., 1989).

In the social amoeba *D. discoideum*, the central peptide of AcbA is known as spore differentiation factor (SDF)-2, and is required for terminal spore encapsulation (Anjard and Loomis, 2005). Under nutrient-rich conditions, *D. discoideum* grows as a unicellular organism. However, upon nutrient starvation, a developmental switch occurs and cells differentiate into so-called prespore and prestalk cells, converting *D. discoideum* into a multicellular organism (Anjard et al., 1998; Shaulsky and Loomis, 1996). Nutrient-starvation also triggers the release of AcbA from the prespore cells. AcbA will get cleaved by ABC transporter/serine protease TagC, which is localized to the plasma membrane of prestalk cells, to give rise to the central active peptide SDF-2 (Anjard and Loomis, 2005). SDF-2 then binds to the histidine kinase receptor DhkA at the prespore membrane. DhkA is a member of two-component signal transduction systems. SDF-2 binding to DhkA generates a positive feedback loop enhancing further release of SDF-2 into the medium and also induces an intracellular cascade leading to increased activity of PKA and thereby inducing terminal spore encapsulation

(Alex and Simon, 1994; Anjard and Loomis, 2005; Wang et al., 1999). Kinseth et al. were able to demonstrate that the unconventional secretion of the highly abundant cytosolic protein AcbA requires the GRASP ortholog GrpA as *grpA*⁻ cells fail to secrete SDF-2 into the medium, using a spore viability assay. This defect is specific to AcbA and it is not due to improper localization of TagC or DhkA (Kinseth et al., 2007). Using the *D. discoideum*-based bioassay for spore viability and the more powerful tool of yeast genetics, the secretion of SDF-2-like material derived from the yeast ortholog Acb1 was shown in *S. cerevisiae* and *P. pastoris* and also the involvement of GRASP (Grh1) in this process was reported (Duran et al., 2010; Manjithaya et al., 2010). Duran et al. collected medium from nutrient-starved budding yeast, processed it to concentrate Acb1-derived SDF-2-like material and analyzed it for the ability to induce sporulation of *D. discoideum* (Anjard and Loomis, 2005; Duran et al., 2010). SDF-2-like activity could be detected in the medium after 2.5h of starvation, reaching a plateau within 30min indicating that the secretion is a regulated process occurring in a single burst (Duran et al., 2010). Acb1 is not following the conventional ER-Golgi-dependent transport pathway as its secretion is unaffected in strains expressing temperature-sensitive alleles of the COPII component *SEC23*, the Arf GEF *SEC7,* or *SEC1*, a protein involved in fusion of vesicles with the plasma membrane (Barlowe et al., 1994; Carr et al., 1999; Duran et al., 2010; Sata et al., 1998). However, the secretion process has been shown to require the NSF factor Sec18, which is involved in several intracellular SNARE-mediated membrane fusion events, indicating that secretion of Acb1 requires a vesicular intermediate (Duran et al., 2010; Graham and Emr, 1991).

Interestingly, several proteins involved in autophagosome formation (like Atg7, Atg8, or Atg12) are required suggesting that Acb1 might be specifically captured into a forming autophagosome upon nutrient starvation (Figure 6) (Duran et al., 2010). On the other hand, these autophagosomes must have the potential to evade fusion with the vacuole to protect its content from degradation. Consistently, strains lacking factors involved in fusion of autophagosomes with the vacuole, like Vam3 or the small GTPase Ypt7, are not defective in secretion of SDF-2-like material (Duran et al., 2010; Haas et al., 1995).

The fact that proteins required for the fusion of membranes with the endosomes, like the t-SNARE Tlg2 or the Rab-GTPase Ypt6, are involved in secretion of Acb1, suggests that the autophagosome could potentially fuse with an endosome or endosome-like structure. Duran et al. also suggested that this endosome-like structure could mature into a MVB, since different ESCRT mutants, like *vps23*Δ (ESCRT-I complex) or *vps4*Δ (ESCRT disassembly machinery), are required for secretion of Acb1 (Babst et al., 1997; Duran et al., 2010; Katzmann et al., 2001). It has been reported for some mammalian lymphoid cells that MVB can fuse directly with the plasma membrane to release exosomes into the extracellular space (Simons and Raposo, 2009; Thery et al., 2009). Consistently, one of the two plasma membrane t-SNAREs in yeast, Sso1, is required for the secretion of Acb1 whereas Sso2 is dispensable (Aalto et al., 1993; Duran et al., 2010). These two SNAREs are highly homologous and redundant for most fusion events. However, it has been reported that Sso1 is, in addition to Acb1 release, also specifically required for sporulation (Jantti et al., 2002).

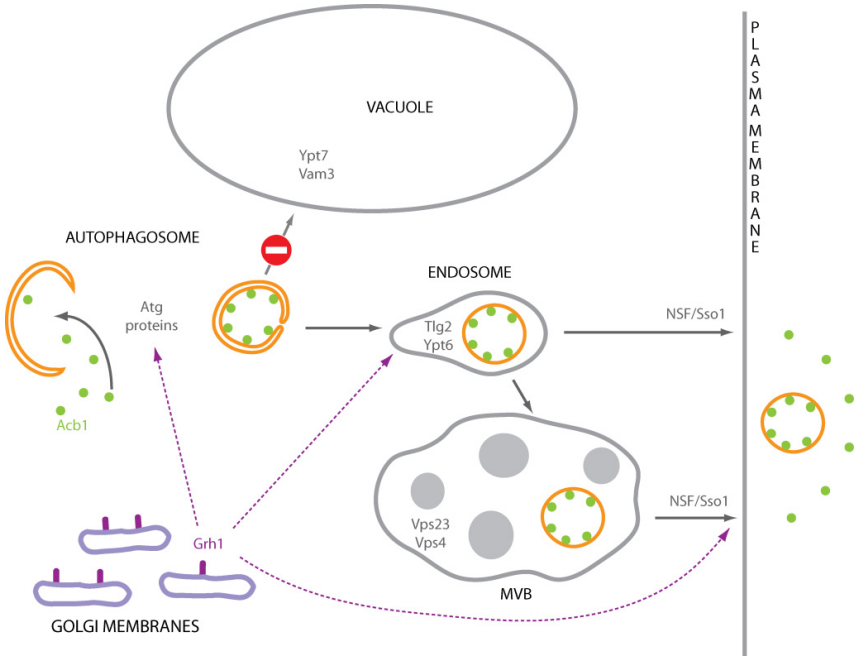

Figure 6: Proposed model for the unconventional secretion of Acb1 (adapted from: Duran et al., 2010). The nutrient starvation-induced secretion of Acb1 requires Atg proteins suggesting that Acb1 might be captured into a forming autophagosome. This autophagosome must evade fusion with the vacuole in order to prevent degradation of its content. Consistently, proteins involved in this fusion step, like Ypt7 and Vam3, are not required for Acb1 secretion. Instead, the autophagosome could fuse with an endosome-like structure in a Tlg2/Ypt6-dependent manner. This endosomes would mature into a MVB requiring the ESCRT machinery (like Vps23 and Vps4) or either of these compartments could fuse with the plasma membrane in a NSF/Sso1-dependent reaction to release Acb1-containing exosomes from the cell. The secretion process also requires the GRASP ortholog Grh1, which role and place of action still has to be identified (Duran et al., 2010).

As mentioned before, the unconventional release of IL-1ß from the cell was reported to occur via secretory lysosomes. However, the mechanistic details remain elusive (Bonaldi et al., 2003; Rubartelli et al., 1990). Recent findings from Dupont et al. highlight the involvement of autophagy and GRASP55 in IL-1ß secretion (Dupont et al., 2011). Basal levels of autophagy repress the release of IL-1ß (Nakahira et al., 2010; Zhou et al., 2011) whereas starvation-

induced autophagy enhances the unconventional secretion of IL-1ß (Dupont et al., 2011). In the latter case, IL-1ß was found in LC3 (mammalian homolog of Atg8)-containing structures representing autophagosomes. Depletion of GRASP55 by RNAi or deletion of Atg5 by excision resulted in reduced secretion of IL-1ß (Dupont et al., 2011). However, how exactly GRASP55 plays a role in the secretion of IL-1ß and how IL-1ß would be captured into an autophagosome-like structure are still open questions. In addition, although the yeast GRASP ortholog Grh1 is required for the secretion of Acb1 from *S. cerevisiae* and *P. pastoris,* as mentioned above, the exact role of Grh1 in this process also remains elusive (Duran et al., 2010; Manjithaya et al., 2010).

2 Objectives

GRASP proteins were initially identified in the late nineties as factors required for stacking of mammalian Golgi membranes to the Golgi ribbon *in vitro*, a supposed function which remains highly debatable, as mentioned above (see chapter 1.4). However, in the past five years, GRASP orthologs in different organism –from the budding yeast to mammals– were identified as key players in the unconventional secretion of several proteins suggesting a conserved role for GRASP in this process. Nevertheless, the exact role and site of action of GRASP remain elusive.

The specific aim of this thesis was focused on elucidating the precise role of the yeast GRASP ortholog Grh1 in the unconventional secretion process of proteins such as Acb1. Specifically, we aimed (1) to determine where Grh1 is localized during conditions promoting unconventional protein secretion, (2) to identify what other factors are interacting with Grh1 under these conditions and (3) to develop a novel assay to monitor unconventional secretion in the budding yeast.

3 Results

3.1 Identification and characterization of a novel compartment for unconventional protein secretion

3.1.1 Re-localization of Grh1 upon nutrient starvation

Grh1 is essential for the unconventional secretion of Acb1 (Duran et al., 2010; Manjithaya et al., 2010), however, its exact role in this process still needs to be unraveled. To monitor its localization under nutrient-rich conditions and nutrient-starvation, the condition known to promote unconventional release of Acb1, endogenous Grh1 was tagged at the C terminus with GFP. In rich medium, Grh1-GFP was found in several punctae equally distributed throughout the cytoplasm whereas upon starvation, Grh1-GFP re-localized to 1-3 larger structures and occasionally smaller ones. These structures were clearly visible within 2h of starvation and remained stable for up to 8h. When 4h-starved yeast were further cultured in rich medium, Grh1-GFP was found to redistribute into numerous small punctae, resembling the growth condition phenotype. These starvation-dependent changes in localization were independent of new protein synthesis as cycloheximide treatment had no effect on them (Figure 7A).

GRASP proteins are usually attached to Golgi membranes and a Golgi-specific localization of Grh1 in *S. cerevisiae* has also been reported (Barr et al., 1997; Behnia et al., 2007; Shorter et al.,

1999). However, more recently, Grh1 was found on ER exit sites based on fluorescence microscopy analysis (Levi et al., 2010) and also the *Drosophila* ortholog *d*GRASP localizes to ER exit sites and the Golgi apparatus at steady state (Kondylis et al., 2005). Consistent with Levi et al., Grh1-GFP was found in close proximity to and in some cases co-localizing with a subset of the ER exit site-specific protein Sec13-RFP (Figure 7B).

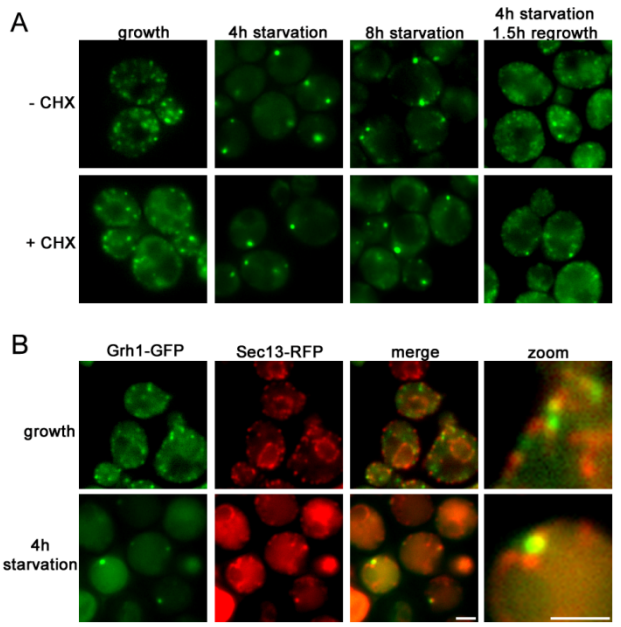

Figure 7: Starvation-dependent re-localization of Grh1-GFP. (A) Grh1-GFP expressing yeast were grown in rich medium, washed and cultured in starvation medium with or without cycloheximide (CHX) for the indicated times and visualized by fluorescence microscopy. (B) Grh1-GFP and Sec13-RFP co-expressing yeast were cultured in rich condition or nutrient starved for 4h and visualized by fluorescent microscopy. Bars: 2µm.

To further define the localization of Grh1 with respect to Sec13, immunoelectron microscopy of ultrathin cryosections was performed (Figure 8). Under nutrient-rich conditions Grh1-GFP was detected close to, but clearly separated from Sec13-RFP-labeled structures (Figure 8A-C). Upon nutrient starvation Grh1-GFP-

containing membranes were in few cases found to co-localize with Sec13-RFP (Figure 8K and L) and therefore 20 random images were analyzed for quantification. Distances of the gold conjugated antibodies against Sec13-RFP [6nm] and Grh1-GFP [12nm] were grouped as being <10nm (virtually exact co-localization limited only by steric hindrance), <50nm (approximately the diameter of each gold probe [Au = 6 or 12nm, 1st IgG = 15nm, 2nd IgG= 15nm]), <100nm (roughly the size of a cisternae or transport vesicle), or >100nm: 18 particles of Grh1 and Sec13 were found at >10nm from each other, 87 Grh1 and 129 Sec13 were found at <50nm, 53 Grh1 and 89 Sec13 gold particles were found at <100nm, and 588 Grh1 and 1908 Sec13 particles were found at >100nm. Taken together, approximately 80% of Grh1 was found more than 100nm apart from Sec13, suggesting that they are localizing in close proximity but not to the same membrane structures under starvation conditions. More interestingly, upon nutrient-starvation, Grh1-containing structures were larger in size and fewer in number. They appeared to be cup-shaped and morphologically similar to the classical PAS/forming autophagosomes (Figure 8F, H, and K) (Baba et al., 1994; Kirisako et al., 1999).

3.1.2 Molecular characterization of the starvation-induced Grh1-containing compartment

Since the Grh1-GFP-containing structures were found close to a subset of ER exit sites but not completely co-localizing with them, we analyzed Golgi apparatus-specific marker proteins for their presence at the Grh1-GFP-containing membranes.

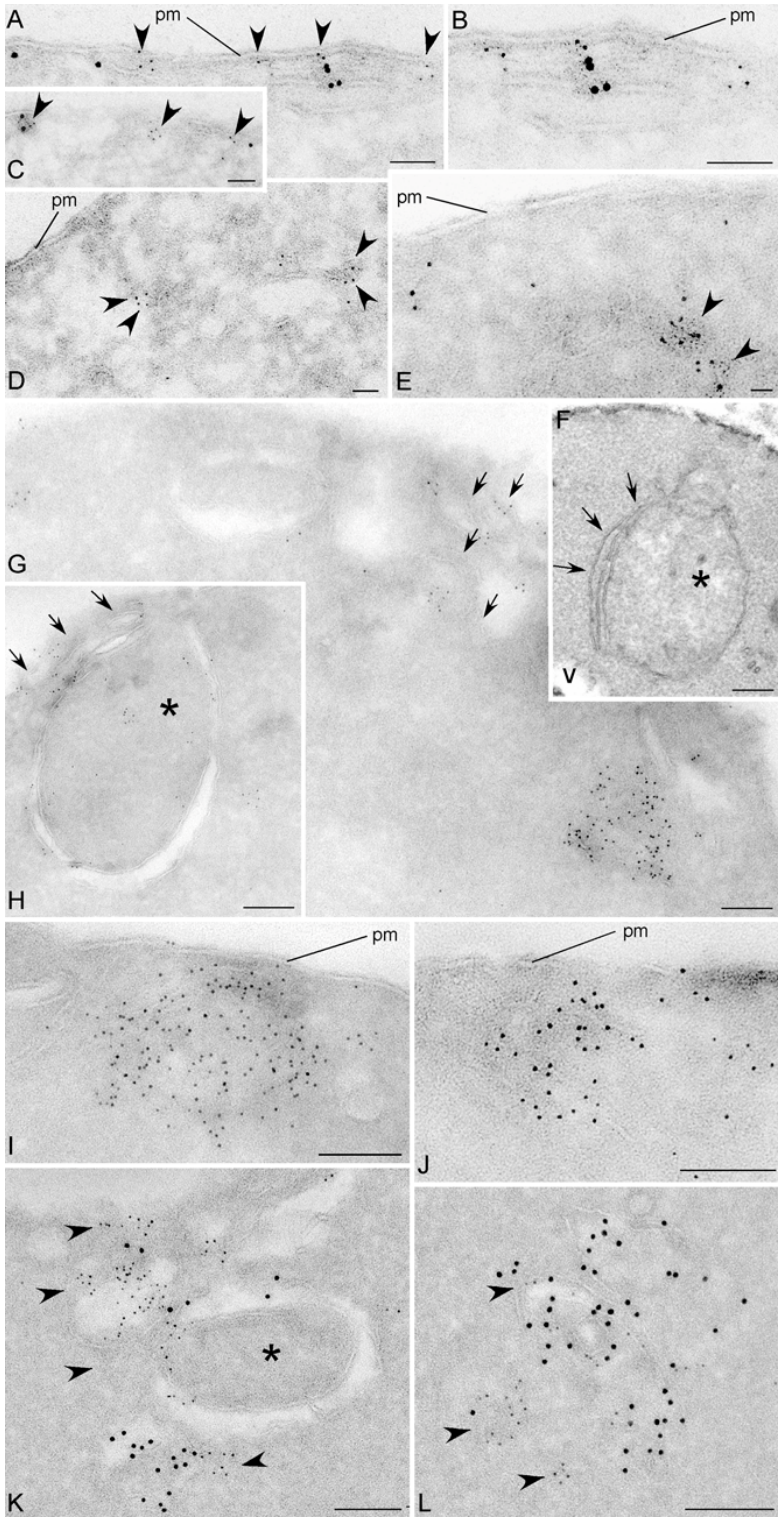
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Figure 8: Grh1 is found near ER exit sites and re-localizes to cup-shaped structures upon starvation. (A-L) Immunoelectron microscopy of ultrathin cryosections. (A-E) Nutrient rich conditions, (G-L) 4h starvation, and (F) conventional morphology after 4h starvation are shown. (A-C, K-L) Immunogold labeling of Grh1-GFP (12nm) and Sec13-RFP (6nm). (D, G-J) Single labeling of Grh1-GFP. (E) Single labeling of Sec13-RFP. Arrowheads indicate 6nm particles. Arrows indicate membrane lamellae. Asterisks indicate PAS. pm: plasma membrane; v: vacuole. Bars: (A–D, G–I, K, and L) 200nm; (F) 500nm; (E and J) 100nm.

A co-localization with Grh1-GFP was not observed for early (CopI-RFP; Anp1-RFP) or late (Sec7-dsRed) Golgi markers, neither under nutrient-rich conditions nor upon nutrient starvation (Figure 9A and B). The cisternae of the *S. cerevisiae* Golgi apparatus, unlike the mammalian Golgi apparatus, are neither stacked nor connected and are found distributed throughout the cytosol (Papanikou and Glick, 2009). Therefore, a missing co-localization with these Golgi markers does not completely exclude a Golgi apparatus localization of Grh1-GFP. However, immunoelectron microscopy showed Grh1-GFP localization to structures not resembling Golgi membranes. Thus, we propose that Grh1 is most likely not a Golgi-associated protein in the budding yeast.

We then asked whether endosomal membrane proteins, of which some are required for the secretion of Acb1, are co-localizing with the Grh1-GFP-containing membranes under nutrient-rich or starvation conditions. No co-localization was observed for mCherry-tagged early (Tlg1) and late (Pep12) endosomal t-SNAREs, neither under growth conditions nor upon starvation (Figure 9C and D). Grh1-GFP-containing structures were not only devoid of these endosomal proteins, they also did not incorporate the endocytosed, lipophilic dye FM4-64. Yeast cells were labeled for 15min with FM4-64, subjected to starvation and monitored by fluorescence microscopy at various time points for the uptake of FM4-64 (Figure 9E).

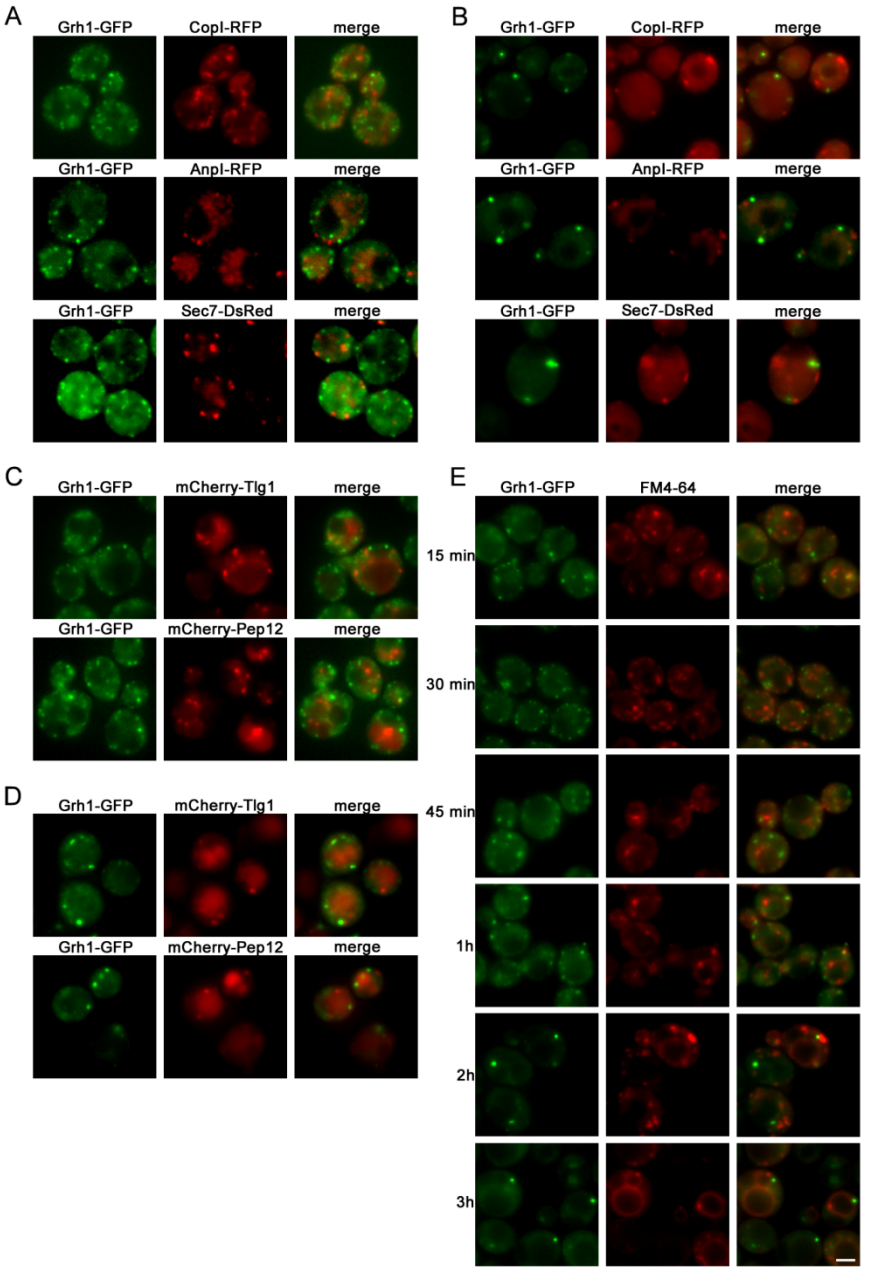


Figure 9: Grh1-GFP does not co-localize with proteins of the Golgi apparatus or the endosomes under growth or starvation conditions. (A-D) Grh1-GFP was co-expressed with the early Golgi markers CopI-RFP and Anp1-RFP and the late Golgi marker Sec7-DsRed under growth conditions (A) or starvation (B) or with mCherry-Tlg1 and mCherry-Pep12 in growth conditions (C) or starvation (D) and visualized by fluorescence microscopy. (E) Grh1-GFP-expressing cells were

labeled with FM4-64 and starved for up to 3h to visualize the localization of the endocytosed FM4-64 with reference to Grh1-GFP. Bar: 2µm.

Previous work identified proteins of the ESCRT complexes, which are usually involved in MVB biogenesis, as requirements for the release of Acb1 upon nutrient starvation, for instance Vps23 of the ESCRT-I complex and Vps4 of the ESCRT disassembly machinery (Babst et al., 1997; Duran et al., 2010; Katzmann et al., 2001). Therefore, we analyzed the distribution of Vps23-mCherry under growth and starvation conditions with respect to Grh1-GFP. No co-localization was observed under rich conditions. However, upon starvation, 88% of Grh1-GFP-containing punctae were positive for Vps23-mCherry (of which 87% of punctae were positive for Grh1-GFP; Figure 10A and Figure 11C). To analyze if the entire ESCRT-machinery is recruited to the starvation specific Grh1-GFP-containing structures, we monitored the distribution of a member of another ESCRT complex, namely Snf7 of the ESCRT-III complex (Teis et al., 2008). However, no co-localization could be detected in yeast cultured in rich or starvation medium (Figure 10B).

Various proteins involved in autophagy inhibited the secretion of Acb1 upon nutrient starvation, thus we asked if two marker proteins of the PAS, Atg8 and Atg9, are also present at the starvation-specific Grh1-GFP-containing compartment. In yeast grown in rich medium, Grh1-GFP was not found to co-localize with Atg8 or Atg9. However, a starvation specific co-localization was evident for these two proteins (Figure 11A and B). Grh1-GFP punctae were found to contain Atg8 in 59% and Atg9 in 68% (Figure 11C).

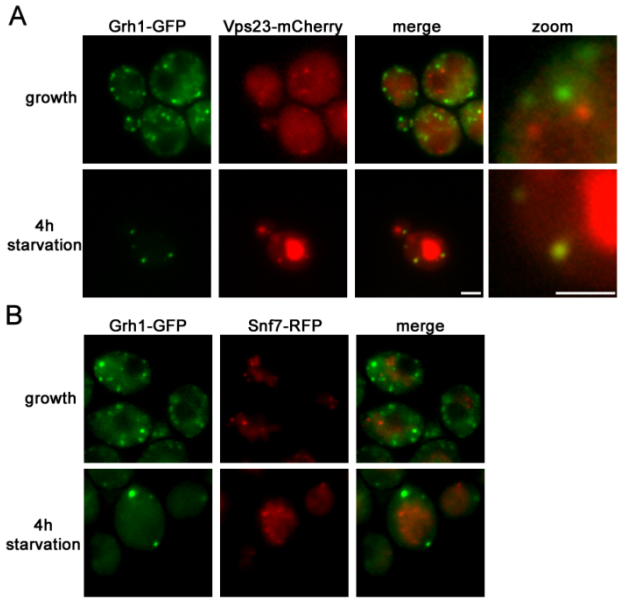
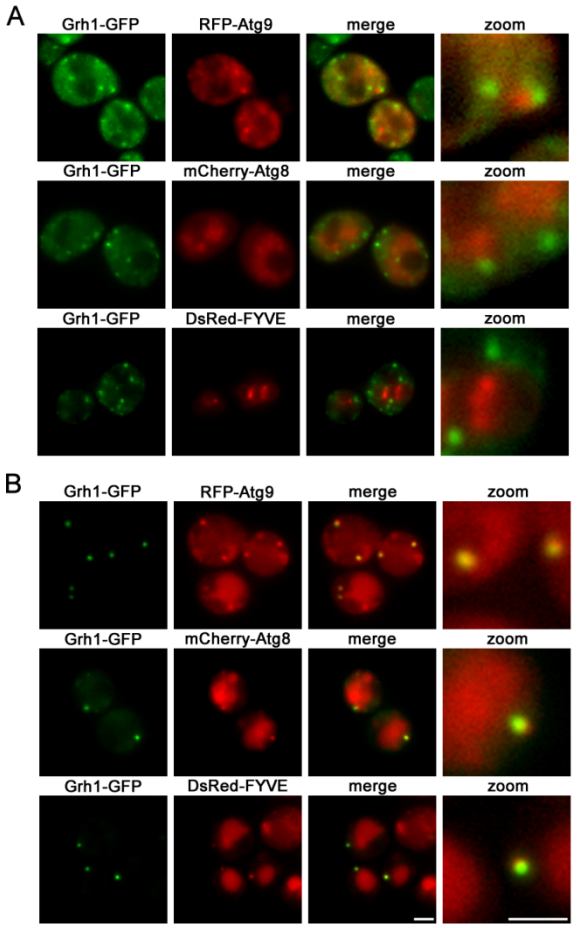


Figure 10: Grh1-GFP co-localization with Vps23 of the ESCRT-I is starvation-specific, but not the entire ESCRT complexes are recruited to Grh1-GFP-containing membranes. (A and B) Grh1-GFP was co-expressed with Vps23-mCherry (A) and Snf7-RFP (B) and visualized by fluorescence microscopy under nutrient-rich conditions and 4h nutrient starvation. Bars: 2µm.

Recently, a structure termed omegasomes has been identified in mammalian cells. Omegasomes form close to the ER upon amino acid starvation, they contain PI(3)P and are thought to act as scaffold for the formation of autophagosomes (Axe et al., 2008; Matsunaga et al., 2010). We therefore tested whether the Grh1-GFP-containing structures were enriched in PI(3)P by co-expressing a DsRed-tagged FYVE domain, a known PI(3)P-sensor (Stenmark et al., 2002). Under nutrient-rich conditions, Grh1-GFP was not co-localizing with DsRed-FYVE, however, upon starvation, 56% of Grh1-GFP structures contained DsRed-FYVE and 76% DsRed-FYVE punctae were co-localizing with Grh1-GFP, indicating the presence of PI(3)P at the starvation-specific Grh1-GFP membranes (Figure 11).



C

% Grh1 co-localized	Protein marker	% marker protein co-localized
88.2 ± 5.41	Vps23-mCherry	85.12 ± 2.22
59.21 ± 8.85	mCherry-Atg8	76.63 ± 5.40
68.74 ± 5.22	RFP-Atg9	66.12 ± 6.54
56.60 ± 3.44	DsRed-FYVE	76.51 ± 3.11

Figure 11: Grh1-GFP co-localizes with components of the autophagy machinery upon starvation. (A and B) Grh1-GFP was co-expressed with RFP-Atg9, mCherry-Atg8, and a DsRed-tagged FYVE domain and cultured in rich (A) or starvation medium (B) and visualized by fluorescence microscopy. Bars: 2µm. (C) The percentages of co-localization were quantified with respect to Grh1 or the indicated marker. A minimum of 60 cells per marker were assessed, and errors were represented as SEM.

The results showed so far sustain the conclusion that Grh1-GFP, under growth conditions, localizes to a compartment in close proximity to the Sec13-RFP-containing ER exit sites. Grh1-GFP-

containing membranes are devoid of various Golgi and endosomal marker proteins. Upon starvation, Grh1-GFP concentrates in 1-3 larger punctae per cell, which are enriched in PI(3)P, contain the ESCRT-I protein Vps23 and the autophagy-related proteins Atg8 and Atg9. Based on its content and its cup-shape, we believe that this is a novel organelle which we named CUPS (*C*ompartment for *U*nconventional *P*rotein *S*ecretion).

To further characterize the properties of the Grh1-GFP-containing membranes under rich conditions and of the CUPS, we fractionated cell lysates of growing or nutrient-starved yeast. Yeast cultured in rich medium or starved for 3h were homogenized and loaded on top of a continuous sucrose gradient. The fractions were probed with several antibodies to different compartment-specific marker proteins (Figure 12). Western blotting with antibodies against the ER marker protein Kar2 and the Golgi marker protein Mnn9 revealed a similar distribution under growth and starvation conditions (Figure 12A). Under nutrient-rich conditions, Grh1-GFP was found in fractions overlapping with the ones containing Kar2 and Mnn9, respectively (Figure 12B and C, II). However, upon nutrient starvation, a second pool of Grh1-GFP was detected in lighter fractions, which were well separated from the ER/Golgi membrane-containing denser fractions (Figure 12B and C, I). We assume that these fractions are enriched in CUPS. To further determine their identity, we probed for Vps23. Importantly, upon starvation, Vps23 was also contained in lighter fractions which were overlapping with the Grh1-GFP-containing fractions (Figure 12B and C, I). These results support the previously detected co-localization of Grh1-GFP and Vps23-mCherry at the CUPS under starvation conditions.

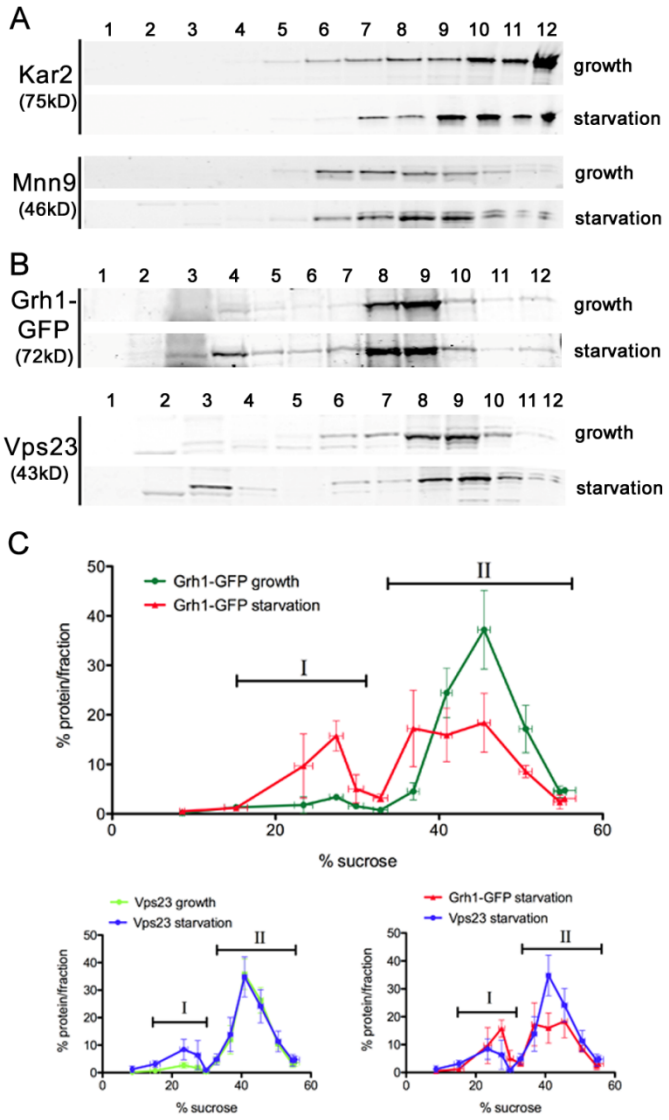


Figure 12. A starvation-specific fraction of Grh1-GFP co-migrates with Vps23. (A and B) Yeast cultured in rich medium or starved for 3h were lysed and fractionated on a continuous 15–60% sucrose gradient for 18h. The fractions were western blotted with αKar2 (ER) and αMnn9 (Golgi) (A) or αVps23 and αGFP to monitor Grh1-GFP (B). The numbers indicate the fractions. (C) The percentage of protein contained in each fraction was plotted against the sucrose concentration, and the error bars represent SEM from three independent experiments. The fractions marked 'I' represent the starvation-specific pool of Grh1 and Vps23, and the denser fractions 'II' represent the ER-Golgi pool of Grh1-GFP and the endosomal pool of Vps23.

3.1.3 Requirements for the biogenesis of CUPS

GRASP proteins are peripheral membrane proteins and the membrane association of the yeast ortholog depends on an N-terminal acetylation and the interaction with a protein called Bug1, the yeast ortholog of GM130 (Behnia et al., 2007). Additionally, Bug1 is also essential for the starvation-induced secretion of Acb1 (Duran et al., 2010). We therefore tested if Bug1 is involved in the recruitment of Grh1 to CUPS under nutrient starvation. Upon deletion of *BUG1*, Grh1-GFP was completely cytosolic and its localization to any membranous structures under nutrient-rich and starvation conditions was abolished (Figure 13).

With our previous observation that CUPS are enriched in PI(3)P, we asked whether the presence of PI(3)P is a prerequisite for the recruitment of Grh1-GFP. A strain deleted for the sole PI 3-kinase in yeast, *vps34*Δ, was analyzed for its ability to form CUPS. The localization of Grh1-GFP under growth conditions was unaffected whereas the starvation-specific re-localization of Grh1-GFP to the CUPS was abolished and it remained present in small punctae distributed throughout the cell (Figure 13).

Previous studies identified several Atg proteins as essential for the secretion of Acb1 (Duran et al., 2010; Manjithaya et al., 2010) and two of these Atg proteins, Atg8 and Atg9, were present on CUPS. Hence we analyzed if their deletion has an effect on the starvation-induced re-localization of Grh1-GFP to the CUPS. The deletion of various Atg protein coding genes, which are involved in several steps of autophagy (induction/regulation, autophagosome nucleation, expansion or completion; *atg1*Δ, *atg5*Δ, *atg7*Δ, *atg8*Δ, *atg9*Δ, *atg11*Δ, *atg14*Δ, *atg17*Δ, *atg18*Δ, *atg11*Δ/*atg17*Δ) (Yang and

Klionsky, 2009) did not abolished the recruitment of Grh1-GFP to the CUPS. However, upon deletion of *ATG8* or *ATG18*, larger structures presumably representing the CUPS were formed, but Grh1-GFP was additionally found in smaller punctae (Figure 13). Interestingly, Atg18 is known to bind to PI(3)P (Dove et al., 2004).

In the same line, as components of the ESCRT complexes are essential for the secretion of Acb1 (Duran et al., 2010) and as Vps23 was found to be contained in the CUPS, we analyzed proteins of the ESCRT complexes for a putative role in CUPS formation. Vps27 is a member of the ESCRT-0 complex, known to bind to ubiquitinated cargo as well as to PI(3)P and has also been shown to interact with Vps23 of the ESCRT-I complex (Katzmann et al., 2003). Deletion of *VPS27* did not abolished CUPS formation but Grh1-GFP was in addition to CUPS still present in a larger number of smaller punctae indicating that there might be a kinetic delay in CUPS formation (Figure 14). Deletion of the second component of the ESCRT-0 complex, *HSE1* (Bilodeau et al., 2002), and members of the ESCRT-I complex (*VPS23*, *MVB12*, and *VPS28*) (Curtiss et al., 2007; Katzmann et al., 2003) had no effect on CUPS biogenesis. Upon deletion of components of the ESCRT-II (*VPS25* and *VPS36*) (Babst et al., 2002b) and ESCRT-III complexes (*VPS2* and *VPS20*) (Babst et al., 2002a), Grh1-GFP was found to re-distribute to larger structures presumably representing the CUPS. However, a part remained localized to smaller punctae also indicating a possible effect on the kinetics similar to what had been observed upon deletion of *VPS27* or a failure in recruiting the entire amount of Grh1-GFP to the CUPS. Finally, deletion of *VPS4* did not affect the re-localization of Grh1-GFP to the CUPS (Figure 14).

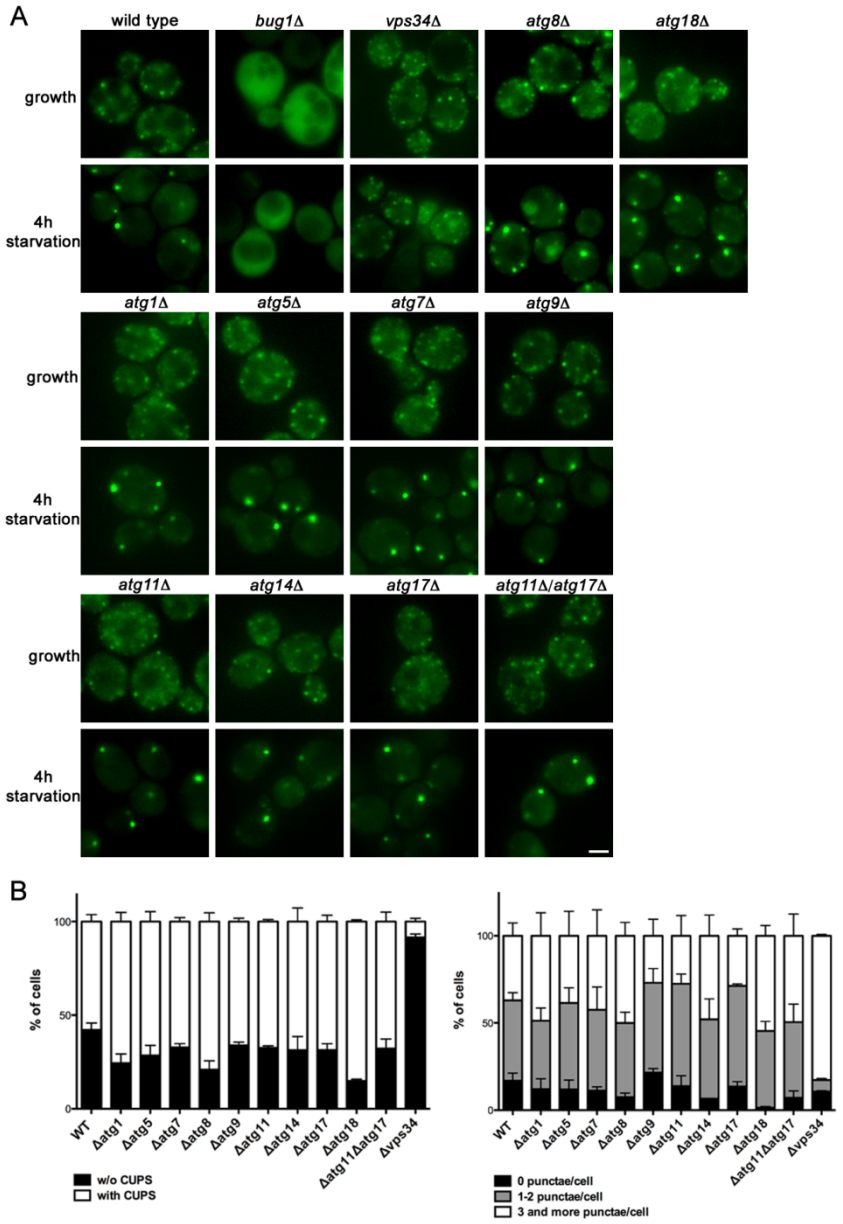


Figure 13: CUPS formation requires Bug1 and PI(3)P but is independent of autophagy-related proteins. (A) Wild type and yeast deleted for *BUG1, VPS34, ATG1, 5, 7, 8, 9, 11, 14, 17, 18, or 11/17* and expressing Grh1-GFP were cultured in rich medium or nutrient starved for 4h and analyzed by fluorescence microscopy. Bar: 2µm. (B) The indicated strains were assessed for the presence or absence of CUPS or counted for the number of punctae per cell. At least 60 cells/experiment in three independent experiments were counted and percentages of CUPS, no CUPS or punctae were plotted with GraphPad Prism; error bars represent SEM.

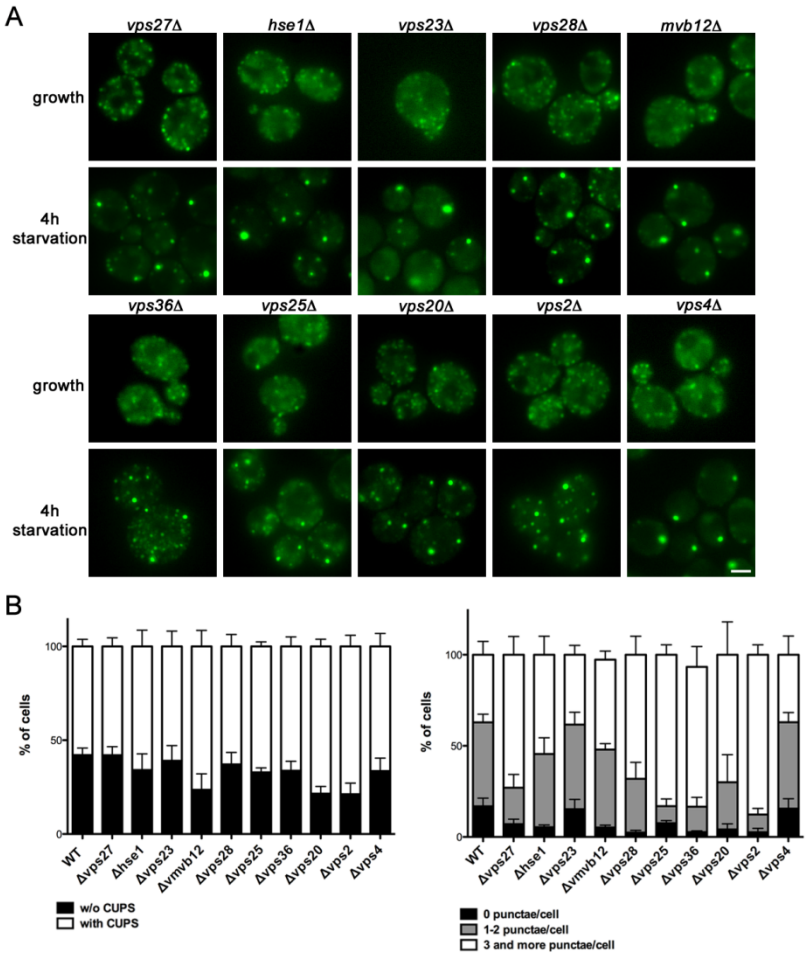


Figure 14: CUPS formation is partially affected by deletion of ESCRT-components. (A) Yeast strains deleted for *VPS27, HSE1, VPS23, VPS28, MVB12, VPS36, VPS25, VPS20, VPS2, or VPS4 and* expressing Grh1-GFP were cultured in rich medium or nutrient starved for 4h and analyzed by fluorescence microscopy. Bar: 2µm. (B) The indicated strains were assessed for the presence or absence of CUPS or counted for the number of punctae per cell. At least 60 cells/experiment in three independent experiments were counted and percentages of CUPS, no CUPS or punctae were plotted with GraphPad Prism; error bars represent SEM.

Besides the analysis of deletion strains with respect to the localization of Grh1, we tested whether Grh1 itself has a role in recruiting factors to the CUPS. Upon starvation of wild type yeast, Vps23-mCherry was found in punctate elements representing the CUPS

and also in the vacuole, whereas deletion of *GRH1* completely abolished the recruitment of Vps23 to the CUPS and its transport into the vacuole. On the other hand, deletion of *VPS23* did not affect Grh1-GFP localization (Figure 14 and 15A). RFP-Atg9 under growth conditions is present on several punctate elements close to the mitochondria that are considered as a reservoir of Atg9 (Mari et al., 2010). Upon starvation, 59% of wild type cells contained RFP-Atg9 in punctate structures whereas this number was reduced to 28% of cells in *grh1*Δ strains, indicating that Grh1 not only is required for the trafficking of Vps23, but might also be involved in Atg9-cycling to the CUPS (Figure 15B).

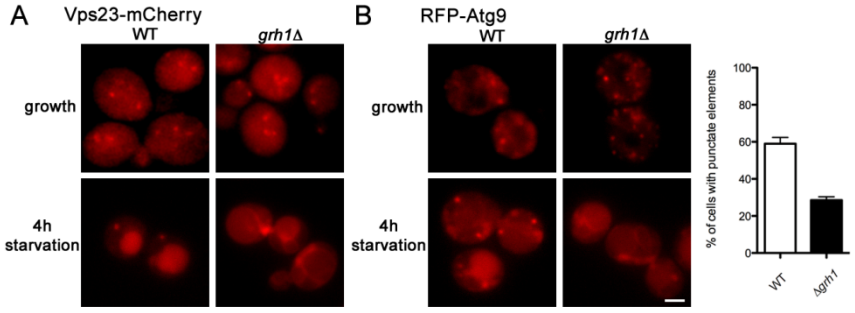


Figure 15: Grh1 is involved in recruiting additional factors to CUPS. (A and B) Wild type (WT) and *grh1*Δ yeast expressing Vps23-mCherry (A) or RFP-Atg9 (B) were cultured in rich medium or starved for 4h and analyzed by fluorescence microscopy. RFP-Atg9 starvation specific punctae representing the CUPS were counted. 59 ± 3.3% of WT cells showed a localization of Atg9 to CUPS compared with only 28.5 ± 1.8% upon deletion of *GRH1* (statistically significant with $P < 0.0001$; error bars represent SEM). Bar: 2µm.

Subsequently, we asked if CUPS formation is mediated by fusion of Grh1-GFP-containing structures or by growth of a preexisting site close to the ER. To obtain information on how CUPS form, we analyzed different temperature-sensitive mutants of essential genes or deletion strains of non-essential genes involved in vesicular transport steps for their ability to form CUPS. Sec12, a GEF for Sar1, is required for the biogenesis of COPII-coated vesicles at the

ER exit sites (Barlowe and Schekman, 1993). We monitored the localization of Grh1-GFP in a temperature-sensitive *sec12-4* yeast strain. Yeast were grown at permissive temperature (25°C) in rich medium and shifted to starvation medium at the same temperature or the non-permissive temperature (37°C) and visualized by fluorescence microscopy. A temperature-sensitive mutant in *SEC12* did not affect re-localization of Grh1-GFP upon starvation, indicating that Sec12 activity is not needed for CUPS biogenesis (Figure 16A). The NSF factor Sec18 is required for membrane fusion events and also essential for the unconventional secretion of Acb1 (Duran et al., 2010; Graham and Emr, 1991). A strain carrying the temperature-sensitive *sec18-1* mutation was analyzed at permissive (25°C) or non-permissive (37°C) temperature for its ability to form CUPS by fluorescence microscopy, and normal CUPS biogenesis was detected at either temperature (Figure 16A). Tlg2 and Ypt6, which are both required for the fusion of membranes/vesicles with the endosomes, have been identified as factors involved in the unconventional secretion of Acb1 (Duran et al., 2010; Luo and Gallwitz, 2003; Nichols et al., 1998). Interestingly, deletion of neither gene impaired the starvation-induced re-localization of Grh1-GFP to the CUPS, but their deletion had a slight effect comparable to the *atg8*Δ strain, as CUPS are formed but residual Grh1-GFP was retained in smaller punctate structures (Figure 16B). Similarly, deletion of the gene encoding the plasma membrane t-SNARE *SSO1*, which has also been shown to be involved in the secretion of Acb1, did not alter the starvation-specific localization of Grh1-GFP (Figure 16B) (Duran et al., 2010).

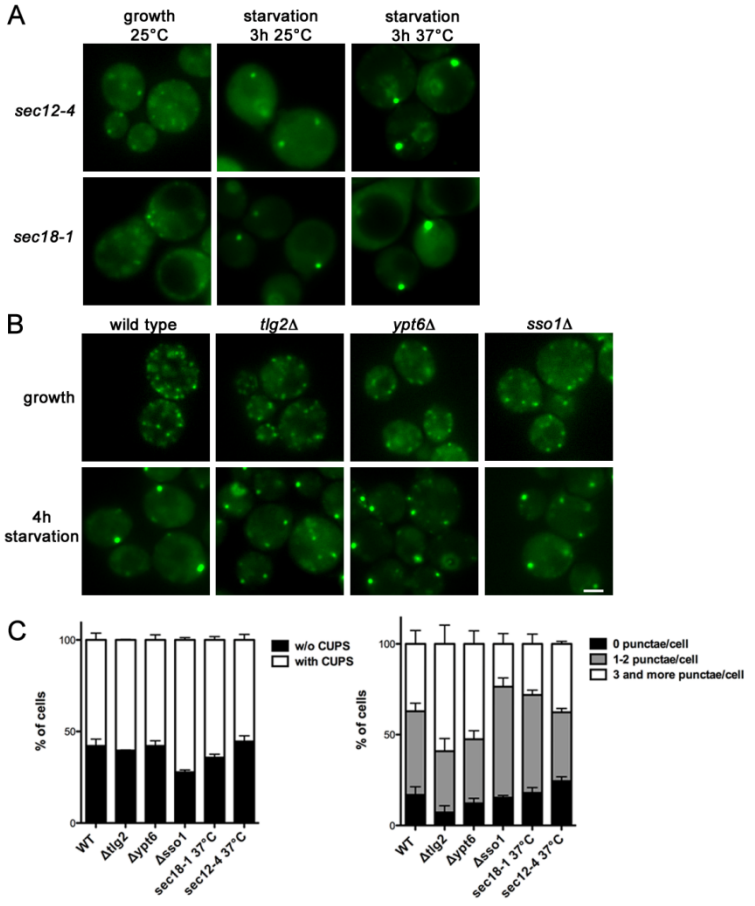


Figure 16: NSF and SNAREs are not essential for CUPS biogenesis. (A) Grh1-GFP was expressed in *sec12-4* and *sec18-1* strains and cells were grown at permissive temperature (25°C) in rich medium and starved for 3h at either 25°C or 37°C (non-permissive temperature) and visualized by fluorescence microscopy. (B) Wild type, *tlg2*Δ, *ypt6*Δ, and *sso1*Δ strains expressing Grh1-GFP were cultured in growth or starvation medium and visualized by fluorescence microscopy. Bar: 2µm. (C) The indicated strains were assessed for the presence or absence of CUPS or counted for the number of punctae per cell. At least 60 cells/experiment in three independent experiments were counted and percentages of CUPS, no CUPS or punctae were plotted with GraphPad Prism; error bars represent SEM.

3.1.4 CUPS are distinct from the classical PAS

As a consequence of the starvation-induced formation of CUPS, their cup-shaped morphology as well as the specific contents of CUPS, all of which partially overlap with the induction of autophagy and the shape and content of autophagosomes, it was necessary to discriminate CUPS from the classical PAS. The drug rapamycin inhibits the TOR kinase by binding to Fpr1 thereby allowing the induction of autophagy independent of nutrient starvation (Noda and Ohsumi, 1998). Grh1-GFP and mCherry-Atg8 co-expressing yeast were grown in rich medium and subsequently either subjected to nutrient starvation for 4h or treated with 0.4µg/ml rapamycin for 3h. Fluorescence microscopy-based analysis revealed that upon rapamycin treatment in rich medium mCherry-Atg8 re-located from the cytosol to the PAS/forming autophagosomes and into the vacuole as expected. However, under these conditions Grh1 did not assemble into CUPS as detected by nutrient starvation. This indicates that by induction of their formation, CUPS are distinct from the PAS/autophagosomes (Figure 17A).

To further investigate the relation between CUPS and PAS, we tested whether the Cvt pathway cargo Ape1 is present at CUPS. Grh1-GFP was co-expressed with Ape1-mCherry and visualized by fluorescence microscopy. Under growth conditions, Grh1-GFP was present in various punctae distributed throughout the cell whereas Ape1-mCherry was visible predominantly in one single structure representing the Cvt vesicle. Upon nutrient starvation, Ape1-mCherry was clearly visible in autophagosomes and in the vacuole. The Ape1-mCherry-containing degradative autophagosomes did not co-localize with the Grh1-GFP-defined CUPS, further indicating that CUPS are distinct from PAS (Figure 17B).

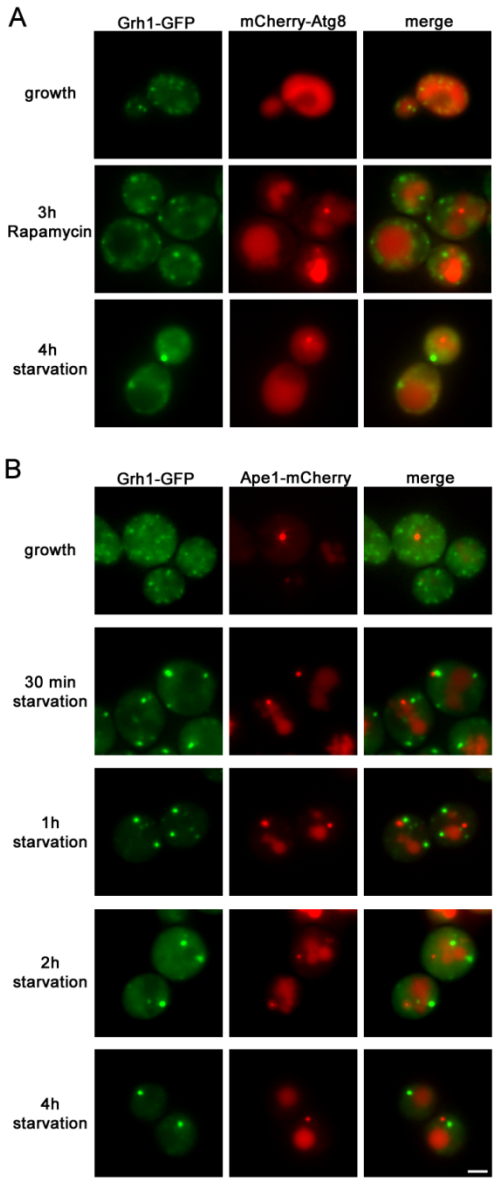


Figure 17: CUPS are distinct from PAS by formation and content. (A) Grh1-GFP and mCherry-Atg8 co-expressing yeast were cultured in rich medium and either treated with 0.4µg/ml rapamycin in nutrient-rich medium for 3h or nutrient starved for 4h. (B) Grh1-GFP and Ape1-mCherry co-expressing yeast were cultured in rich medium or starved for the indicated time points and visualized by fluorescence microscopy. Bar: 2µm.

3.1.5 CUPS formation is nutrient starvation specific

CUPS formation was induced by nutrient starvation, which is also the stress trigger known to release Acb1 from the cells. In general, unconventional protein secretion can be induced by different stress triggers depending on the extracellular function of the secreted protein. Therefore, we analyzed if CUPS biogenesis is nutrient-starvation specific or if it could also be induced by applying different stress triggers. As shown in Figure 18, shifting yeast for increasing time intervals to 37°C to induce heat shock response did not provoke formation of CUPS, and neither did salt/sodium stress (treatment with 1M sodium chloride) nor osmotic stress (treatment with 1M sorbitol). Induction of oxidative stress response by treatment with hydrogen peroxide (0.3mM H_2O_2) revealed no changes in the localization of Grh1-GFP whereas induction of reductive stress by treatment with DTT led to the appearance of a larger number of even smaller Grh1-GFP-containing punctae. DTT treatment blocks the formation of disulfide bonds and therefore leads to the accumulation of unfolded proteins within the ER and induction of ER stress (Bertolotti et al., 2000). Treatment of yeast with sodium azide (0.5% NaN_3) leads to the inhibition of cytochrome oxidase and thereby impairs mitochondrial function (Rikhvanov et al., 2002). This is known to inhibit translation which subsequently induces accumulation of mRNA into RNA-protein granules named P-bodies or stress granules (Buchan et al., 2011). Importantly, sodium azide treatment did not alter the distribution of Grh1-GFP. Taken together, CUPS formation appears to be starvation-specific as no additional stress triggers we tested induced CUPS biogenesis (Figure 18).

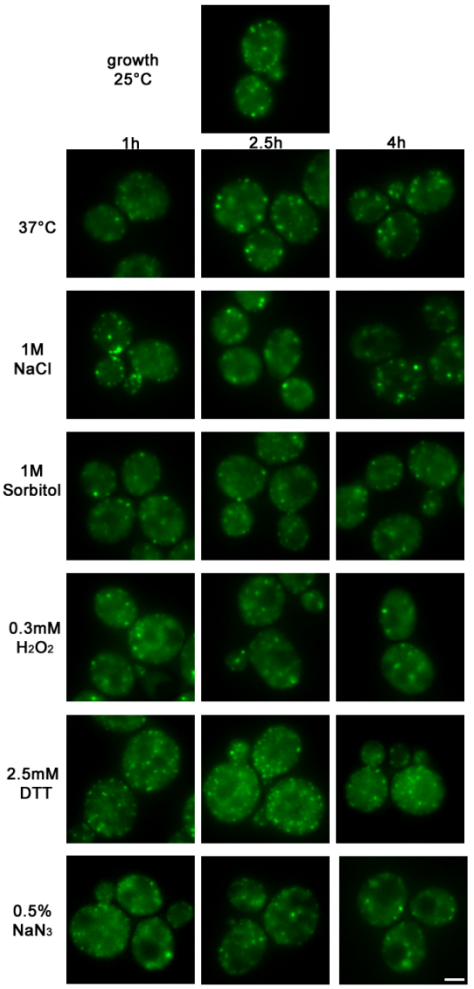


Figure 18: CUPS are starvation specific. Grh1-GFP expressing yeast were cultured in rich medium and subsequently treated with the indicated stress trigger (in YPDA) for 1h, 2.5h or 4h and visualized by fluorescence microscopy. Bar: 2µm.

Besides our standard starvation approach, namely culturing yeast in 2% potassium acetate, selective removal of specific nutrient components is possible. In minimal medium containing the required salts and ions, the nitrogen source or carbon source was selectively removed. Interestingly, only glucose depletion was capable of inducing CUPS formation (Figure 19).

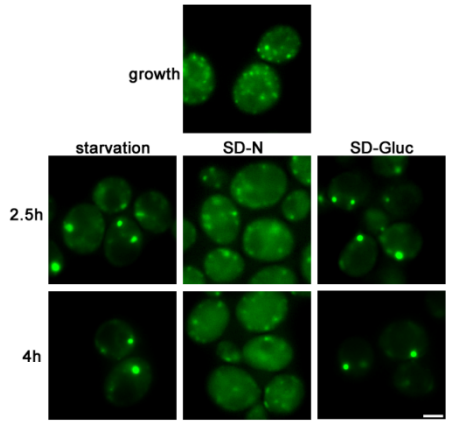


Figure 19: CUPS are specific for glucose starvation. Grh1-GFP expressing yeast were cultured in rich medium, washed and cultured in the indicated minimal media for 2.5h or 4h and visualized by fluorescence microscopy. SD-N: synthetic defined media without nitrogen source; SD-Gluc: synthetic defined media without glucose. Bar: 2µm.

The results obtained from the different starvation media were consistent with our previous results regarding the rapamycin treatment as rapamycin inhibits the TORC1 complex, thereby inducing autophagy. However, it did not elicit CUPS formation (Figure 17A). TORC1 is also thought to be the primary kinase complex responding to amino acid or nitrogen starvation and as starvation for those components did not induce Grh1-GFP redistribution, CUPS formation seems to be independent of TORC1. Additionally, the major glucose-sensing enzyme in the budding yeast is the AMP kinase (AMPK) Snf1 (Hardie et al., 1998). We therefore analyzed a strain deleted for *SNF1* for its ability to form CUPS in response to nutrient starvation. We clearly detected incomplete CUPS formation further indicating that they are glucose starvation-specific (Figure 20). However, it is still unknown how direct the effect of *SNF1* deletion on Grh1-GFP re-localization is. Both Grh1 and its mammalian orthologs are known to be phosphorylated (Lin et al., 2000; Sutterlin et al., 2002), but there is no evidence yet that Snf1 is directly involved in the phosphorylation of Grh1.

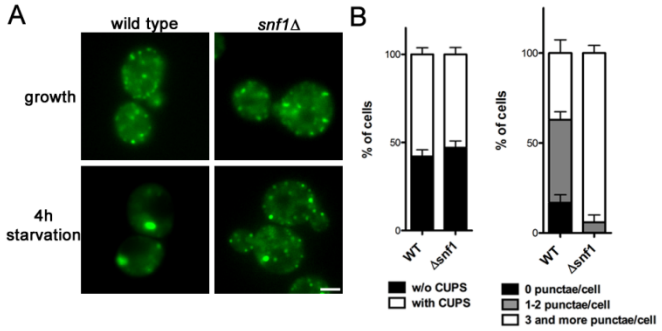


Figure 20: The AMPK Snf1 is required for CUPS assembly upon starvation. (A) Wild type and *snf1*Δ strains expressing Grh1-GFP were cultured in rich medium or nutrient starved for 4h and the localization of Grh1-GFP was analyzed by fluorescence microscopy. Bar: 2µm. (B) The indicated strains were assessed for the presence or absence of CUPS or counted for the number of punctae per cell. At least 60 cells/experiment in three independent experiments were counted and percentages of CUPS, no CUPS or punctae were plotted with GraphPad Prism; error bars represent SEM.

3.1.6 Grh1 interacts with different proteins upon nutrient starvation

In response to nutrient starvation, we observed a change in the localization of Grh1 from various smaller punctae distributed throughout the cytoplasm into 1-3 larger structures which we named CUPS. Besides Grh1, we also localized Vps23, PI(3)P, Atg8, and Atg9 to this nutrient-starvation induced structure. However, we wondered how direct the connection between Grh1 and those factors was and also if Grh1 could be present in different protein complexes under nutrient-rich conditions compared to nutrient starvation. We therefore used a strain expressing endogenous Grh1 with a C-terminal TAP-tag to purify Grh1-TAP-containing complexes under growth and starvation conditions (Figure 21). After SDS-PAGE followed by silver-staining, protein bands detected more intense in starvation conditions were cut out and the

polypeptide composition was analyzed by mass spectrometry (Table 1). Parental strains without TAP-tag were used as control for non-specific interactions with the column material.

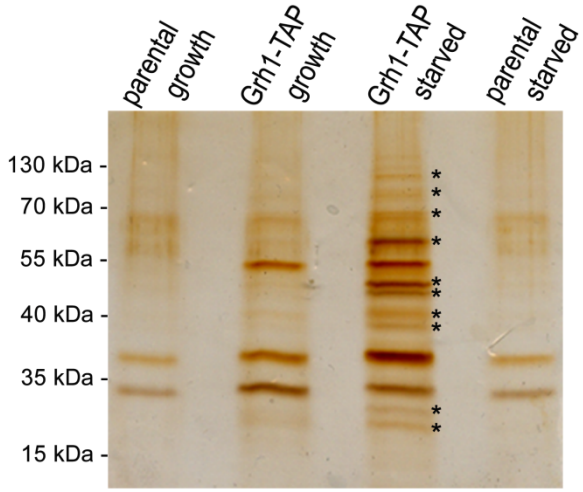


Figure 21: Identification of starvation-specific interacting partners of Grh1. Parental and Grh1-TAP expressing strains were cultured in rich medium or nutrient-starved for 4h, lysed, and the immunoprecipitated protein complexes were analyzed by SDS-PAGE, followed by mass spectrometry. Asterisks mark protein bands more prominent upon starvation. Parental strains were used as control.

Table 1: Starvation-specific interacting proteins of Grh1-TAP.

Protein	Description	Number Peptides
Bug1	*cis*-Golgi localized protein	67
Pgk1	3-phosphoglycerate kinase	64
Cdc19	pyruvate kinase	26
Pfk2	beta subunit of heterooctameric phosphofructokinase	10
Gvp36	BAR domain-containing protein, localizes to early and late Golgi vesicles	6
Mck1	kinase related to mammalian glycogen synthase kinases of the GSK-3 family	4
Stm1	protein required for optimal translation under nutrient stress	4
Rtn1	reticulon protein that stabilizes membrane curvature	3
Cmk1	calmodulin-dependent protein kinase; may play a role in stress response	3
Scs2	integral ER membrane protein, VAP homolog	2
Sso2	plasma membrane t-SNARE	1

Some of the proteins detected upon starvation (Gvp36, Rtn1, Scs2, Stm1, Mck1, Sso2) –except for Bug1, whose effect on Grh1-GFP localization was shown previously (Figure 13)– were analyzed for their role in recruiting Grh1-GFP to CUPS. None of the new interacting partners had a strong effect in CUPS biogenesis as monitored by Grh1-GFP. Yeast deleted for *STM1* showed an increased number of smaller Grh1-GFP-containing punctae besides CUPS (Figure 22).

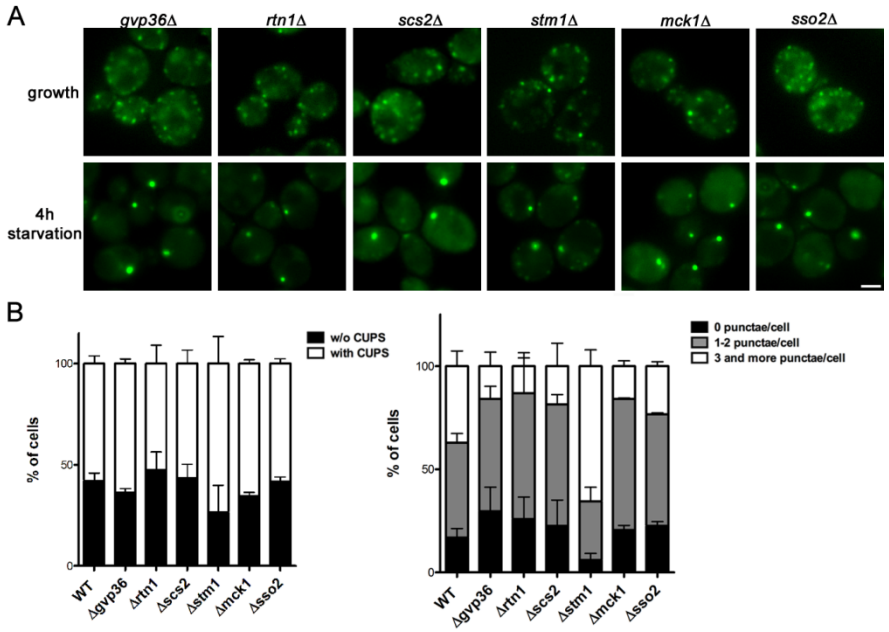


Figure 22: New interacting proteins do not affect Grh1 localization. (A) Strains deleted for *GVP36*, *RTN1*, *SCS2*, *STM1*, *MCK1* and *SSO2* expressing Grh1-GFP were cultured in rich medium or nutrient starved for 4h and the localization of Grh1-GFP was analyzed by fluorescence microscopy. Bar: 2µm. (B) The indicated strains were assessed for the presence or absence of CUPS or counted for the number of punctae per cell. At least 60 cells/experiment in three independent experiments were counted and percentages of CUPS, no CUPS or punctae were plotted with GraphPad Prism; error bars represent SEM.

Additionally, the localization of some of these proteins cultured in rich or starvation medium in wild type and *grh1*Δ strains was analyzed by fluorescence microscopy (Figure 23A). Gvp36-GFP was found in small punctae and cytosolic under nutrient-rich and starva-

tion conditions. No effect on the localization was observed upon deletion of *GRH1* (Figure 23A). Furthermore, the small Gvp36-GFP-containing punctae did not co-localize with the CUPS as stained with Grh1-mCherry (Figure 23B). Rtn1 and Scs2 are integral ER-membrane proteins. Upon starvation the perinuclear ER staining of Rtn1-GFP was less pronounced whereas the localization to the cortical ER was unaltered. Furthermore, deletion of *GRH1* did not affect the localization of Rtn1-GFP under either condition. Compared to Rtn1-GFP, GFP-Scs2 was found at the perinuclear and cortical ER in wild type cells under growth and starvation conditions. Its localization under nutrient-rich condition was unaltered upon deletion of *GRH1* whereas GFP-Scs2 seemed to partially lose its cortical ER localization under starvation conditions in *grh1*Δ strains (Figure 23A). Additionally, these proteins were not found to be contained in CUPS, whereas Bug1-GFP clearly was co-localizing with Grh1-mCherry at the CUPS (Figure 23B).

As these new proteins were found to interact with Grh1-TAP upon starvation, we asked if they are –like Grh1 and Bug1– also involved in the unconventional secretion of Acb1. Unfortunately, due to the lack of an assay to monitor Acb1 release (see chapter 3.2), their role in the secretion of this specific cargo could not be tested yet and remains elusive. Nevertheless, we analyzed them for an involvement in the unconventional release of the a-factor mating pheromone, which was unaffected. Also the α-factor mating pheromone, a conventionally secreted cargo, was released normally upon deletion of those genes (Figure 24). To finally be able to link them to the unconventional secretion of Acb1 or to even identify

them as potential cargoes following the same pathway, it is required to establish a new secretion assay.

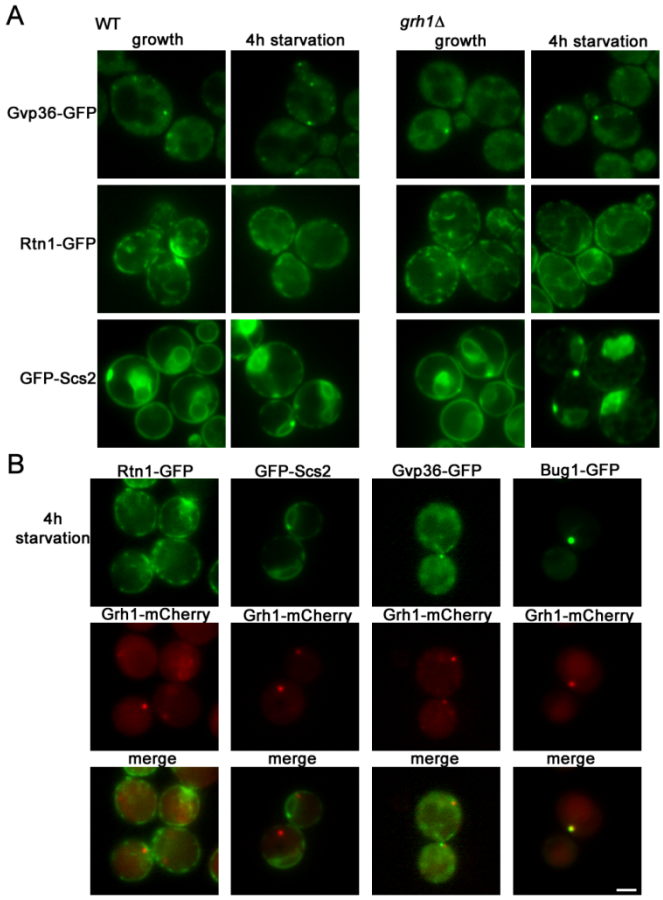


Figure 23: Localization of new Grh1-interacting proteins does not depend on Grh1 and they are not contained in CUPS. (A) Grh1-interacting proteins were tagged endogenously with GFP and their localization under nutrient-rich and starvation condition in wild type (WT) or *grh1*Δ strains was monitored by fluorescence microscopy. (B) The starvation-specific localization of GFP-tagged proteins of Gvp36, Rtn1, Scs2, and Bug1 was analyzed in yeast cells co-expressing Grh1-mCherry to stain the CUPS. Bar: 2µm.

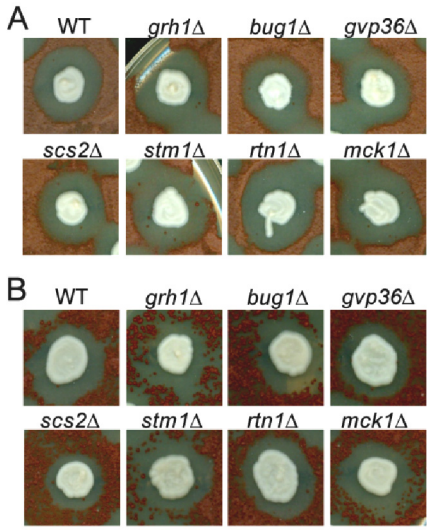


Figure 24: New Grh1-interacting proteins without role in secretion of a- and α-factor pheromones. Wild type (WT) and the indicated deletion strains were analyzed for their role in (A) α-factor and (B) a-factor pheromone secretion using the halo assay. A halo of space around the pinned strain indicated secretion of the mating pheromone. No deletion strain was defective in secretion of either pheromone.

3.2 Identification of novel cargoes for unconventional protein secretion in S. cerevisiae

The major problem we are currently facing regarding the unconventional protein secretion in the budding yeast is the lack of a powerful and straightforward detection method. Not only is the *Dictyostelium*-based bioassay for spore viability complicated, indirect, and not suitable for testing multiple candidates with highly reproducible results, it is also no longer available for us. Therefore, we needed to establish a novel assay to monitor unconventional secretion directly in the budding yeast. The most obvious detection method one could think of could to be a sporulation-based assay as AcbA in *Dictyostelium* is required for terminal spore encapsulation and also Acb1 in *P. pastoris* was reported to be involved in

sporulation (Anjard and Loomis, 2005; Kinseth et al., 2007; Manjithaya et al., 2010).

3.2.1 Extracellular Acb1 peptide is not required for spore viability but does alter the yeast transcriptome

It is known that deletion of *ACB1* in budding yeast leads to reduced levels of very-long-chain fatty acids (C_{26}) and that this affects membrane assembly (Fabrizio et al., 2010; Faergeman et al., 2004; Gaigg et al., 2001). Cells carrying a deletion of *ACB1* are genetically very unstable and revert into suppressed normal growing yeast with altered membrane compositions (Gaigg et al., 2001). For these reasons, we decided to use diploid *grh1*Δ cells and not *acb1*Δ strains in a sporulation assay to measure spore viability as a consequence of unconventional secretion of SDF-2-like material.

Sporulation of diploid wild type yeast or diploid strains deleted for *GRH1* or *ATG8* (as a control) was induced by culturing the yeast for 5 days at 30°C in 2% potassium acetate plus required amino acids for auxotrophies. Preparation of spores was performed with increasing concentrations of detergent (0.5% Triton-X100 [TX100], 1% SDS, 2% SDS) and the spore viability was then assessed by counting spores plated on YPDA agar. *atg8*Δ yeast were entirely sporulation-deficient and no colonies were detected even in very low dilutions of spores treated with 0.5% TX100 (standard assay; Figure 25). WT and *grh1*Δ diploid strains were able to sporulate and increasing concentration of detergents lowered the survival rate as less spores were able to form colonies. However, *grh1*Δ yeast were even less sensitive than WT yeast.

In the yeast *P. pastoris*, it was shown that the *acb1*Δ strain was unable to form viable spores and that this defect could be restored to around 20% of wild type by addition of 4nM recombinant AcbA (Manjithaya et al., 2010). Based on this finding we also sporulated *grh1Δ* strains in the presence of 4nM of a synthesized peptide containing the central 34 amino acids of Acb1 (representing the SDF-2-like material). However, this did not increase the number of spores by a significant amount (Figure 25) which indicates that either the role of Acb1 regarding the formation of more resistant spores is not conserved in the budding yeast or that the assay conditions were not suitable to detect a possible role of Acb1 in spore viability.

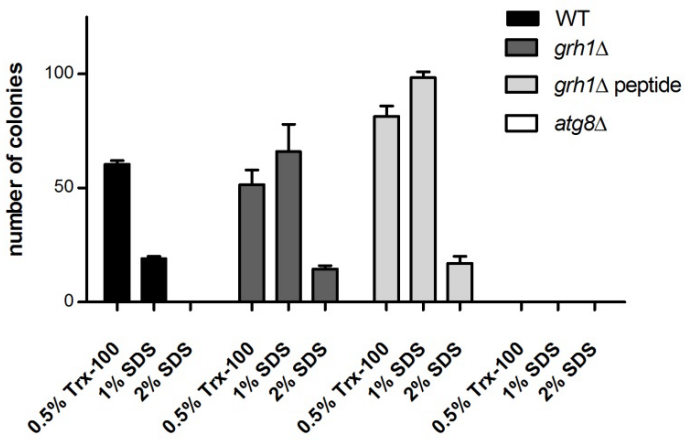


Figure 25: Acb1 peptide does not significantly increase spore viability. WT, *grh1*Δ (with or without 4nM Acb1 peptide added into the medium), and *atg8*Δ were sporulated, the spores were plated on YDPA plates in appropriate dilutions and the numbers of spores were counted. WT: 0.5% TX100 = 60.5 colonies, 1%SDS = 19, 2%SDS = 0; *grh1*Δ: 0.5% TX100 = 51.5 colonies, 1%SDS = 60, 2%SDS = 14.5; *grh1*Δ (+ peptide): 0.5% TX100 = 81.5 colonies, 1%SDS = 98.5, 2%SDS = 17; *atg8*Δ: 0 for all conditions.

Nevertheless, the central peptide, when added to the starvation medium in excess, did have an effect as it influenced the yeast transcriptome as analyzed by microarray. WT and *grh1*Δ yeast

were starved for 4h in 2% potassium acetate in the presence or absence of the synthesized Acb1 peptide (10nM) and total RNA levels were extracted and analyzed. Proteins identified to be down- or up-regulated were grouped by function (Figure 26).

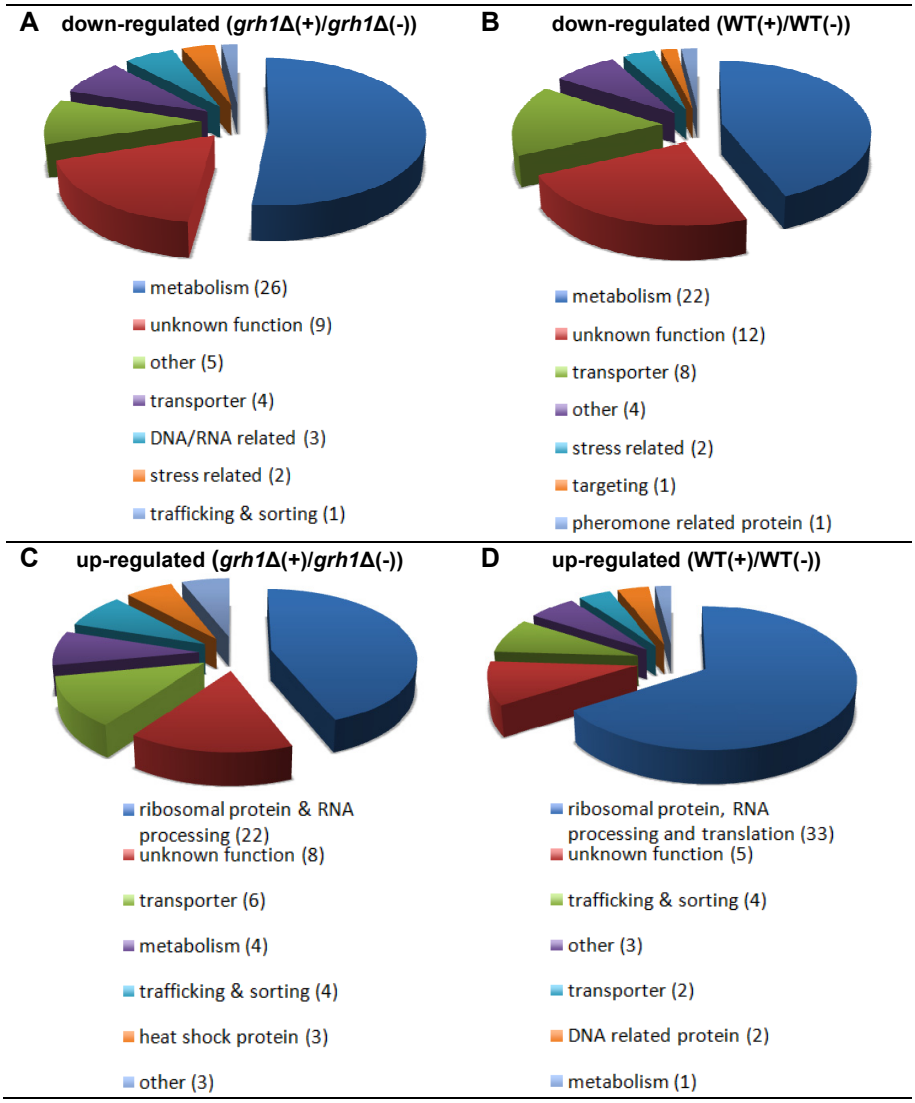


Figure 26: Acb1 central-peptide in the medium influences total RNA levels. Total RNA were extracted upon starvation and levels were analyzed by microarray. Proteins were grouped according to their proposed function. Gene expression down-regulated in the presence of the peptide: (A) *grh1*Δ(+)/ *grh1*Δ(-); (B) WT(+)/WT(-). Gene expression up-regulated in the presence of the peptide: (C) *grh1*Δ(+)/*grh1*Δ(-) and (D) WT(+)/WT(-).

Interestingly, several metabolic enzymes, particularly enzymes involved in the citrate cycle (TCA cycle) and beta oxidation of fatty acids, were down-regulated when the peptide was added in excess to the medium. Conversely, RNA of ribosomal and RNA-processing proteins was found in higher levels. Complete lists of the 50 most up- or down-regulated genes represented in Figure 25 are shown in Table 2-3.

Table 2: Genes which expression levels are down-regulated by addition of the SDF-2-like peptide to the medium.

Gene	Ratio WT(+)/ WT(-)	Ratio *grh1*Δ(+)/ *grh1*Δ(-)	Description
Acs1	-25,20	-4,58	acetyl-CoA synthetase
Adh2	-40,75	-10,89	glucose-repressible alcohol dehydrogenase II
Adh3		-4,42	mitochondrial alcohol dehydrogenase isozyme III
Ady2	36,24	-4,73	acetate transporter
Aro10		-4,58	phenylpyruvate decarboxylase
Ato3	-25,94	-7,88	putative ammonium transporter
Atp3		-3,95	mitochondrial F1F0 ATP synthase
Cat2	-12,33		carnitine acetyl-CoA transferase
Cit2	-13,05		citrate synthase
Cit3	-21,70	-4,62	citrate synthase
Crc1	-42,47	-3,96	membrane carnitine transporter
Csm4		-4,42	chromosome segregation in meiosis
Cta1	-14,57		catalase A
Cup2		-3,65	cupper binding transcription factor
Dcs2		-3,96	decapping mRNA
Eci1	-12,76		enoyl-CoA isomerase
Edc2		-3,83	enhancer of mRNA decapping
Fbp1	-16,70		fructose-1,6-bisphosphatase
Fdh1	-11,17		NAD(+)-dependent formate dehydrogenase
Fum1		-3,76	fumarase
Gap1		-3,67	amino acid permease
Gdh3		-3,80	glutamate dehydrogenase
Gln1		-5,24	glutamine synthetase
Glo4		-3,70	mitochondrial glyoxalase II
Gpg1		-4,16	gamma subunit of the heterotrimeric G protein
Gre1	-22,40	-4,79	hydrophilin essential in dessication-rehydration process
Hxt5	-34,92	-4,11	hexose transporter
Icl1	-46,43	-6,63	isocitrate lyase

Idp2	-27,95	-5,63	isocitrate dehydrogenase
Jen1	-35,57	-4,74	monocarboxylate/proton symporter of the plasma membrane
Leu4	-18,60	-5,87	alpha-isopropylmalate synthase
Mep2		-5,56	ammonium permease
Mls1	-40,02	-5,14	malate synthase
Nde2	-21,03	-4,70	mitochondrial external NADH dehydrogenase
Om14	-21,70	-3,65	integral mitochondrial outer membrane protein of unknown function
Om45	-10,86	-3,75	mitochondrial outer membrane protein of unknown function
Pai3	-16,61	-5,18	cytoplasmic proteinase A (Pep4p) inhibitor
Pck1	-19,41	-4,86	phosphoenolpyruvate carboxykinase
Pdh1	-20,29	-4,83	mitochondrial protein that participates in respiration
Pex18	-14,76		peroxin required for targeting of peroxisomal matrix proteins
Pns1	-14,89		protein of unknown function
Prm4	-14,00		pheromone regulated membrane protein
Put4	-17,10		proline permease
Qcr6	-13,34	-4,38	subunit 6 of the ubiquinol cytochrome-c reductase complex
Reg2	-20,60	-4,35	regulatory subunit of the Glc7p type-1 protein phosphatase
Sdh4	-13,18		succinate dehydrogenase
Sfc1	-39,09	-4,41	mitochondrial succinate-fumarate transporter
Smf3	-11,59		putative divalent metal ion transporter involved in iron homeostasis
Spg4	-14,00	5,96	required for survival at high temperature
Sps19	-12,79		peroxisomal 2,4-dienoyl-CoA reductase
Stl1	-12,99		glycerol proton symporter of the plasma membrane
Sue1		-5,20	required for degradation of Cytochrome C
Tma10	-11,26		protein of unknown function
Tma17		-8,87	protein of unknown function
Ubc8	-14,41	-3,88	ubiquitin-conjugating enzyme
Ugx2	-18,21	-4,04	protein of unknown function
Yat1	-16,64		outer mitochondrial carnitine acetyltransferase
Yat2	-23,95	-3,84	carnitine acetyltransferase
Ybl048w	-10,88	-4,44	protein of unknown function
Yfr017c	-11,81		protein of unknown function
Ygr067c	-15,98		zink finger motif, unknown function
Yil057c	-46,68	-5,00	protein of unknown function
Yjl045w		-3,72	minor succinate dehydrogenase isozyme
Ylr164w	-13,32	-4,98	protein of unknown function
Ynl195c	-19,11		protein of unknown function
Yor289w		-3,94	putative protein of unknown function

Ypt53		-4,50	Rab family GTPase; required for vacuolar protein sorting and endocytosis
Ytp1	-12,66		probable type-III integral membrane protein of unknown function

Table 3: Genes which expression levels are up-regulated by addition of the SDF-2-like peptide to the medium.

Gene	Ratio WT(+)/ WT(-)	Ratio *grh1*Δ(+)/ *grh1*Δ(-)	Description
Acc1		6,01	acetyl-CoA carboxylase
Ai2		6,55	reverse transcriptase
Ai5_α		6,03	endonuclease I-SceIV
Btn2		9,25	v-SNARE binding protein
Cbf5	17,85	8,45	pseudouridine synthase catalytic subunit of box H/ACA small nucleolar ribonucleoprotein particles
Cha1	13,40		catabolic L-serine (L-threonine) deaminase
Dbp3	18,01		RNA-dependent ATPase
Drs2	13,61		aminophospholipid translocase (flippase)
Dys1	14,32	6,28	deoxyhypusine synthase
Ecm16	16,63		DEAH-box ATP-dependent RNA helicase
Gar1	13,94		component of the H/ACA snoRNP pseudouridylase complex
Gic2		6,15	rho-like GTPase Cdc42p effector
Hap4		5,83	transcriptional activator and global regulator of respiratory gene expression
Hxt3		7,94	hexose transport
Krr1	13,76		nucleolar protein required for the synthesis of 18S rRNA
Laa1		6,14	AP-1 accessory protein
Lcb2	13,29		component of serine palmitoyltransferase
Lsg1	15,16		putative GTPase involved in 60S ribosomal subunit biogenesis
Lys2		6,26	alpha aminoadipate reductase
Mak5	17,33		putative DEAD-box RNA helicase
Mdn1	29,59	13,93	huge dynein-related AAA-type ATPase (midasin)
Myo2	14,17		type V myosin motor involved in actin-based transport of cargos
Ncl1	16,06	6,14	tRNA: m5C-methyltransferase
Nop14	15,79		nucleolar protein
Nop4	14,32		nucleolar protein
Nop58	18,72	6,79	protein involved in pre-rRNA processing
Nop7	18,34	6,01	component of several different pre-ribosomal particles
Npl3	14,84		RNA-binding protein; promotes elongation, regulates termination

Pdr5		26,75	plasma membrane (ABC) transporter
Pds5	16,57		establishment and maintenance of sister chromatid condensation and cohesion
Pet122		9,91	mitochondrial translational activator
Pol2	18,91	6,40	catalytic subunit of DNA polymerase (II) epsilon
Prp43	22,01		RNA helicase in the DEAH-box family
Pwp1	19,90	6,90	protein with WD-40 repeats involved in rRNA processing
Pxr1	18,26		protein involved in rRNA and snoRNA maturation
Rbg1	13,54		GTP binding, associates with translating ribosomes
Rok1	19,96		RNA-dependent ATPase
Rpl14a		5,88	ribosomal 60S subunit protein
Rpl24b	14,10	6,38	ribosomal 60S subunit protein
Rpl25	18,58	6,40	ribosomal 60S subunit protein
Rpl29		6,21	ribosomal 60S subunit protein
Rpl30		6,03	ribosomal 60S subunit protein
Rpl31b	23,27	6,02	ribosomal 60S subunit protein
Rpl33a		6,04	ribosomal 60S subunit protein
Rpl37a		5,98	ribosomal 60S subunit protein
Rpl43a		6,85	ribosomal 60S subunit protein
Rps16b		6,19	protein component of the small (40S) ribosomal subunit
Rps18b	14,26		protein component of the small (40S) ribosomal subunit
Rps22a		7,46	protein component of the small (40S) ribosomal subunit
Rps22b		7,85	protein component of the small (40S) ribosomal subunit
Rrp12	13,85		protein required for export of the ribosomal subunits
Sac1	16,99	6,74	phosphatidylinositol 4-phosphate (PtdInsP) phosphatase
Shm2	17,55		cytosolic serine hydroxymethyltransferase
Snq2		17,00	plasma membrane (ABC) transporter
Spb1	15,39		AdoMet-dependent methyltransferase
Sro9	15,00		cytoplasmic RNA-binding protein
Srp40	15,87		nucleolar, serine-rich protein with a role in preribosome assembly
Ssa2		9,11	ATP binding protein involved in protein folding and vacuolar import of proteins
Ssa4		11,30	heat shock protein that is highly induced upon stress
Ste12	13,27		transcription factor that is activated by a MAP kinase signaling cascade
Ste6	15,76	6,16	plasma membrane (ABC) transporter, a-factor export
Svl3	14,95		protein of unknown function

Tcb2	14,12	6,30	ER protein involved in ER-plasma membrane tethering
Tdh3		6,77	glyceraldehyde-3-phosphate dehydrogenase, isozyme
Tif4631	13,63		translational initiation factor
Tpo1	15,59	11,03	polyamine transporter
Trm11	13,92		catalytic subunit of an adoMet-dependent tRNA methyltransferase complex
Utp5	13,97		subunit of U3-containing small subunit (SSU) processome complex
Utr2	15,37		chitin transglycosylase
Ycr013c	19,40		protein of unknown function
Yef3		5,74	gamma subunit of translational elongation factor eEF1B
Ygl182c		5,97	cellular response to starvation, regulation mitotic cell cycle
Ygr035c	13,61	34,77	protein of unknown function
Ygr109w-a	14,24		protein of unknown function
Yhr033w		7,54	protein of unknown function
Yhr093w		7,94	protein of unknown function
Yil100w		5,92	protein of unknown function
Ykl153w	21,56	7,15	protein of unknown function
Ykr011c		6,15	protein of unknown function
Ylr162w	16,24		protein of unknown function
Ymr173w-a		16,91	protein of unknown function
Ypr174c		6,79	protein of unknown function
Zpr1		7,42	protein of unknown function

3.2.2 Exogenous expression of known unconventional secreted cargoes from higher eukaryotes in yeast

Previous work done by Cleves et al. reported the exogenous expression and subsequent unconventional secretion of galectin-1 in yeast. Galectin-1 is a small protein of 14kDa with an acetylated N terminus and no further post-translational modifications (Cleves et al., 1996). Its secretion in *S. cerevisiae* was independent of the a-factor mating pheromone transporter Ste6 and did not require the NSF factor Sec18 suggesting that secretion of galcetin-1 does not

require vesicular intermediates (Cleves et al., 1996). This indicates that it does not follow the same pathway as Acb1. However, these results imply that it is possible to express proteins from higher eukaryotes in the budding yeast and to study their unconventional release from the cells.

Similarly to galectin-1, also IL-1ß and macrophage migration inhibitory factor (MIF) are relatively small cytosolic proteins and secreted unconventionally (Flieger et al., 2003; Rubartelli et al., 1990). Secretion of IL-1ß was reported to require autophagy-related proteins as well as GRASP55 suggesting a similar secretion route compared to Acb1 (Dupont et al., 2011) whereas MIF was found in vesicular intermediates within the cell that also contained LC3 suggesting that MIF is present in some class of autophagosomes (Josse van Gaalen, unpublished data). We therefore decided to examine those two proteins in yeast. Exogenous expression of MIF could not be detected in whole yeast cell lysates whereas IL-1ß was expressed and even found to be secreted into the medium by yeast cells. However, this was not starvation specific nor dependent on the presence of Grh1 as a *grh1*Δ strain was not defective in secretion, indicating that at least when expressed exogenously in yeast, IL-1ß does not require a similar secretion mechanism as Acb1 (data not shown).

3.2.3 Analysis of the starvation-specific secretome

The expression of unnatural cargoes could lead to artificial or misleading results as it does not represent the natural situation. We therefore tested if we could identify additional unconventionally secreted proteins in the yeast secretion medium in response to

nutrient starvation. A radioactive approach was used to establish the conditions. WT and *grh1*Δ yeast as well as a strain carrying a temperature-sensitive mutation in *SEC1, sec1-1,* and a double mutant strain, *sec1-1 grh1*Δ, were pulsed for 45min with ^{35}S-methionine at 25°C or 37°C. The cells were harvested, starved for 30min at 25°C or 37°C, harvested and further starved for 4h in fresh starvation medium at 25°C or 37°C. The secreted proteins were precipitated, separated by SDS-PAGE and subsequently analyzed by autoradiography. In the secretion medium from the pulse sample of WT, *grh1*Δ strains and *sec1-1* strains incubated at 25°C, several protein bands were detected whereas protein secretion at the non-permissive temperature in *sec1-1* strains was greatly reduced, as expected. Upon starvation, protein secretion of all strains was similar at both temperatures (25°C and 37°C) indicating that secretion of most proteins is blocked during starvation even in strains usually not affecting the conventional secretory pathway (WT and *grh1*Δ; Figure 27). We therefore decided to use WT and *grh1*Δ strains for the analysis of the starvation-specific secretome.

As we only detected a very small number of proteins in the secretion medium with a very low protein concentration in general, we purified the proteins/peptides secreted upon starvation by passing the medium over a C18- or a C8-column instead of TCA precipitation that produced non-reproducible results (data not shown). To identify proteins secreted upon nutrient starvation in a Grh1-dependent manner, we used quantitative stable isotope labeling by amino acids in cell culture (SILAC) mass spectrometry. WT cells were grown in heavy lysine-containing medium whereas *grh1*Δ yeast were cultured in medium containing unlabeled lysine.

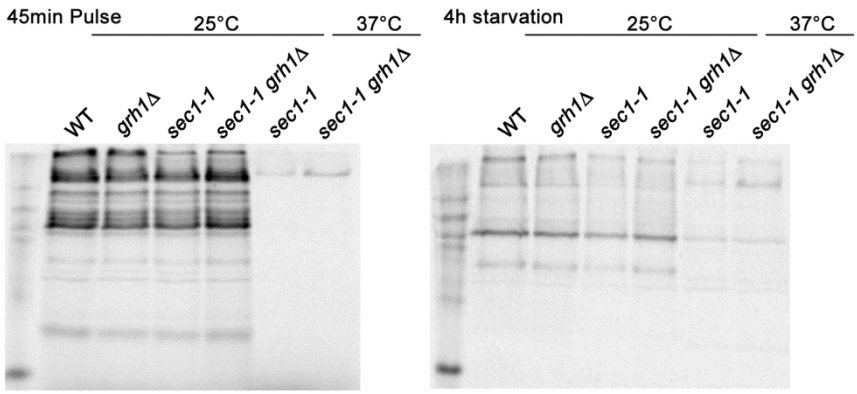


Figure 27: Protein secretion is strongly reduced upon starvation. WT, *grh1*Δ, *sec1-1* and *sec1-1 grh1*Δ strains were pulsed for 45min at the indicated temperature to label newly synthesized proteins with ^{35}S-methionine. The cells were harvested, incubated for 30min in starvation medium, harvested again and further starved for 4h. The secretion medium after 4h starvation was collected and the proteins were precipitated, separated on an SDS-PAGE and analyzed by autoradiography.

The same OD of yeast was subjected to starvation and incubated for 4h. The cells were harvested by centrifugation and the secretion medium was passed through a 0.22µm-filter to ensure complete removal of cells. The secretion media of WT and *grh1*Δ strains were pooled and processed as described in chapter 5.3.6. The detected peptides are listed in Tables 4-7. Several peptides and proteins were identified, however, a large number of them are known cell wall-associated proteins with a classical signal sequence. Additionally, a larger number of metabolic enzymes were found in the secretion medium, of which several had been detected outside before. However, their secretion mechanism remains elusive on most cases (Nombela et al., 2006).

Unfortunately, we did not identify proteins/peptides without a signal peptide that were secreted only in WT strains and absent in *grh1*Δ strains. However, we did detect Stm1 in the secretion medium, a protein previously pulled-out with Grh1-TAP upon starvation (Figure 21 and Tables 4-7) and its secretion mechanism is currently

being investigated. Nevertheless, more effort needs to be spend to either identify a new cargo molecule to analyze unconventional protein secretion in the budding yeast or a different strategy needs to be developed to be able to detect unconventionally secreted Acb1 outside the cell.

Table 4: Most abundant peptides found in the secretion medium. SP: signal peptide, -: no SP; +: SP present; M: mitochondrion, T: transmembrane domains

Protein	SP	Description	Number Peptides	Ratio (H/L) WT/*grh1*Δ
Pdc1	–	alpha subunit of fatty acid synthetase	278	0,49
Tdh3	+	3-phosphoglycerate kinase	204	0,49
Eno2	–	enolase II, a phosphopyruvate hydratase	209	0,45
Eno1	–	ATPase; member of HSP70 family	167	0,65
Tdh2	+	ATP binding protein involved in protein folding and vacuolar import of proteins	136	0,41
Hsp150	+	major of three pyruvate decarboxylase isozymes	123	3,13
Tdh1	+	cytoplasmic chaperone of the Hsp90 family	94	0,64
Pgk1	–	Hsp90 chaperone	88	0,45
Fba1	–	cystathionine beta-synthase	88	0,48
Cdc19	–	ATPase; component of the heat shock protein Hsp90 chaperone complex	75	0,48
Ssa1	–	glyceraldehyde-3-phosphate dehydrogenase, isozyme 3	79	0,47
Tef2	–	cytoplasmic ATPase	80	0,51
Ssa2	–	enolase I, a phosphopyruvate hydratase	73	0,39
Gpm1	M	pyruvate kinase	63	0,51
Ssb2	–	glyceraldehyde-3-phosphate dehydrogenase, isozyme 2	67	0,39
Pir1	+	beta subunit of fatty acid synthetase	68	2,97
Ssb1	–	cytoplasmic ATPase	69	0,47
Tpi1	–	triose phosphate isomerase	42	0,48
Stm1	–	protein required for optimal translation under nutrient stress	36	0,48
Pma1	T	plasma membrane H^+-ATPase	42	0,46

Table 5: Most abundant proteins found in the secretion medium.
SP: signal peptide, -: no SP, +: SP present

Protein	SP	Description	Number Peptides	Ratio (H/L) WT/*grh1*Δ
Fas2	–	alpha subunit of fatty acid synthetase	20	0,53
Pgk1	–	3-phosphoglycerate kinase	19	0,47
Eno2	–	enolase II, a phosphopyruvate hydratase	18	0,48
Ssa1	–	ATPase; member of HSP70 family	17	0,55
Ssa2	–	ATP binding protein involved in protein folding and vacuolar import of proteins	16	0,45
Pdc1	–	major of three pyruvate decarboxylase isozymes	14	0,51
Hsc82	–	cytoplasmic chaperone of the Hsp90 family	14	0,59
Hsp82	–	Hsp90 chaperone	14	0,42
Cys4	–	cystathionine beta-synthase	13	0,46
Sse1	–	ATPase; component of the heat shock protein Hsp90 chaperone complex	13	0,51
Tdh3	–	glyceraldehyde-3-phosphate dehydrogenase, isozyme 3	12	0,70
Ssb1	–	cytoplasmic ATPase	12	0,50
Eno1	–	enolase I, a phosphopyruvate hydratase	11	0,70
Cdc19	–	pyruvate kinase	11	0,53
Tdh2	+	glyceraldehyde-3-phosphate dehydrogenase, isozyme 2	10	0,55
Fas1	–	beta subunit of fatty acid synthetase	10	0,52

Table 6: Proteins secreted less in *grh1*Δ yeast compared to WT.
SP: signal peptide, -: no SP, +: SP present

Protein	SP	Description	Number Peptides	Ratio (H/L) WT/*grh1*Δ
Hsp150	+	O-mannosylated heat shock protein	20	0,53
Pir1	+	O-glycosylated protein required for cell wall stability	19	0,47
Ygp1	+	cell wall-related secretory glycoprotein	18	0,48
Met17	–	methionine and cysteine synthase	17	0,55
Bgl2	+	endo-beta-1,3-glucanase	16	0,45
Crh1	+	chitin transglycosylase	14	0,51
Prb1	+	vacuolar proteinase B	14	0,59

Table 7: Peptides secreted less in *grh1*Δ yeast compared to WT.
SP: signal peptide, -: no SP, +: SP present

Protein	SP	Description	Number Peptides	Ratio (H/L) WT/*grh1*Δ
Hsp150	+	O-mannosylated heat shock protein that is secreted	123	3,13
Pir1	+	O-glycosylated protein required for cell wall stability	68	2,97
Pep4	+	vacuolar aspartyl protease	12	2,63
Prb1	+	vacuolar proteinase B	12	1,65
Ygp1	+	cell wall-related secretory glycoprotein	9	2,97
Egt2	+	glycosylphosphatidylinositol (GPI)-anchored cell wall endoglucanase	7	6,62
Ecm33	+	GPI-anchoring on the plasma membrane crucial to function	7	1,43
MF(α)1	+	mating pheromone alpha-factor, made by alpha cells	5	1,56
Ino1	–	inositol-3-phosphate synthase	4	2,53
Anp1	+	subunit of the alpha-1,6 mannosyltransferase complex; type II membrane protein	4	1,40
Exg1	+	major exo-1,3-beta-glucanase of the cell wall	4	1,45
Ktr6	+	probable mannosylphosphate transferase	3	13,41
Ybr139w	+	putative serine type carboxypeptidase with a role in phytochelatin synthesis	3	3,04

4 Discussion

Even though GRASP proteins were initially identified as factors required for stacking the Golgi cisternae into the Golgi ribbon *in vitro* (Barr et al., 1997; Shorter et al., 1999), their conserved *in vivo* function throughout eukaryotes appears to be in the process of unconventional protein secretion. Plants do not possess a GRASP ortholog yet they have a stacked Golgi apparatus; by contrast the Golgi cisternae of the budding yeast are not stacked and distributed throughout the cell even in the presence of the GRASP protein Grh1. Unconventional secretion of proteins in mammalian cells, *Drosophila* S2 cells, *D. discoideum*, and the yeasts *S. cerevisiae* and *P. pastoris* requires a GRASP protein (Dupont et al., 2011; Duran et al., 2010; Gee et al., 2011; Kinseth et al., 2007; Manjithaya et al., 2010; Schotman et al., 2008). However, the exact role as well as the specific site of its involvement remain elusive and have so far not been deeply investigated.

The main aim of this thesis was therefore to investigate the role of Grh1 in the unconventional secretion of Acb1. The work presented here shows that Grh1 redistributes to unique membrane structures, the CUPS, in close proximity to ER exit sites, specifically under conditions that promote Acb1 secretion. Due to the properties, morphology, and specific content of the CUPS, we suggest that they serve as a starting point or a sorting station for the biogenesis of cargo-containing vesicular intermediates which are used for unconventional protein secretion.

4.1 CUPS as a novel compartment for unconventional protein secretion

CUPS form close to the ER exit sites specifically under conditions of glucose starvation, are stable compartments during the time of starvation, and contain a characteristic cup-shape as observed by immunoelectron microscopy. CUPS do not seem to fuse or mix with Golgi membranes or endosomes and they do not contain cargoes destined for a degradative pathway. We identified Grh1, Bug1, Vps23, Atg8, Atg9, and the phosphoinositide PI(3)P as starvation-specific content of the CUPS and showed that Bug1 as well as the phosphatidylinositol 3-kinase Vps34 are essential to recruit Grh1-GFP to the CUPS.

We analyzed CUPS assembly by monitoring Grh1-GFP localization in several deletion strains and strains carrying temperature-sensitive mutations in genes previously linked to Acb1 secretion, most of which had no effect on Grh1-GFP redistribution. We noticed improper CUPS formation in an *atg8*Δ strain, whereas deletion of other genes coding for proteins involved in autophagy did not affect Grh1-GFP re-localization at the level of fluorescence microscopy. We cannot rule out, however, that deletion of these genes does not affect the generation of the characteristic cup-like shape and therefore could have an effect on its function. In addition, strains deleted for ESCRT-II and ESCRT-III complex coding genes failed to concentrate the entire amount of Grh1-GFP to the CUPS. This cannot be simply explained by a failure in MVB biogenesis, as deletion of *VPS23* for example, did not affect Grh1-GFP recruitment. Some ESCRT deletion strains, for instance *vps20*Δ and *vps27*Δ, were reported to have a decreased starvation resistance (Davey et al., 2012). Thus, the partial effect on CUPS

biogenesis could also be an effect of dying cells and this would need to be investigated further.

A genome-wide screen for CUPS formation (consisting of the entire yeast deletion collection and a collection of temperature-sensitive strains of essential genes) has just been performed in collaboration with the laboratory of Brenda Andrews (University of Toronto). The query strain used for this high-content synthetic genetic array is co-expressing Grh1-GFP with a nuclear and a cytoplasmic marker to facilitate automated image analysis and the data analysis is currently ongoing. Once the entire data set has been analyzed and validated, we expect to have a deeper understanding in how this compartment is formed, where the membranes are coming from and potentially how the characteristic cup-like shape is generated and maintained.

We observed that CUPS formation is induced specifically upon glucose starvation and that a strain deleted for the AMP kinase *SNF1*, the major glucose-sensing enzyme in the budding yeast, failed to redistribute a large amount of Grh1-GFP into CUPS. Therefore we are also planning to determine how the formation of CUPS is induced by attempting to identify downstream effector proteins of Snf1.

CUPS share similarities with omegasomes, an amino acid starvation-induced compartment in mammalian cells known to form close to the ER, to be enriched in PI(3)P, to contain the mammalian Atg8-ortholog LC3, and thought to be a scaffold for the formation of autophagosomes (Axe et al., 2008; Matsunaga et al., 2010). CUPS however are not involved in a degradative pathway and their formation is not induced by treatment with the autophagy-inducing drug rapamycin. Thus, CUPS are most likely not the same com-

partment in yeast as omegasomes are in mammalian cells. Moreover, CUPS are stable and not consumed during the time of starvation and form independent of new protein synthesis. Additionally, as CUPS contain several factors which were previously identified as requirements for the secretion of Acb1 (Grh1, Vps23, and Atg proteins), we therefore propose that they have a secretory function.

Duran et al. reported that proteins involved in fusion of membranes with endosomes, proteins of the ESCRT machinery and proteins involved in fusion of vesicles with the cell surface are a prerequisite for the secretion of Acb1 (Duran et al., 2010). It was suggested that Acb1 is captured into autophagosome-like structures which would eventually fuse with endosomes to give rise to MVBs. The final fusion step with the cell surface would then rely on the plasma membrane t-SNARE Sso1 (Duran et al., 2010). However, deletion of key genes involved in these steps (*YPT6*, *TLG2*, and *SSO1*) and a temperature-sensitive mutant of the NSF factor *SEC18* did not affect CUPS biogenesis indicating that they likely act post-CUPS assembly for Acb1 secretion. Are CUPS then involved in cargo recognition/selection and subsequent transport or attachment of Acb1 to vesicular intermediates? ACBPs are known to bind to acyl-CoA and mutating the crucial residues for this binding prevents membrane attachment of ACBP (Hansen et al., 2008). Additionally, mutation of a putatively acetylated N-terminal phenylalanine in *D. discoideum* AcbA inhibits the secretion of SDF-2 into the medium, suggesting an involvement of this residue in unconventional secretion (Christophe Anjard, unpublished data). Could acetylation and/or acyl-CoA binding of Acb1 be involved in targeting the protein to CUPS? Clearance of misfolded huntingtin protein by autophagy has been shown to rely on acetylation of a specific residue

(Jeong et al., 2009). It is therefore possible that the unconventional secretion of Acb1, which also requires autophagy-related proteins, indeed relies on acetylation as a sorting motif.

Assuming that Acb1 is targeted by acetylation/acyl-CoA binding to the CUPS, its subsequent secretion will require the formation of a vesicular intermediate as CUPS are stable compartments, thus CUPS themselves are unlikely to represent a vesicular intermediate promoting the secretion of Acb1. Duran et al. suggested the formation of an autophagosome-like double-membrane vesicular intermediate (Duran et al., 2010). Alternatively, the formation of a vesicular intermediate surrounded by a single lipid bilayer is also reasonable (Figure 28). In this situation, CUPS could be the membrane source for a vesicle that buds from it and this vesicular intermediate would contain Acb1 attached to its surface (Figure 28A (1) and (2)). Upon fusion with an endosome (mediated by Tlg2), Acb1 would remain on the outside of the endosome (Figure 28A (3)) and would need to be invaginated into the lumen of a vesicle by the ESCRT complexes. The generic cargoes found in these intralumenal vesicles (ILV) are ubiquitinated transmembrane domain-containing proteins (Duncan et al., 2006; Hicke and Dunn, 2003; Piper and Katzmann, 2007). Interestingly, Acb1 was found to be ubiquitinated *in vivo* (Starita et al., 2011) and therefore, a membrane-attached and ubiquitinated Acb1 could potentially be a cargo for the ESCRT machinery. Furthermore, this vesicular intermediate generated from the CUPS could be enriched in PI(3)P and contain Vps23 and thus, could be already primed for subsequent ESCRT-mediated invagination prior to a possible deacetylation and detachment of Acb1 from the outside of the endosomes (Figure 28A

(4)). The generated MVB could subsequently fuse with the plasma membrane to release Acb1-containing exosomes (Figure 28A (5)).

Additionally, the generation of an autophagosome for the release of Acb1 is feasible (Figure 28B). Formation of a so-called secretory autophagosome –as CUPS are not involved in a degradative pathway and unlikely form generic autophagosomes– could occur similarly to the proposed generation of autophagosomes from omegasomes (Hayashi-Nishino et al., 2009; Tooze and Yoshimori, 2010; Yla-Anttila et al., 2009). This could potentially explain the role of Atg proteins at the CUPS and why deletion of *ATG*s does not affect CUPS formation. This secretory autophagosome could contain Acb1 attached to the inner and outer membrane similar to the distribution of Atg8 at autophagosomes. Also, in this situation, specific membrane attachment of Acb1 could occur by acetylation and/or acyl-CoA binding as described above (Figure 28B (1) and (2)). This secretory autophagosome would be enriched in PI(3)P and could contain Vps23, and could therefore as well be applicable for the subsequent fusion with and invagination into specific endosomes. Upon fusion with an endosome-like structure, a part of Acb1 would be directly delivered into the lumen in ILVs (Acb1 previously bound to the inner membrane) whereas Acb1 attached to the outer membrane would stay on the outside of the endosomes (Figure 28B (3)) and requires the ESCRT complexes for its import into ILVs (Figure 28B (4)). Moreover, granted that only fully developed MVBs and not structures containing remaining Acb1 on the surface are able to fuse with the plasma membrane to release Acb1-containing exosomes (Figure 28B (5)), the ESCRT complexes would be needed for the unconventional secretion of Acb1 as identified previously (Duran et al., 2010).

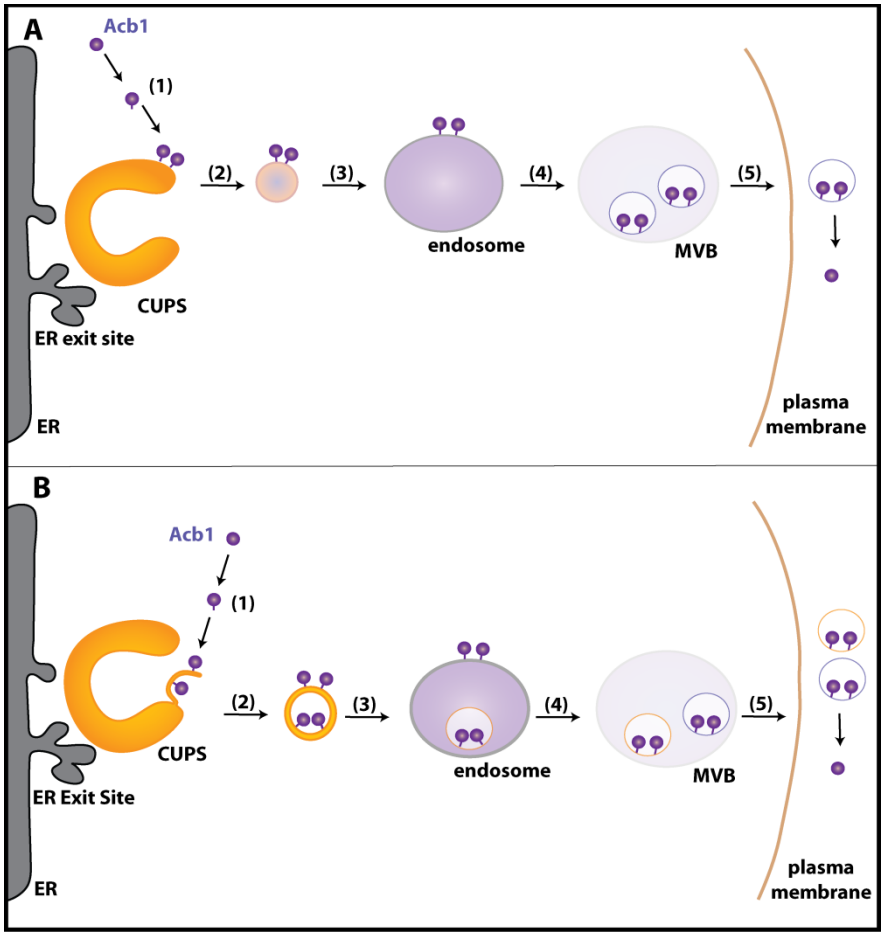


Figure 28: Proposed models for CUPS-mediated unconventional secretion of Acb1. (A) Attachment of Acb1 to the CUPS is mediated by N-terminal acetylation and/or acyl-CoA binding (1). Subsequently, a vesicular intermediate (single lipid bilayer) containing Acb1 attached to the outside buds from the CUPS (2) and fuses with an endosome (mediated by Tlg2 and Ypt6) (3). At this step Acb1 is ubiquitinated and subsequently imported into intralumenal vesicles (ILVs) by the ESCRT complexes (4). The generated MVB fuses with the plasma membrane to release Acb1-containing exosomes from the cell (5) which will eventually lyse to release Acb1. (B) Acb1 will be attached to a membrane extending from the tip of the CUPS via N-terminal acetylation and/or acyl-CoA binding (1). Upon closure, an autophagosome-like vesicular intermediate (double lipid bilayer) will contain Acb1 attached to its inner and outer membrane (2). This vesicular intermediate will fuse with an endosome by Tlg2/Ypt6 and Acb1 attached to the outer membrane will remain attached to the surface whereas Acb1 attached to the inner membrane will directly be released into the lumen as ILV (3). Remaining Acb1 attached to the surface is imported into ILVs by the ESCRT machinery (4). Subsequently, the MVB fuses with the plasma membrane to release Acb1-containing vesicles from the cell (5).

As proposed above, Acb1 might be captured into vesicular intermediates which would eventually fuse with endosomes in a fusion step requiring the endosomal t-SNARE Tlg2. Deletion of this gene should allow Acb1-containing intermediates to accumulate within the cell, provided that they are not rerouted for instance to the vacuole in case of secretory autophagosomes. Similarly, blocking the fusion step with the cell surface by deletion of *SSO1* should accumulate Acb1-containing MVB-like structures. However, an *sso1*Δ strain does not contain higher amounts of proteinase K-protected Acb1 (unpublished results). This indicates that the blockade of certain transport routes in yeast does not necessarily cause the accumulation of downstream intermediates. A similar situation has been reported, for example, for the conventional transport of cargo molecules from late Golgi cisternae to the cell surface (Harsay and Bretscher, 1995). Yeast are known to generate two different classes of vesicles for Golgi to cell surface transport –low-density secretory vesicles (LDSVs) and high-density secretory vesicles (HDSVs)–, which are specific for a subset of secretory proteins. LDSVs are known to transport Pma1 to the plasma membrane or the cell wall protein Bgl2, whereas HDSVs contain cargoes such as invertase (Harsay and Bretscher, 1995). Interestingly, in clathrin mutants where the biogenesis of HDSV is inhibited invertase transport still occurred and this cargo could be detected in LDSV, indicating that secretory cargo had been rerouted (Gurunathan et al., 2002; Harsay and Schekman, 2002). Even though these events are not related to unconventional protein secretion, they emphasize that yeast are capable of redirecting cargo molecules into different intermediates and therefore the possible rerouting of Acb1-containing intermediates from a secretory to a degradative pathway cannot be excluded.

Additionally, to obtain a deeper understanding in the lipid and polypeptide composition of CUPS, we considered immunoisolating them from the lighter gradient fractions using Grh1 as a marker protein. At the level of fluorescence microscopy, virtually all Grh1-GFP is recruited to the CUPS. In contrast, when fractionated on a continuous sucrose gradient, only of about 20% of Grh1-GFP shifted to lighter fractions. Thus, it could be possible that the CUPS are attached to the ER and that the part of Grh1 that shifts in the gradient was separated during preparation complicating the ability to determine where the CUPS migrate on the gradient.

Our fluorescence and immunoelectron microscopy-based analysis of Grh1-GFP localization under nutrient-rich conditions showed that Grh1 is not a Golgi-associated protein in the budding yeast even though GRASP proteins in mammals are known to localize to early Golgi membranes (Barr et al., 1998; Barr et al., 1997; Short et al., 2001; Shorter et al., 1999). We could also not detect a clear localization of Grh1 to the ER, neither the ER exit sites as it has been reported by the laboratory of Ben Glick at the level of fluorescence microscopy (Levi et al., 2010). Therefore, we propose that under nutrient-rich conditions Grh1 might associate to membranes similar to an ER-Golgi intermediate compartment (ERGIC) that has not yet been described in yeast or to specific sub-domains of the ER. The ERGIC in higher eukaryotic cells is defined as vesicular-tubular system and thought to facilitate cargo sorting prior to their entry into the Golgi apparatus or their transport back to the ER (Appenzeller-Herzog and Hauri, 2006). Interestingly, Grh1 has been identified to interact physically and/or genetically with several COPII components, the β-coat protein of the COPI coatomer and other factors involved in trafficking between the ER and the Golgi, like Uso1 and

Sly1, yet no role for Grh1 conventional secretion has been observed (Behnia et al., 2007; Collins et al., 2007; Costanzo et al., 2010; Tarassov et al., 2008). Therefore it is tantalizing to speculate that Grh1 could localize to an ERGIC-like compartment in the budding yeast under nutrient-rich conditions. This would indicate an even higher degree of conservation of the classical secretory pathway from yeast to human that has yet to be unraveled.

4.2 Grh1 as chaperone for specific proteins at the level of the CUPS

We identified that one of the roles of Grh1 at the level of the CUPS is the recruitment of factors like Vps23 and Atg9 and therefore it is most likely involved in establishing and/or maintaining the identity of the CUPS. If we assume that Atg proteins are involved in the formation of an Acb1-containing vesicular intermediate destined for unconventional protein secretion and that Vps23 is needed to achieve the specific properties of this intermediate to direct it towards endosomes and to avoid fusion with the vacuole as described above, deletion of *GRH1* would severely affect the secretion of Acb1. We also observed that, upon starvation, Grh1 interacts with different and probably more proteins compared to growth conditions. Nevertheless, the proteins we were focusing on did not affect Grh1 re-localization nor were they found to be present in the CUPS, except for Bug1, so that the impact of their interaction with Grh1 still needs to be determined. However, we suggest that additional starvation-specific interacting proteins have yet to be identified and serve crucial roles in recruiting Grh1 to the CUPS. Deletion strains defective in Grh1-GFP recruitment to the CUPS, which

will be identified in our genome-wide screen, will therefore also be validated for a starvation-specific localization to CUPS.

In a strain deleted for *BUG1*, Grh1-GFP remained entirely cytosolic under growth and nutrient-starvation conditions. Furthermore, a *bug1*Δ strain is also defective in secretion of SDF-2-like material (Duran et al., 2010), indicating that Grh1 needs to be membrane-attached, most likely to the CUPS, in order to achieve its function for the unconventional release of Acb1. However, if this is the only function of Bug1 in unconventional secretion of Acb1 remains unanswered so far.

Both, Grh1 and GRASP proteins contain PDZ-domains at their N-terminal regions. PDZ-domains are known protein-protein interacting motifs; they either bind to PDZ domains of other proteins or to hydrophobic C-terminal motifs with the consensus sequence S/T/Y-X-V/I-COO^- (Lee and Zheng, 2010; Ranganathan and Ross, 1997; Songyang et al., 1997). The interaction of the C-terminal valine-containing motif with GRASPs was shown to be required for the transport of TGFα or CD8α along the Golgi stack (D'Angelo et al., 2009; Kuo et al., 2000). Does Grh1 also interact with specific cargo molecules following an unconventional protein secretion process and could potentially act as a chaperone? Our mass-spectrometry data of the yeast starvation specific secretome support this hypothesis, as two of the Grh1-interacting proteins, Stm1 and Scs2, were found in the secretion medium.

Stm1 is a yeast-specific protein with a putative PDZ-interacting motif at its C terminus (the last three amino acids are SLA). Even though this motif does not exactly match with the consensus sequence mentioned above, the last amino acid is hydrophobic. Atypical PDZ-interacting motifs have been described, for example

Syntenin-PDZ binds to the C terminus of Syndecan with the atypical motif FYA (Grootjans et al., 1997). Furthermore, secreted Stm1 contains an acetylated N terminus as we detected by mass spectrometry.

A second interesting observation was the detection of Scs2 in the secretion medium. Scs2 contains a transmembrane domain close to its C terminus, is an integral ER membrane protein and is known to be involved in phospholipid metabolism and ceramide transport (Loewen et al., 2003). Additionally, Scs2 contains an amino-terminal major sperm protein (MSP)-domain that is conserved from yeast to mammals. MSP was originally identified in *Caenorhabditis elegans*, where it is a sperm-derived hormone, that binds to the Epherine receptor and is required to induce oocyte maturation (Calo et al., 2006). Its secretion mechanism is not understood, however it has been detected in double-membrane vesicular intermediates resembling autophagosomes (Kosinski et al., 2005). Processed forms of the human and the *Drosophila* ortholog, the N-terminal MSP domain of VAPB and *d*VAP respectively, have been detected extracellularly (Tsuda et al., 2008) which let us wonder if a processed N-terminal part of Scs2 might be secreted in yeast as well. Not only does Stm1 contain an acetylated N terminus, this modification was also present in the secreted N-terminal part of Scs2. In addition to the putative role of acetylation in secretion of Acb1, the acetylation of these two proteins strengthens our hypothesis that this modification could at least in part be involved in cargo selection for unconventional protein secretion and Grh1 could have a role in guiding these proteins for their unconventional release.

4.3 No generic cargo for unconventional secretion

Unconventional secretion of cytoplasmic proteins is suggested to follow four different main secretion routes: (1) secretion by secretory lysosomes; (2) direct translocation across the plasma membrane; (3) secretion of exosomes; and (4) export of microvesicles through plasma membrane blebbing (Nickel, 2005). However, many open questions remain. For example, what are the cargo properties that determine which specific route they will follow? What are the required signals for their entry into the unconventional secretion pathway? Are the factors regulating the secretion process shared amongst all unconventionally secreted proteins? As the mechanisms of unconventional protein secretion in most cases are just beginning to be deciphered, no generic cargo to study this secretion process has been identified and it has not been possible yet to engineer a cargo molecule similar to signal sequence-containing proteins for the study of the conventional pathway.

The only known unconventionally secreted cargo in the budding yeast, whose release involves a vesicular intermediate, is Acb1 (Duran et al., 2010). However, we have not been able to establish a biochemical assay to monitor the steps of unconventional secretion of Acb1 inside the cell nor to detect it in the yeast secretion medium due to various technical problems: First, Acb1 is a small (~10kDa), highly abundant cytosolic protein conserved from yeast to humans and the secreted part, the central SDF-2-like peptide, is only of about 4kDa in size (Kinseth et al., 2007; Mandrup et al., 1993; Mogensen et al., 1987; Rosendal et al., 1993), therefore detection by standard methods of western blot or ELISA have been unsuccessful. Second, only a small percentage of Acb1 is thought to be secreted from the cells upon nutrient starvation (Duran et al.,

2010; Kinseth et al., 2007) and possible cell lysis could thus mask the unconventionally secreted pool of Acb1. Third, as Acb1 serves important intracellular functions, mutating specific sites of the protein or introducing internal tags to monitor the SDF-2-like peptide negatively affects protein stability and therefore further complicated our studies (unpublished results). Fourth, whereas it is known, that in *D. discoideum*, AcbA is secreted from the prespore cells and then cleaved by the protease TagC –which is only expressed on the plasma membrane of the prestalk cells– into the central SDF-2 peptide (Anjard and Loomis, 2005; Kinseth et al., 2007), the protease and its localization in the yeasts *S. cerevisiae* and *P. pastoris* remain elusive as they do not express a TagC ortholog yet they generate SDF-2-like material (Duran et al., 2010; Manjithaya et al., 2010). Fifth, even though in *D. discoideum* and *P. pastoris* SDF-2 is involved in spore viability/sporulation (Anjard and Loomis, 2005; Kinseth et al., 2007; Manjithaya et al., 2010), we did not observe this role for Acb1 in *S. cerevisiae* and could thus far not determine a physiological role of Acb1 outside the cell. We therefore suggest that the extracellular role of Acb1 in the budding yeast is different than in *P. pastoris* or the conditions we used to set up the sporulation assay were not suitable for *S. cerevisiae*. Furthermore, the secreted ACBP peptides TTN and ODN in mammals are known to bind to GABA (γ-aminobutyric acid)$_A$ receptors and modulate their response to GABA (Ferrero et al., 1986; Slobodyansky et al., 1989). *D. discoideum* possesses a GABA$_B$-like receptor (GrlJ) (Prabhu et al., 2007), and treatment with GABA further enhances the secretion of AcbA (Kinseth et al., 2007). However, no orthologs of GABA or GABA-like receptors have been identified in yeast. Therefore the question of functional relevance of Acb1 secretion in the budding yeast remains unresolved. However, we know that,

when the central peptide was added to the secretion medium in excess, it influences the TCA/glyoxylat cycle and most likely also translation of certain proteins.

Additionally, wash-out of the yeast cytoplasm to visualize Acb1 at the CUPS or in vesicular intermediates within the cell using microscopy-based techniques would require removal of the yeast cell wall. As yeast cells are not surrounded by an extracellular matrix, the remaining plasma membrane is not stabilized and its permeabilization would provoke the cell to burst. Thus, other means to detect Acb1 at the CUPS or in vesicular intermediates are needed.

Photobleaching the cytoplasm of Grh1-YFP and Acb1-CFP co-expressing yeast to remove the cytosolic straining of Acb1-CFP did not give rise to an increased fluorescence signal of Acb1-CFP at CUPS (unpublished data). Also the use of the rapamycin-dependent anchor-away technique (Haruki et al., 2008) which uses the rapamicin-induced heterodimerization of FKBP12 and FRB (Muthuswamy et al., 1999) did not allow the visualization of Acb1-containing vesicles within the cell: co-expression of FRB-GFP-tagged Acb1 with the plasma membrane-localized Pma1-FKBP12 allowed the shift of cytosolic Acb1 to the inner leaflet of the plasma membrane, however, it did not reveal any Acb1-GFP-containing structures in the cytoplasm (Juan Duran, unpublished data).

In order to obtain deeper insights into the mechanism of how Acb1 might be captured into a vesicular intermediate and what the downstream events are, we are currently establishing an *in vitro* assay. Using the Promega system for quick-coupled transcription/translation in combination with fluorescent BODIPY-modified lysine, fluorescently labeled Acb1 ($Acb1_{FL}$)-STREP will be transla-

ted *in vitro* and subsequently purified. $Acb1_{FL}$-STREP will then be added to whole yeast cell lysates of starved wild type cells and the membrane attachment/incorporation determined by differential centrifugation and subsequent proteinase K digestion. A combination of membranes and cytosol prepared from different deletion strains will then shed light on the mechanism of $Acb1_{FL}$ transport into putative vesicular intermediates prior to its release from the cell.

Besides Acb1 and the previously mentioned putatively unconventionally secreted proteins Stm1 and Scs2, no other cargo proteins are available in the budding yeast. Even though yeast has the advantage of using complete gene deletions and not RNAi-based partial knockdown of the proteins to monitor their effects on a particular process, the lack of commercially available antibodies to yeast proteins requires tagging and thereby the alteration of the putative cargo protein. As a result, the possibility to switch to a different organism, for instance to *Drosophila* S2 cells, to identify the mechanism of unconventional protein secretion and to generate a generic cargo protein is currently being considered.

4.4 Conclusion

We have identified a novel glucose starvation-specific compartment in yeast that forms close the ER exit sites. This organelle, CUPS– *C*ompartment for *U*nconventional *P*rotein *S*ecretion–, has a characteristic cup-shape and contains Grh1, Vps23, Atg proteins, and the phosphoinositide PI(3)P. Further characterization of CUPS as a cellular organelle will help define its specific function in processes such as unconventional protein secretion. Additionally, studying the release of CUPS upon re-growth in rich medium will further expand our understanding of this structure.

We obtained first insights in the requirements to recruit Grh1 to the CUPS –Vps34 and Snf1 (amongst others) as prerequisites– and we will have a deeper understanding of Grh1-GFP re-localization after thorough analysis and subsequent validation of the genome-wide screen.

CUPS are stable compartments during starvation and we suggest that Acb1-containing vesicular intermediates form at the CUPS. Future work will need to focus on the identification of the vesicular intermediate and the downstream events to promote Acb1 secretion to confirm our hypothesis that CUPS are the starting point or the sorting station for unconventional protein secretion.

The exact function of Grh1 in unconventional protein secretion still needs to be fully characterized; nevertheless, we suggest that by starvation-specific interaction with different proteins Grh1 might act as chaperone for a subset of unconventionally secreted proteins.

5 Material and Methods

5.1 Chemicals and Consumables

Commonly used reagents, chemicals and consumables were purchased from Becton Dickinson Bioscientific, Fisher Scientific, Fluka, Invitrogen, Merck, Millipore, New England Biolabs, Roche, Sarstedt, Sigma Aldrich, Thermo Scientific, and VWR International.

5.2 Yeast media, strains, and plasmids

5.2.1 Yeast media

Yeast cells were grown in rich YPDA (1% yeast extract, 2% peptone, 2% glucose, and 0.004% adenine) or synthetic complete (SC) media (0.67% yeast nitrogen base, 2% glucose supplemented with amino acid drop out mix from Sigma Aldrich [lacking uracil, histidine, leucine and tryptophan], Clonetech [lacking methionine], or MP Biomedicals [lacking uracil, adenine, histidine, lysine, tryptophan, leucine]; with amino acids for auxotrophies and/or plasmid selection added as needed). Sporulation of diploid cells was induced by cultivation in sporulation medium (2% potassium acetate plus required amino acids for auxotrophies), nutrient starvation was induced by washing yeast cells in sterile water and subsequent culturing in 2% potassium acetate at $1OD_{600nm}$/ml (unless otherwise stated) for the indicated time intervals. Starvation for specific nutrients was performed in synthetic defined (SD) media (0.67% nitro-

gen base, 2% glucose, amino acids for auxotrophies) lacking the nitrogen source (SD-N) or the carbon source (SD-Gluc).

Cycloheximide treatment was performed at 250µg/ml in growth or starvation medium, rapamycin (Calbiochem) treatment was performed at 0.4µg/ml in nutrient-rich medium for 3h (Suzuki et al., 2002). Stress treatments were performed by adding 1M sodium chloride, 1M sorbitol, 0.3mM H_2O_2, 2.5mM DTT or 0.5% sodium azide to rich medium. For long term storage, 15% glycerol (final concentration) was added to a fresh culture and 1ml of this solution was frozen at -80°C.

5.2.2 Yeast strains

All yeast strains used in this study are summarized in Table 8. Strains were either purchased from EUROSCARF (deletion strains), Open biosystems (TAP strains) or Invitrogen (GFP-tagged strains; S288C, isogenic to BY4741), generated by gene replacement using homologous recombination (5.2.2.1), by mating and subsequent sporulation (5.2.2.2) or kindly provided by the indicated source.

Table 8: Yeast strains used in this study.
All strains used for this study are listed below with the corresponding genotype and source.

Name	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
BY4742	*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF
grh1Δ	BY4741, *grh1Δ::KanMX*	EUROSCARF
JMD018	BY4742, *GRH1-GFP::HIS3*	Duran et al., 2010
Grh1-GFP	*MATa GRH1-GFP::HIS3*	Invitrogen
RSY11	*MAT alpha, ura3-52 leu2-3,2-112 sec18-1*	Randy Schekman
RSY263	*MAT alpha, ura3-52 his4-619 sec12-4*	Randy Schekman

SC0000	*MATa, ade2Δ arg4Δ,leu2-3,112 trp1-289 ura3-52*	Open Biosystems
SC3523	SC0000, *GRH1-TAP::URA3*	Open Biosystems
Sec13-RFP	*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SEC13-RFP::KanMX6*	Erin O'Shea
Cop1-RFP	*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 COP1-RFP::KanMX6*	Erin O'Shea
Anp1-RFP	*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ANP1-RFP::KanMX6*	Erin O'Shea
Snf7-RFP	*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SNF7-RFP::KanMX6*	Erin O'Shea
CBY012	BY4741, *GRH1-GFP::HIS3 atg5Δ::KanMX4*	This study
CBY013	BY4741, *GRH1-GFP::HIS3 atg7Δ::KanMX4*	This study
CBY014	BY4741, *GRH1-GFP::HIS3 atg8Δ::KanMX4*	This study
CBY015	BY4741, *GRH1-GFP::HIS3 atg9Δ::KanMX4*	This study
CBY016	BY4741, *GRH1-GFP::HIS3 atg11Δ::KanMX4*	This study
CBY017	BY4742, *GRH1-GFP::HIS3 bug1Δ::KanMX4*	This study
CBY022	BY4741, *GRH1-GFP::HIS3 vps23Δ::KanMX4*	This study
CBY018	BY4741, *GRH1-GFP::HIS3 vps34Δ::KanMX4*	This study
CBY019	BY4741, *GRH1-GFP::HIS3 tlg2Δ::KanMX4*	This study
CBY020	BY4741, *GRH1-GFP::HIS3 ytp6Δ::KanMX4*	This study
CBY021	BY4741, *GRH1-GFP::HIS3 sso1Δ::KanMX4*	This study
CBY031	*MATa*, *GRH1-GFP::HIS3 SEC13-RFP*::*KanMX6*	This study
CBY032	*MATa*, *GRH1-GFP::HIS3 ANPl-RFP*::*KanMX6*	This study
CBY033	*MATa*, *GRH1-GFP::HIS3 COPl-RFP*::*KanMX6*	This study
CBY034	*MATa*, *GRH1-GFP::HIS3 SNF7-RFP*::*KanMX6*	This study
CBY080	*MATa, GRH1-GFP::HIS3 atg1Δ::KanMX4*	This study
CBY082	*MATa, GRH1-GFP::HIS3 atg14Δ::KanMX4*	This study
CBY084	*MATa, GRH1-GFP::HIS3 atg17Δ::KanMX4*	This study
CBY062	*MATa, GRH1-GFP::HIS3 atg18Δ::KanMX4*	This study
CBY073	*MATa, GRH1-GFP::HIS3 atg11Δ::KanMX4 atg17Δ::NatMX4*	This study
CBY060	*MATa, GRH1-GFP::HIS3 hse1Δ::KanMX4*	This study
CBY066	*MATa, GRH1-GFP::HIS3 vps27Δ::KanMX4*	This study
CBY067	*MATa, GRH1-GFP::HIS3 vps28Δ::KanMX4*	This study
CBY063	*MATa, GRH1-GFP::HIS3 mvb12Δ::KanMX4*	This study
CBY069	*MATa, GRH1-GFP::HIS3 vps25Δ::KanMX4*	This study
CBY070	*MATa, GRH1-GFP::HIS3 vps36Δ::KanMX4*	This study
CBY064	*MATa, GRH1-GFP::HIS3 vps2Δ::KanMX4*	This study
CBY065	*MATa, GRH1-GFP::HIS3 vps20Δ::KanMX4*	This study
CBY090	*MATa, GRH1-GFP::HIS3 vps4Δ::KanMX4*	This study

CBY091	*MATa, GRH1-GFP::HIS3 snf1Δ::KanMX4*	This study
CBY002	*MATa, GRH1-GFP::HIS3 gvp36Δ::KanMX4*	This study
CBY004	*MATa, GRH1-GFP::HIS3 stm1Δ::KanMX4*	This study
CBY005	*MATa, GRH1-GFP::HIS3 scs2Δ::KanMX4*	This study
CBY006	*MATa, GRH1-GFP::HIS3 mck1Δ::KanMX4*	This study
CBY007	*MATa, GRH1-GFP::HIS3 rtn1Δ::KanMX4*	This study
CBY010	*MATa, GRH1-GFP::HIS3 sso2Δ::KanMX4*	This study
Bug1-GFP	*MATa, BUG1-GFP::HIS3*	Invitrogen
Gvp36-GFP	*MATa*, *GVP36-GFP::HIS3*	Invitrogen
Rtn1-GFP	*MATa*, *RTN1-GFP::HIS3*	Invitrogen
GFP-Scs2	*MATa*, *NatNT2::GFP-SCS2*	Pedro Carvalho
CBY003	*MATa*, *GVP36::GFP::HIS3 grh1Δ::KanMX4*	This study
CBY008	*MATa*, *RTN1::GFP::HIS3 grh1Δ::KanMX4*	This study
CBY009	*MATa*, *GFP-SCS2::NatNT2 grh1Δ::KanMX4*	This study
DBY7730	*MATa, adeΔ his6Δ met1Δ ura1Δ can1Δ cyh1Δ rme1Δ sst1-3*	Jeremy Thorner
DBY7442	*MATalpha, leu- ura- ade- sst2- (exact genotype unknown)*	Jeremy Thorner

5.2.2.1 Yeast transformation

Manipulation of the yeast genome was achieved by transformation of PCR products as described (Janke et al., 2004). Yeast cells were cultured over night in rich medium and diluted to an optical density of 0.2OD_{600nm}/ml in 10ml medium. Cells were harvested at OD_{600nm}/ml ~0.6 by centrifugation for 3min at 3000g. Cells were washed with sterile water and resuspended in 50µl yeast transformation buffer (YTB, 100mM lithium acetate, 10mM Tris, 1mM EDTA, pH 8). 5µl of salmon sperm DNA (10mg/ml; 10min denaturated 100°C, cooled on ice), 3-5µl plasmid DNA or 30-40µl PCR product, and 300µl PEG buffer (YTB, 50% PEG-3350) were added to the cells. The mixture was incubated on a rotating platform for 30min at 25°C, heat-shocked for 15min at 42°C and centrifuged for 3min at 3000g. Cells were resuspended in 100µl sterile water. Selection for auxotrophic marker was carried out directly by plating cells on appropriate selection plates. In case of antibiotic

resistance, cells were cultured for 6 or more hours in rich medium before plating on a selection plate (G418 [Invitrogen], 200mg/l; Nourseothricin [WERNER BioAgents], 100mg/l). Conformation of the integration was performed by genomic DNA extraction following the instructions of the Y-PER Yeast DNA Extraction Kit (Thermo Scientific) and subsequent analysis by PCR.

5.2.2.2 Random spore preparation

Spore preparation was performed as described previously (Herman and Rine, 1997) with slight modifications. Diploid yeast were generated by mating haploid strains on YPDA plates for 4-6h and subsequent transfer to selection plates ensured successful mating. Diploid cells were cultured over night in rich medium and diluted to 0.2OD_{600nm}/ml in 10ml YPDA containing 4% glucose. Cells were harvested at 0.8OD_{600nm}/ml by 3min spin at 3000g, resuspended in sporulation medium at 1-2OD_{600nm}/ml and cultured for 3-5 days. 5OD of cells and spores were collected by 3min centrifugation at 3000g, resuspended in softening buffer (100mM Tris-H_2SO_4, pH 9.4, 10mM DTT) at 5OD_{600nm}/ml and incubated for 10min at 30°C. Cells were harvested by 5min spin at 3000g, washed in spheroplasting buffer (2.1M sorbitol, 10mM phosphate buffer, pH 7.2), and resuspended in spheroplasting buffer at 25OD_{600nm}/ml. Zymolyase-20T (ICN Immunobiologicals) was added at 0.5mg/OD_{600nm} and cells were incubated for 30min at 30°C with minimal shaking. Speroplasts and spores were harvested by 5min centrifugation at 3000g, washed once in 0.5% Triton-X100 and resuspended at 50OD_{600nm}/ml. Complete speroplast lysis and disruption of the asci was assured by brief sonication (Bioruptor

[Diagenode], medium strength, 30sec burst, 30sec pause; 5min total). Appropriate dilutions of spore preparations were plated on selection plates for haploid selection.

Haploidity was confirmed using the halo mating type assay (Sprague, 1991). 100µl of an overnight culture of the tester strain was spread on YPDA plates. The strains of interest were pinned to the lawn of tester strain and scored for the presence of a halo of space around them, which indicates that the lawn strain responded to the pheromone secreted by the pinned strain (opposite mating type; a- or α-factor).

For assaying spore viability of deletion strains, cells were resuspended in solutions containing increasing concentrations of detergents (0.5 – 2% SDS) before sonication. 100µl of appropriate dilutions were plated on YPDA plates and the number of spores was counted.

5.2.3 Yeast plasmids

For generation of the Grh1-GFP fusion protein under the control of its endogenous promoter, the coding sequence of Grh1 without the stop codon including 1kb upstream of the start codon was amplified by PCR and cloned as a ClaI-BamHI fragment into pRS416 plasmid. Yeast enhanced GFP (yeGFP) was amplified from pYM26 and inserted as BamHI-SacI fragment into the Grh1-containing pRS416 to generate the pRS416 Grh1-GFP plasmid. To express the mCherry-tagged Grh1-fusion protein under the control of its endogenous promoter, the coding sequence of Grh1 without the stop codon including 1kb upstream of the start codon was amplified by PCR and cloned as SacI-HindIII fragment into pRS416 plasmid

containing the mCherry coding sequence for the generation of the pRS416 Grh1-mCherry plasmid. The coding sequence of Ape1 without the stop codon including 1kb upstream of the start codon to include its own promoter was amplified by PCR and cloned as a Sac1-Spe1 fragment into pRS416 plasmid containing the mCherry coding sequence to generate pRS416 Ape1-mCherry plasmid.

mCherry-Atg8 was cloned into pRS316 plasmid under the control of its endogenous promoter and kindly provided by Y. Ohsumi (National Institute for Basic Biology, Okazaki, Japan). RFP-Atg9 was cloned into the pRS416 plasmid (Chang and Huang, 2007) and provided by W.P. Huang (Department of Life Science, National Taiwan University, Taipei). Vps23-mCherry was cloned into pRS416 under the control of its endogenous promoter (Curtiss et al., 2007) and provided by M. Babst (Department of Biology, University of Utah, Salt Lake City, USA). mCherry-Tlg1 and mCherry-Pep12 were cloned into pRS416 under the control of the Vps21 promoter and provided by D. Katzmann (Biochemistry and Molecular Biology, Mayo Clinic, Rochester, USA). Plasmids for the expression of Sec7-DsRed (Calero et al., 2003) and DsRed-FYVE (Katzmann et al., 2003) were provided by S. Emr (Cornell University, Weill Institute for Cell and Molecular Biology, Ithaca, USA).

The coding sequence of MIF or the cleaved, mature IL-1ß coding sequence (Keller et al., 2008) were introduced as a Spe1-Xho1 fragment into pRS415 under the control of the ADH promoter.

5.3 Biochemical protein analysis

5.3.1 TAP-tag purification of yeast protein complexes

The tandem affinity purification (TAP)-tag was designed for 2-step purification of the TAP-fusion protein allowing the isolation of protein complexes (Puig et al., 2001; Rigaut et al., 1999). The TAP-tag consists of a calmodulin binding peptide (CBP) followed by a tobacco etch virus (TEV) protease cleavage site and a Protein A domain at the C-terminal end. A yeast strain expressing Grh1-TAP and the corresponding parental strain without TAP-tag as a control were used. Yeast strains were grown in 2l YPDA to an OD_{600nm} ~0.8, harvested by 15min centrifugation at 1000g, washed in sterile water and either directly frozen at -80°C (growth sample) or resuspended in 1.5l 2% potassium acetate and starved for 4h. The starved cells were then harvested by 15min centrifugation at 1000g, washed with sterile water and immediately frozen at -80°C. The cells were rapidly thawed and keep at 4°C. 1 packed cell volume (PCV) but not less than 15ml of buffer A (10mM K-Hepes, pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM DTT, 0.5mM PMSF, 2mM benzamidine, 1µM leupeptin, 1µM pepstatin, 1µM aprotinin) were added and the cells were lysed by passing 3 times through a Frensh press (EmulsiFlex-C5, Avestin). The KCl concentration of the lysate was adjusted to 2M and the lysate was subsequently cleared by 30min spin at 25000rpm. The extract buffer was adjusted to 10mM Tris-HCl, pH 8.0, 0.1% NP-40 and added to 200µl (50% slurry) of washed (10ml IPP150; 10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.1% NP-40) IgG Agarose (Sigma). The extract-beads mixture was incubated for 2h at 4°C on a rotating platform and the elution was done by gravity flow. The beads were washed

3 times with 10ml IPP150 buffer and once with 10ml TEV cleavage buffer (IPP150 adjusted to 0.5mM EDTA, 1mM DTT). TEV cleavage was performed in 1ml TEV cleavage buffer and 100µl TEV protease (AcTEV™ Protease [Invitrogen]) for 2h at 16°C. 200µl (50% slurry) calmodulin agarose (Sigma) were washed with 10ml IPP150 CBB (calmodulin binding buffer; 10mM Tris-HCl, pH 8.0, 10mM β-mercaptoethanol, 150mM NaCl, 1mM MgAc, 1mM imidazole, 2mM $CaCl_2$, 0.1% NP-40). The eluate was recovered by gravity flow. 3ml of IPP150 CBB and 3µl 1M $CaCl_2$ were added to the eluate. The eluate was then added to the washed calmodulin beads and incubated for 1h at 4°C on a rotating wheel. Elution was done by gravity flow and the beads were washed 3 times with 10ml IPP150 CBB. Elution was performed by adding 1ml IPP150 CEB (calmodulin elution buffer; 10mM Tris-HCl, pH 8.0, 10mM β-mercaptoethanol, 150mM NaCl, 1mM MgAc, 1mM imidazole, 0.1% NP-40, 2mM EGTA). The eluate was incubated with 15% trichloroacetic acid (TCA; final concentration) for 10min at 4°C and proteins were precipitated by 15min centrifugation at 13000g. The pellet was resuspended in 1x SDS sample buffer (5% SDS, 200mM Tris, pH 6.8, 1mM EDTA, 1.5% DTT, bromophenol blue as pH indicator) and one half of the sample was run on a 4-20% Tris-HEPES-SDS gel (Thermo Scientific). The gel was silver-stained; protein bands were cut and subsequently analyzed by mass spectrometry.

5.3.2 Silverstaining

After separation of proteins on a SDS-PAGE, the gel was placed in a glass dish and incubated in fixing solution (50% methanol, 10% acetic acid) for 45min. The gel was washed twice with water for

15min and once for 1h up to overnight. The gel was then covered with sodium thiosulfate (0.1g/500ml) and shaken for 90sec. The gel was rinsed 3 times with water and then covered with silver nitrate solution (0.1g/50ml) for 25min. After washing 3 times with water, the gel was covered with developing solution (3g sodium carbonate in 98ml water, 2ml sodium thiosulfate solution, 50µl formaldehyde) for 5-8min. The solution was removed and the gel was covered with 6% acetic acid for 10min and then washed in water. Silver stained bands were cut with a sterile scalpel and the protein content was analyzed by mass spectrometry.

5.3.3 Subcellular fractionation of yeast cell lysates

250OD_{600nm} of yeast expressing Grh1-GFP were grown in rich medium or nutrient starved for 3h. Cells were collected by 5min centrifugation at 3000g, washed with ice-cold 10mM NaN_3/NaF, resuspended at 20OD_{600nm}/ml in pre-spheroplasting buffer (10mM NaN_3, 10mM NaF, 100mM Tris-H_2SO_4, pH 9.4, 0.36µl/ml β-mercaptoethanol) and incubated for 20min at 25°C. Cells were collected by centrifugation, washed in gradient spheroplasing buffer (40mM HEPES-NaOH, pH 7.5, 1.4M sorbitol, 2µl/ml β-mercaptoethanol) and spheroplasted with 50U/OD_{600nm} Zymolyase-100T (ICN Immunobiologicals) for 45min at 35°C at 50OD_{600nm}/ml in spheroplasting buffer. Spheroplasts were harvested by 5min centrifugation at 3000g, resuspended in 1.5ml gradient lysis buffer (10mM HEPES-NaOH, pH 7.5, 1mM $MgCl_2$, 0.3M sorbitol, 1µM leupeptin, 1µM pepstatin, 1µM aprotinin) and lysed by using a Dounce homogenizer. Lysates were cleared twice by centrifugation for 3min at 600g. Equal amounts of proteins were loaded on top of a continu-

ous sucrose gradient (10ml of 15-60% sucrose in 10mM HEPES-NaOH, pH 7.5, 1mM $MgCl_2$) and centrifuged for 18h at 100000g at 4°C. 1ml fractions were taken from the top and analyzed either directly or after TCA precipitation by western blotting using antibodies against GFP (Santa Cruz), Vps23 (a gift from Dr. S. Emr), Mnn9 (a gift from Dr.Y. Noda), and Kar2 (a gift from Dr. M Rose). Statistical analysis of the signal obtained in each fraction was performed by quantifying the band intensity of 3 independent experiments with the Odyssey 2.1 software and the percentages were plotted using Graph Pad Prism.

5.3.4 Protein analysis by western blot

Protein analysis was performed according to Laemmli (Laemmli, 1970). Volumes corresponding to equal amounts of proteins were separated on a SDS-PAGE (for gradient fractions: Criterion Tris-HCl 4-20% polyacrylamide gradient gels [Biorad]) and for subsequent western blot analysis the proteins were transferred onto nitrocellulose membrane using the iBlot transfer system (Invitrogen, standard protocol 3). Membranes were blocked with 2.5% BSA/PBS for 30min. Primary antibodies were diluted in 2.5% BSA/PBS and incubated for 1h at RT or at 4°C over night. Primary antibodies were washed away with PBS-0.05% Tween. Secondary antibodies (IgG linked to Alexa-Fluor680 [Invitrogen]) were used at 1/10000 dilution in 2.5% BSA/PBS. Secondary antibodies were incubated for 1h at RT, washed away with PBS-0.05% Tween as before and the membranes were analyzed using an Odyssey LI-COR infrared imaging system (Bioscience).

5.3.5 ^{35}S-methionine labeling of yeast cells

Yeast were grown over night in SC media and diluted to 0.2OD_{600nm}/ml and grown to approximately 0.8OD_{600nm}/ml. 10OD of cells were harvested by 5min centrifugation at 3000g, washed in sterile water and resuspended in SC medium without methionine (Sc-Met) at 5OD_{600nm}/ml (pre-warmed to the desired temperature, 25°C for or 37°C [for temperature-sensitive strains]) incubated for 15min. Cells were then labeled with ^{35}S-methionine (Hartmann Analytic, 300µcr/2ml) for 45min at 25°C or 37°C, harvested by 1min spin at 10000g, and the medium of time-point P (pulse) was collected. Cells were washed with 2ml pre-warmed starvation buffer, resuspended in 1ml starvation buffer and incubated for 30min at 25°C or 37°C. Cells were harvested by 1min spin at 10000g, time-point 30min was collected, the entire supernatant was removed and cells were resuspended in 1ml starvation buffer and starved further for 3.5 more hours (25°C or 37°C). Cells were harvested by 5min spin at 3000g and time-point 4h was collected. Secretion media were passed through a 0.22µm-filter to ensure complete removal of cells and the proteins were precipitated over night in the presence of 10µg BSA and 20% TCA. Samples were resuspended in sample buffer, boiled 5min at 100°C. 1/4th was loaded on a 10% polyacrylamide SDS-PAGE and labeled secreted proteins were visualized by autoradiography.

5.3.6 SILAC of starvation specific yeast secretome

WT and *grh1*Δ cells were grown in SC medium (WT in medium containing heavy lysine 6x^{13}C, 2x^{15}N) to 1OD_{600nm}/ml. 1000OD of cells were harvested by 5min centrifugation at 3000g, washed with

sterile water, resuspended in starvation medium at $10OD_{600nm}$/ml, cultured for 30min, harvested as before, resuspended in fresh starvation medium as before and cultured for 4 more hours at 25°C. The secretion medium was separated from the cells by 10min centrifugation at 3000g and then filtered through a 0.22µ-filter to ensure removal of residual cells. Secretion medium from WT and *grh1*Δ cells were mixed 1:1 and containing peptides and proteins were further purified by passing over a C18 or a C8 column. Proteins retained in the C8 column were after elution digested with trypsin, and the polypeptide composition of either sample (C8 and C18) was analyzed by mass spectrometry.

5.3.7 Total RNA extraction and microarray

Large scale isolation of total RNA was performed as described (Schmitt et al., 1990) with modifications. WT and *grh1*Δ yeast were grown over night in rich medium and diluted to $0.2OD_{600nm}$/ml in 200ml. Cells were harvested at OD_{600nm}/ml ~0.8, washed and nutrient starved for 2h. 10nM of synthesized Acb1 peptide (containing the central 34 amino acids representing SDF-2-like material) was added to the (+) cultures; (-) cultures were left without peptide and the cells were starved for 4 more hours, then harvested by centrifugation, and stored over night at -80°C. Cell pellets were thawed and resuspended in 10ml sodium acetate buffer (50mM sodium acetate, 10mM EDTA, adjusted to pH 5.0 with acetic acid). 1.5ml glass beads, 1ml 10% SDS and 12ml hot phenol (65°C, liquified phenol equilibrated with 1 volume sodium acetate buffer over night to adjust pH to 5.0) were added and the mixture was incubated for 4min at 65°C in a water bath shaker at maximum speed. Cells were

immediately cooled down to RT and harvested by 10min spin at 2500g. The lower organic phase was removed, and the remaining aqueous phase including the interpahse and cell pellets were treated with 12ml hot phenol as before and centrifuged for 10min at 2500g. After separation, the uppor aquous phase was transfered into a new tube. 10ml chloropane (50% liquified phenol; 50% chloroform; 0.5% hydrohychinoline, equilibrate with ANE buffer [ANE buffer: 10mM sodium acetate, 100mM NaCl, 1mM EDTA, pH 6.0] was added and the mixture was vortexed for 2min and centrifuged for 10min at 2500g. The upper aqueous phase was transfered into a new tube, 1ml of 3M sodium acetate (pH 5.3) and 30ml of ethanol were added, the mixture was briefly vortexed and then stored over night at -20°C to precipitate the RNA. The precipitated RNA was collected by centrifugation (2500g, 4°C, 30min) and the pellet was washed once with 20ml 70% ethanol, briefly dried and dissolved in 1200µl nuclease-free water. Six 200µl aliquots were treated with 20µl 3M sodium acetate (pH 5.3) and 600µl ethanol and stored for 1h at -80°C. RNA was pelleted at 15000g at 4°C for 20min, washed with 70% ethanol, dried and dissolved in nuclease-free water (3 tubes in 100µl). The RNA was stored at -80 and small samples were used for determination of quality and quantity.

The *S. cereviaise* Gene Expression Microarray v2 (Agilent, [8x15K format, G4813] containing 6,256+ *S. cerevisiae* probes of the S288C strain) was used to analyze 8 samples per array (in duplicates; WT(+), WT(-), Δ*grh1*(+), Δ*grh1*(-)). 1µg pure total RNA was used for the hybridization with a 1-color labeling method. AFM (Array file maker) 4.0 toolbox for microarray analysis was used (Breitkreutz et al., 2001).

5.4 Microscopy based analysis

5.4.1 Fluorescence microscopy of living yeast cells

Yeast cells were grown in the appropriate medium before imaging. Cells were harvested at 3000g for 5min, spotted on a polylysine coated microscopy slide (VWR) and immediately live imaged with Leica DMI6000B microscope equipped with a DFC 360 FX camera using a HCX PI APO 100X 1.4 objective. Images were taken using Leica LAS AF software with the same exposure times for Grh1-GFP; co-localization analysis with different marker proteins was performed with lower exposure times. Figures were assembled in Adobe Photoshop with only linear adjustments. Statistical analysis was performed by counting at least 60 cells from 3 independent experiments and the statistical significance was tested in an un-paired Student's t test using Graph Pad Prism. Compared datasets were termed as statistically significant when $p < 0.05$.

5.4.1.1 FM4-64 uptake

Staining of endocytic intermediates from endosomal to vacuolar membranes with the lipophilic dye FM4-64 was performed as described previously (Vida and Emr, 1995) with slight modifications. Cells were grown to 0.8OD_{600nm}/ml at 25°C. A 10ml culture was centrifuged and resuspended at 30OD_{600nm}/ml. FM4-64 (Invitrogen) was added at a final concentration of 30µM and cells were incubated for 15min at 25°C. Cells were harvested by centrifugation for 5min at 3000g, resuspended at 1OD_{600nm}/ml in starvation medium and cultured at 25°C. At different time points, cells were collected by centrifugation and immediately visualized.

5.4.2 Conventional and immunoelectron microcopy

Yeast strains expressing Grh1-GFP or Grh1-GFP and Sec13-RFP were cultured in rich medium or further nutrient starved for 4h at 30°C. Sample preparation for conventional and immunoelectron microscopy was performed as described (Rieder et al., 1996) with slight modifications. For conventional electron microscopy, yeast were harvested and fixed for 1h at 25°C in glutaraldehyde buffer (0.1M sodium cacodylate, pH 7.4, 5mM $CaCl_2$, 5mM $MgCl_2$, 3% glutaraldehyde, 2.5% sucrose). Cells were spheroplasted and embedded in 2% ultra-low-gelling-temperature agarose, cooled and cut into small pieces (~1mm^3). After a second fixation step for 30min at 25°C, the agarose blocks were washed 4 times 2.5min with water, transferred into 1% thiocarbohydrazide for 3min at 25°C, washed 4 more times for 1min in water and transferred into 1% OsO4/1% potassium ferrocyanide contained in 0.1M sodium cacodylate, pH 7.4, 5mM $CaCl_2$ for additional 3min at 25°C. The blocks were washed 4 times 4min with water, stained in Kellenberger's uranyl acetate (UA) for 2h to overnight, dehydrated through a graded series of ethanol, and subsequently embedded in Spurr resin. Sections were cut on a Reichert Ultracut T ultramicrotome, post-stained with UA and lead citrate, and observed on an FEI Tecnai 12 TEM at 100kV. Images were recorded with a Soft Imaging System Megaview III digital camera and figures were assembled in Adobe Photoshop with only linear adjustments in contrast and brightness.

For immunoelectron microscopy, cells were fixed in suspension for 15min by adding an equal volume of freshly prepared 8% formaldehyde contained in 0.1M potassium phosphate buffer, pH 7.4. The cells were pelleted and again resuspended in fixing solution for 18–

24h at 4°C. The cells were washed in PBS and resuspended in 1% low-gelling-temperature agarose. The blocks were cut into pieces of 1mm^3, cryoprotected by infiltration with 2.3M sucrose/30% polyvinyl pyrrolidone (10,000MW)/PBS (pH 7.4) for 2h, mounted on cryo-pins and rapidly frozen in liquid nitrogen. Ultrathin cryosections were cut on a Leica UCT ultramicrotome equipped with an FC-S cryo-attachment and collected onto formvar/carbon-coated nickel grids. The grids were washed through several drops of PBS containing 2.5% fetal calf serum (FCS) and 10mM glycine (pH 7.4), then blocked in 10% FCS for 30min, and incubated overnight in chicken anti-GFP antibody (abCam), or chicken anti-GFP + rabbit anti-RFP (abCam). After washing, the grids were incubated for 2h in 12nm gold donkey anti-chicken conjugate or a mixture of 12nm donkey anti-chicken + 6nm donkey anti-rabbit (Jackson ImmunoResearch). The grids were washed through several drops of PBS followed by several drops of water. Grids were embedded in an aqueous solution containing 3.2% polyvinyl alcohol (10,000MW)/0.2% methyl cellulose (400 centiposes)/0.1% uranyl acetate. The sections were examined and photographed on a Tecnai 12 transmission electron microscope at 100 kV, and images collected with a Soft Imaging System Megaview III digital camera. Figures were assembled in Adobe Photoshop with only linear adjustment of contrast and brightness.

6 Appendix

The thesis contains the article that resulted from the presented work:

Bruns C, McCaffery JM, Curwin AC, Duran JM, Malhotra V. Biogenesis of a novel compartment for autophagosome-mediated unconventional protein secretion J Cell Biol. 2011; 195(6):979-92.

7 References

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