Functional characterisation of the yeast tumour suppressor homologue Sro7p

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‘I am thankful for laughter, except when milk comes out of my nose’

- Woody Allen

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ABSTRACT

Correct targeting of newly synthesized proteins to appropriate domains of the cell membrane is crucial to cellular architecture, polarity and function, making it no surprise that many proteins of the secretory machinery are conserved throughout evolution. This work presents a functional characterisation of the *Saccharomyces cerevisiae* cell polarity protein and tumour suppressor homologue, Sro7p. This protein, and its paralogue Sro77p, belong to the Lgl-family of WD-40 repeat proteins that are conserved from yeast to human. Deletion of *Lgl* genes produces different phenotypes that all seem to share the common denominator of defective targeting of critical cell surface proteins. Yeast cells lacking *SRO7* become sensitive to NaCl and we here show that this defect is due to mis-targeting of the sodium transporter Ena1p. In *sro7* mutants Ena1p becomes routed to the vacuole for degradation via the multi-vesicular body (MVB) pathway, instead of being properly expressed at the cell surface. Isolation and analysis of post-Golgi secretory vesicles showed a defective sorting of Ena1p into these vesicles from *sro7* mutants, implying mis-sorting in late Golgi or early endosomes. The diversion of Ena1p into the MVB pathway further required ubiquitylation by the ubiquitin ligase Rsp5p. Isolation of suppressors of the *sro7* salt sensitivity identified two genes of unknown function, *RSN1* encoding a trans-membrane protein, and *ART5* (*RSN2*), encoding an arrestin-like protein. Deletion of either gene in *sro7* mutants re-establishes salt tolerance and retargets Ena1p to the cell surface. Previous proteomic studies have shown that Art5p interacts with Rsp5p and we showed that deletion of *ART5* in *sro7* mutants inhibits ubiquitylation of Ena1p. Our data are consistent with Art5p being a selective adaptor protein that helps Rsp5p recruiting Ena1p for ubiquitylation. To identify further candidate proteins for mis-sorting in salt stressed *sro7* mutants we performed the first proteomic analysis of purified yeast post-Golgi vesicles (PGVs), using quantitative proteomics techniques. By this analysis we could identify 107 genuine vesicle residents in control yeast cells, including a number of cargo proteins not previously identified in PGVs. Vesicles derived from *sro7* mutants contained essentially the same list of proteins but were depleted of a subset of proteins, thus being candidates for mis-routing. The present study finally analysed possible Lgl conservation in plants by characterising two *Arabidopsis thaliana* Lgl homologues. Sequence based modelling showed that both proteins can fold into the twin β-propellers shown by the published Sro7p crystal structure. However, only one of the proteins, AtLGL1, could partially substitute for the yeast Sro7/77 proteins. The other, AtLGL2 showed structural similarities with tomosyn that is known to regulate vesicle fusion in mammals. Homozygous T-DNA insertion mutants in *A. thaliana* exhibited defects in lateral root formation, a phenotype associated with changed cell- and tissue polarity.

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

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IV: **Forsmark, A**., I. Wadskog, E. Krogh Johansson and L. Adler. The arrestin-like protein Art5p is required for Rsp5p mediated ubiquitylation and mis-sorting of the sodium transporter Ena1p. *Manuscript*

Table of contents

1. Introduction 9
1.1 The eukaryotic cell... 9
1. 2 Yeast 9

2. The secretory pathway.. 11
2.1 Protein secretion 11
2.2 From ER through the Golgi 12
2.3 From Golgi to the plasma membrane 13
2.4 The endosomal pathway 16

3. Protein sorting 18
3.1 Identification of the *SEC* genes.. 18
3.2 Different classes of post-Golgi carriers 19
3.3 Cargo selection and adaptors 19
3.3 Proteomics of secretory vesicles.. 21

4. Cell polarity 22
4.1 Polar growth in yeast 22
4.2 Polarity in epithelial cells 23
4. 3 Cell polarity complexes 24

5. The *LGL* family 25
5.1 *Lgl in* Drosophila*;* a tumor suppressor 25
5.2 Lgl, a mammalian tumor suppressor?....................................... 26
5.3 Lgl in the maintenance of cell polarity....................................... 27
5.4 Tomosyn: Lgl homologues that regulate SNARE assembly........ 28

6. The *SRO* genes 29
6.1 Identification of the *SRO* genes 29
6.2 The crystal structure of Sro7p 33
6.3 Localisation of Sro7p 35
6.4 Suppressors of *sro7Δ* phenotype 36

7. Quality control and regulation 40
7.1 Protein quality control in the ER (ERAD) 40
7.2 Quality control at the Golgi 40
7.3 Endocytosis 41

8. Ubiquitylation 42
8.1 Steps in ubiquitylation by E1, E2, E3 enzymes 42
8.2 Modes of ubiquitylation 42
8.3 Deubiquitylation enzymes (DUBs) 43
8.4 E3 adaptors in yeast 43
8.5 Regulation by ubiquitylation 45

9. Concluding remarks 47

10. Acknowledgements 50

11. References 52

1. Introduction

1.1 The eukaryotic cell

The cell is the smallest entity of life. It harbours the entire blueprint of the organisms' genetic code. Cells progressing as single units constitute the group of unicellular organisms, further divided in prokaryotes and eukaryotes. The name eukaryote stems from the Greek words for true (eu) and kernel (karyon), describing the main feature separating eukaryotes from prokaryotes; the nucleus. Eukaryotic cells are composed of an aqueous cytoplasm, in which the nucleus and the different specialised organelles are embedded. The protein filaments of the cytoskeleton extend throughout the cytoplasm, constituting the structural framework of the cell. The cytoplasm is bounded by the plasma membrane, which is a highly dynamic organelle providing the cell with information about outside conditions or from neighbouring cells. In response to outside cues, there are rapid cellular adjustments reflected in remodelling of e.g. the lipid and protein composition of the plasma membrane. Such changes are crucial for a quick adaptation of the cell to a stochastically fluctuating environment.

Unicellular eukaryotes gave rise to multi-cellular organisms somewhere between 0.4 – 1 billion years ago (Rokas, 2008), leading to present day diversity of plants, animals and fungi. The complexity of multi-cellular organisms is truly intriguing, their life-processes depending on elaborate cell communication mechanisms and coordinate co-operation between highly specialised cells. Paradoxical though, the basic cellular features are similar to those of unicellular micro-organisms. This conservation is explained by the fact that multi-cellular organisms originated as single-celled organisms, and most of their fundamental properties were established long before multi-cellularity was developed.

1. 2 Yeast

1.2.1 In history and industry…

Yeasts are the simplest eukaryotes. They belong to a group of unicellular fungi, of which there are about 700 described genera to present date (Walker, 2000). The first microscopic observation of yeast was made by Antonie van Leeuwenhoek in 1680. He considered them as globular structures, but did not recognise them as a living organism. It was not until Louis Pasteur published his ground-braking paper *Mémoire sur la fermentation alcoolique*

that it was convincingly shown that yeast is a growing organism associated with the process of alcoholic fermentation. However, yeast had already been domesticated by mankind for thousands of years in brewing, wine-making and baking (Legras et al., 2007). In fact, brewing of beer has been considered the first application of biotechnology (Walker, 2000). However, the first use of yeast was probably for the fermentation of wine, since this production requires no inoculum of yeast. Yeasts can be isolated from most environments, terrestrial as aquatic (Walker, 2000). Well known is its occurrence on grapes, and plant tissues are in fact the preferred habitat. However, yeasts are also found in for example the intestinal flora and mucus membranes of warm-blooded animals like humans (Naglik et al., 2008) and can occasionally act as opportunistic pathogens (Rupp, 2007; Zisova, 2009).

In baking and alcoholic fermentation, the most commonly used species of yeast is *Saccharomyces cerevisiae*. It is often referred to as “baker’s yeast” and belongs to the division of Ascomycetes. *S. cerevisiae* is seldom isolated from sites distant from human applications. Genetic improvement of yeast strains used in production has traditionally depended on classical genetic techniques and more recently on yeast genetic engineering, and has mainly aimed at improving fermentation performance and the quality of the products of winemaking, brewing and baking (Dequin, 2001). However, there are a growing number of applications for yeast bioengineering in industry, ranging from generating drug precursors for the pharmaceutical industry (Ro et al., 2006; Szczebara et al., 2003) to producing organic acids for use in renewable fuels (Abbott et al., 2009). In short, yeast offers a very versatile tool in biotechnology.

1.2.2 In molecular biology…

In the field of molecular biology, *S. cerevisiae* has long been a well established eukaryotic model system. The yeast genome was the first eukaryotic genome to be fully sequenced in 1996, revealing 6000 candidate genes and making the yeast genome the pioneer eukaryotic genome (Goffeau et al., 1996). Progress in cell biology has been much dependent on studies of bacterial systems, like *Escherichia coli*. Bacteria offer an easily manipulated genetic system and fast growth (Botstein et al., 1997). They have also been pivotal for invention and maturation of experimental techniques in molecular biology. The applicability to eukaryotic systems is limited, though. In common with bacteria, yeast

shares the advantage of offering the full range of advanced molecular genetic techniques combined with a fast generation time. For *S. cerevisiae* the optimal generation time is about 1.5 - 2 hours (Smith and Snyder, 2006). Being a eukaryote, yeast shares with higher multi-cellular organisms basic cell biological features such as subcellular organelles, secretory system, cytoskeleton, chromosome organization and posttranslational protein modification (Cereghino and Cregg, 1999).

The life cycle of *S. cerevisiae* includes a haploid stage, making genetic manipulations fast and straightforward. Consequently, a gene of interest can easily be linked to a certain function in the cell. Such phenotypic studies have established that surprisingly many cellular processes are conserved between yeast and higher eukaryotes. In line with this observation, the amino acid sequences of eukaryotic proteins are also well conserved (Botstein et al., 1997). There are a number of cases where a yeast phenotype has been complemented by a mammalian gene (Lee and Nurse, 1987; Li and Harris, 2005), or the other way around (Bond et al., 1986). Approximately one third of the yeast genes have orthologs in the human genome, a fact that has been used to unravel molecular events in human diseases (Walberg, 2000).

Today, yeast is a well studied system and pieces to the puzzle are constantly being added. Further research is facilitated with the growing amount of information. In addition to the genomic sequence, there are today exhaustive databases like SGD (Saccharomyces Genome Database, www.yeastgenome.org) where up-to-date yeast research is collected in one place. Adding to this is the increasing availability of tools that are shared within the yeast community, like standardized vectors, mutant strains and complete libraries of deleted, tagged or cloned genes. The availability of the entire genome sequence has also paved the way for a variety of approaches for genome-wide screening and for system-level studies of how genes, gene-products and their regulation interact together (Dolinski and Botstein, 2005).

2. The secretory pathway

2.1 Protein secretion

In the mid seventies, microscopic and biochemical studies by George Palade provided the framework for establishing the existence and understanding the function of the eukaryotic

secretory pathway (Palade, 1975). The further task of scrutinizing the molecular mechanisms by which protein secretion is accomplished was taken on by the J. Rothman and R. Schekman labs, which conferred pioneering insights into the molecular biology of eukaryotic protein secretion (Balch et al., 1984; Novick et al., 1980). In addition, painstaking *in vitro* studies revealed that crucial components of the transport machinery are surprisingly conserved between plants, animals and lower eukaryotes (Paquet et al., 1986).

2.2 From ER through the Golgi

Proteins destined for secretion are synthesized on ribosomes bound to the endoplasmic reticulum (ER) and co-translationally translocated into the ER, where they are folded and subjected to initial post-translational modifications (Ponnambalam and Baldwin, 2003). Once correctly folded and assembled, newly synthesized proteins destined to proceed through the secretory pathway are separated from ER resident proteins and enriched at ER exit sites (ERES) (Lippincott-Schwartz et al., 2000). These specialized membrane domains are the sites where secretory cargo is packaged into COPII vesicles for further transport. This is the case for most eukaryotes, with *S. cerevisiae* being an exception. In *Saccharomyces*, the COPII vesicles are formed throughout the ER membrane. The COPII protein complex is assembled upon activation of Sar1p, a Ras-like GTPase. Transition to the GTP-loaded active form of Sar1p is mediated by the effector Sec12p. Effectors of GTPases are named GEFs (guanine nucleotide exchange factors) and transfer the GTPase from its inactive GDP bound form to the activated GTP form (Ortiz et al., 2002; Walch-Solimena et al., 1997). As Sar1p is activated, part of its N-terminus is inserted into the ER membrane, which in turn recruits the Sec23p/Sec24p heterodimer to form the so called pre-budding complex (Sato and Nakano, 2007). Transmembrane cargo proteins are enriched in the pre-budding complex by binding to Sec24p via sorting signals in their cytosolic domains. Eventually, the pre-budding complex binds the heterotetramer Sec13p/Sec31p, which is believed to stabilise the membrane deformation that precedes release of COPII vesicles from the ER membrane. The cargo loaded COPII vesicles continue to the Golgi or the ER-Golgi intermediate compartment (ERGIC) (Bonifacino and Glick, 2004). Moving through the Golgi sub-compartments, N- and O-linked glycosylation of the secretory proteins is completed (Ponnambalam and Baldwin, 2003). The mechanism for progression

through the Golgi stacks is not yet clear, one hypothesis being the theory of cisternal maturation. According to this model, resident Golgi proteins are recycled back to earlier compartments or to the ER concomitant with forward transport of cargo, giving rise to differentiation of the Golgi sub-compartments. Retrograde transport is believed to be mediated by another class of vesicles; COPI coated vesicles (Glick and Nakano, 2009). The COPI coat assembly follows the same principle as for COPII, but involving other proteins. Instead of Sar1p, the Arf1p GTPase governs the COPI coat assembly (Spang, 2008). In fact, Arf1p also functions in the formation of a third type of vesicles; clathrin coated vesicles (CCV:s). The clathrin coat forms a cage-like structure built by clathrin triskelions. These coat proteins mediate budding in endocytosis as well as specific targeting from Golgi to the vacuole/lysosome (Spang, 2008).
Secretion involves the budding of vesicles from a donor compartment and the subsequent docking and fusion with an acceptor compartment (Bonifacino and Glick, 2004). From the Golgi, proteins and lipids are sorted according to their final destination, the plasma membrane, endosomes or the lysosome/vacuole (Sato and Nakano, 2007). This sorting is of critical importance to keep the cell compartmentalized and differentiated.

2.3 From Golgi to the plasma membrane

Proteins destined to the plasma membrane are delivered in secretory vesicles by the process of exocytosis, which mediates cell growth and cell polarity. Exocytosis includes targeted transport, docking and fusion of vesicles from the Golgi with the plasma membrane. In most eukaryotic cells, including yeast, exocytosis is polarized (Roumanie et al., 2005). This means that the delivery of proteins to the cell surface is directed to a specific domain of the plasma membrane, enabling the cell to grow asymmetrically. This is an obvious feature of for example epithelial cells and neurons, which they share with the yeast *S. cerevisiae*. A yeast cell is polarized in the sense that it propagates by growing a bud, which forms a daughter cell upon division. The site for exocytosis and subsequent growth is shifted throughout the cell cycle. At first, the process is directed towards the bud initiation site. As the bud grows, secretion is initially directed to the bud tip but spreads throughout the bud as it enlarges. Eventually growth is re-polarized at the bud-neck before cytokinesis (Brennwald and Rossi, 2007b). A subset of proteins in all cells are secreted

constitutively, while in selected cells of higher eukaryotes secretion can also be regulated (Salminen and Novick, 1987). This is the case for specialised cells of the neuroendocrine tissue. Here, the secretory vesicles are stored and released upon a stimulus in a calcium dependent manner (Loubery and Coudrier, 2008).

2.3.1 Vesicle targeting

Signals within secreted proteins decide their sorting and final destination. However, the mechanisms by which cargo proteins are sorted and packaged into transport carriers are still far from clear (Bard and Malhotra, 2006). As vesicles are detached from the Golgi, they are believed to move to the correct compartment along actin cables by the action of molecular motor proteins (Loubery and Coudrier, 2008). In yeast, a class V myosin, Myo2p, is the candidate for moving the secretory vesicles to the site of exocytosis. In accordance, a *myo2-66* temperature sensitive mutant, which bears a mutation in the motor domain, accumulates vesicles at restrictive temperature and arrests as a large unbudded cell (Johnston et al., 1991). However, impairment of Myo2p actin binding affects only polarized secretion and does not completely abolish growth, which continues at a slower rate. The same effect is seen in mutants lacking actin cables, indicating that neither Myo2p nor intact actin cables are necessary for secretion itself, but essential for its polarization (Karpova et al., 2000; Pruyne et al., 1998). In addition, polarized delivery of vesicles from the Golgi to the plasma membrane depends on the Rab GTPase Sec4p and its effector Sec2p. Rab GTPases belong to the Ras superfamily of small GTPases, and are implicated to have a role at many levels of membrane traffic. In yeast, a model has been proposed in which the GEF Sec2p is recruited to vesicles by another Rab GTPase, Ypt32p. On the vesicles, Sec2p activates Sec4p, mediating the polarized delivery to the plasma membrane (Ortiz et al., 2002). An extension of the model suggests that activated Sec4p in turn recruits another effector, Sec15p, possibly assisting in keeping Sec4p activated (Medkova et al., 2006; Novick et al., 2006b).

2.3.2 Vesicle docking

At the cell surface, vesicle docking with the plasma membrane is mediated by a vesicle-tethering protein complex termed the exocyst (Finger and Novick, 1998; Guo et al., 1999b;

TerBush et al., 1996). Originally, seven proteins were identified as constituents of this complex; Sec6p, Sec8p, Sec15p, Sec3p, Sec5p, Sec10p and Exo70p. Later, Exo84p was identified as an additional component of the exocyst, which is presently considered to be an octameric complex uniquely involved in exocytosis (Guo et al., 1999a; TerBush et al., 1996). Homologues of all exocyst subunits have been identified in mammals, pointing to a strong evolutionary conservation of this complex. Most work in mammalian systems refers to the exocyst as the Sec6p/8p complex (Hsu et al., 1999). The crystal structure of the full length or C-terminal third of four exocyst components have shown that they form helical bundles that arrange end to end into long rods. The rods appear to arrange into flowerlike petals that can attain an open or a closed conformation (Munson and Novick, 2006; Novick et al., 2006b). The localisation of the exocyst varies with the cell cycle, perfectly corresponding to the sites of active growth of the plasma membrane. It has been shown that most of the subunits of the exocyst, except for Sec3p and Exo70p, are carried on vesicles to the exocytic sites (Boyd et al., 2004; Finger and Novick, 1998). This led to the proposal that Sec3p might act as a spatial landmark for exocytosis. Further studies have, however, pointed to an alternative model in which the exocyst is activated at local patches enriched in the GTPases Rho3p and Cdc42p. According to this "localised activation" model, GTP-bound Rho3p or Cdc42p act as allosteric regulators of the exocyst by interacting with the component Exo70p. This interaction could in turn cause a conformational change within the complex, relieving an autoinhibitory interaction. The localised activity of the exocyst would now lead to the subsequent docking of the incoming vesicles carrying exocytic components, hence leading to an increasing polarization of the secretory machinery (Adamo et al., 1999; Brennwald and Rossi, 2007b; Roumanie et al., 2005). Proteins of the Rho family are important regulators of cell polarity, and Rho3p has an established role in actin polarization. The direct interaction with the exocyst defines a second function in cell polarity (Adamo et al., 1999).

2.3.3 Vesicle fusion

Once tethered at the plasma membrane, the fusion between the vesicles and the plasma membrane are mediated by a set of so called SNARE proteins. SNAREs were first identified in neuronal cells, where they mediate the fusion of synaptic vesicles with the

pre-synaptic plasma membrane (Sollner et al., 1993a). The SNAREs in this process were found to involve three proteins. One of them is a vesicle associated protein (VAMP), also termed synaptobrevin. This protein associates with two other proteins in the pre-synaptic membrane called syntaxin and SNAP-25. As components associated with this fusion machinery were found in all cell types, the SNARE hypothesis postulated that all transport vesicles carry their own vesicle SNARE (v-SNARE) that recognizes the matching membrane target SNARE (t-SNARE) (Rothman and Warren, 1994; Sollner et al., 1993b). In an alternative classification based on conserved amino acids essential for SNARE complex formation, SNAREs commonly conferred to the v-SNARE group are termed R-SNARES, while the cognate SNAREs are called Q-SNAREs (Fasshauer et al., 1998). The SNARE hypothesis has gained proof with SNARE proteins identified in all eukaryotes, with specific SNARE pairs mediating fusion events at each step of the exocytic and endocytic pathways (Wickner and Schekman, 2008). In yeast, the fusion of post-Golgi vesicles with the plasma membrane is mediated by the synaptobrevin/VAMP homologues Sncp1/2p (Protopopov et al., 1993), the syntaxin homologues Sso1p/2p (Aalto et al., 1993) and the SNAP-25 homologue Sec9p (Brennwald et al., 1994). SNARE proteins share a heptad-repeat SNARE motif, which takes part in the formation of a four-helix coiled-coil structure (Wickner and Schekman, 2008). The assembly of this structure is thought to provide the free energy needed to bring the two opposing membranes in close proximity to promote fusion (Bonifacino and Glick, 2004). Following the fusion event, α-SNAP binds to the SNARE complex and in turn recruits the ATPase NSF. Hydrolysis of ATP is thought to mediate SNARE disassembly, making the SNAREs available for another round of complex formation (Sollner et al., 1993a). Taken together, SNAREs have two distinct functions in exocytosis; they mediate membrane fusion and govern the specificity of vesicle to membrane targeting.

2.4 The endosomal pathway

Incorporating newly synthesized proteins into the plasma membrane is a prerequisite for growth and requires delivery of biomolecules from the biosynthetic pathway, which provides the material for anabolic processes within the cell. Proteins that are not transported directly to the plasma membrane can take three alternative routes from the

Golgi (Fig. 1). One pathway is directed straight to the vacuole, the yeast equivalent to the lysosome. This route is named the ALP-pathway after the most known cargo, alkaline phosphatase. In a second route, proteins destined to the vacuole pass via a prevacuolar compartment, also termed late endosomes or multivesicular bodies (MVBs). This route is also named the CPY-pathway after a well characterised cargo, carboxypeptidase Y. Finally as a third alternative, proteins can be sorted from the late Golgi to early endosomes, from where they can cycle to the plasma membrane (Bowers and Stevens, 2005).

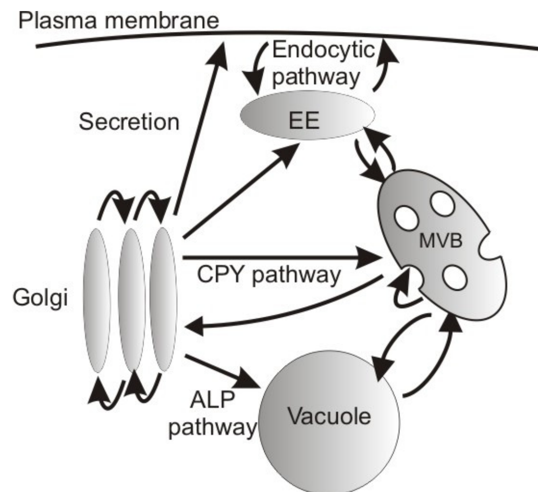

Fig.1 Protein trafficking from late Golgi, adapted from Bowers & Stevens, 2005 (Bowers and Stevens, 2005)

The endosomal and exocytic pathways converge at the endosomal system, from where proteins are sorted for recycling or degradation. In the search for mutants that block the delivery of CPY to the vacuole, about 60 *VPS* (vacuole protein sorting) genes have been identified (Bowers and Stevens, 2005; Raymond et al., 1992). These can in turn be divided in six groups based on the mutant phenotype (Raymond et al., 1992). This clustering corresponds well to genes that function at certain trafficking steps. For instance, proteins

encoded by the class E group are responsible for sorting membrane proteins into the lumen of endosomes. Out of these, four protein complexes named ESCRT (endosomal sorting complex required for transport) have been characterised as responsible for sorting ubiquitylated proteins to the vacuole via MVBs for subsequent degradation. The process and function of ubiquitylation will be described in more detail in section 8. The ESCRT (0-III) complexes act sequentially to sort membrane proteins from the endocytic or biosynthetic pathways into intraluminal vesicles (ILVs) that bud inwards into late endosomes, thereby forming MVBs (Hurley and Emr, 2006; Seaman, 2008; Williams and Urbe, 2007). Early and late endosomes are in part classified by their temporal ordering in the endosomal pathway. However, there is also a change in structure and composition during the maturation process. Selected components of early endosomes are sorted back via recycling endosomes to the plasma membrane or the Golgi. Simultaneously, late endosomes take on the shape of MVBs as ILVs are accumulated. Eventually, MVBs fuse with and empty their contents into the vacuole/lysosome, where it is degraded by vacuolar hydrolases (Bowers and Stevens, 2005; Seaman, 2008; Williams and Urbe, 2007).

3. Protein sorting

3.1 Identification of the *SEC* genes

The yeast *SEC* genes were isolated in the lab of Randy Schekman in 1980. In an elaborate study they identified a subset of conditional mutants that failed to secrete invertase and acid phosphatase at the non-permissive growth temperature (37ºC). The 23 secretory mutants that were isolated continue to synthesize proteins under the restrictive condition, but have all a specific block at a certain step of the secretory pathway. A common and discriminative feature to all of the mutants was the accumulation of secretory organelles. Ten of the *sec* mutants were characterised as accumulating 80-100 nm vesicles. These were identified as post-Golgi vesicles, in agreement with a block between Golgi and the plasma membrane (Novick et al., 1980). A subsequent study, analysing maturation of the enzyme invertase in different *sec* mutants, established the locus for different post-translational modifications and showed that yeast and mammalian secretion systems are very similar (Novick et al., 1981). The hierarchy of Sec proteins involved in the early secretory

pathway was later determined using different combinations of *sec* double mutants (Kaiser and Schekman, 1990). The same approach was used in an extensive examination of the synthetic lethality of post-Golgi *sec* genes, further establishing connections between proteins of the late secretory pathway (Finger and Novick, 2000).

3.2 Different classes of post-Golgi carriers

Protein secretion in *S. cerevisiae* is a fast process. The secretion of invertase is a telling example; this enzyme is released in the periplasmic space just minutes after synthesis. (Novick et al., 1981). For that reason, secretory vesicles are not detected in any significant quantities in wild type cells (Novick and Schekman, 1979). Initial efforts to characterise post-Golgi vesicles (PGVs) therefore took advantage of the vesicle accumulating phenotype of the late s*ec* mutant *sec6-4*. The preparations that were attained proved highly homogenous with few contaminants (Walworth and Novick, 1987). Using the same basic principle, a later study aimed to classify the PGVs in yeast using density gradient centrifugation. Two vesicle subpopulations of different densities could be distinguished, each associated with a distinct set of cargo proteins. The lighter class of vesicles carried the endoglucanase Bgl2p, as well as the plasma membrane ATPase, Pma1p. In the denser vesicles, the periplasmic enzymes invertase and acid phosphatase were both identified. This indicated that at least two separate routes can be used for transport of proteins from Golgi to the plasma membrane in yeast (Harsay and Bretscher, 1995). A third route from the Golgi involves a separate class of 40-50 nm vesicles, which carry soluble and membrane localised proteins destined to the prevacuolar/vacuolar compartment (Horazdovsky et al., 1995). The sorting into either type of vesicle is dependent on targeting determinants of the secreted protein, which often consist of short stretches of amino acid residues in the cytosolic domain of membrane proteins (Bonifacino and Traub, 2003; Keller and Simons, 1997).

3.3 Cargo selection and adaptors

Incorporation of cargo protein into the right type of trafficking vesicle depends on the recognition of the intrinsic sorting signal by specific adaptors. Most signals can be classified as tyrosine-based or dileucine- based. In addition, cytosolic lysine residues can

be conjugated to the small protein ubiquitin that in turn functions as a sorting signal (Bonifacino and Traub, 2003). Protein coats do not seem necessary for the targeting of vesicles straight to the plasma membrane. However, the laboratory of Schekman recently demonstrated the requirement for a coat complex called the exomer for exocytosis of the chithin synthase, Chs3p, from Golgi to the cellsurface (Wang et al., 2006). In sorting to endosomes or the vacuole/lysosome a set of specific adaptors recruit clathrin to coated vesicles. Initially, two such adaptor complexes, AP-1 and AP-2 were identified as co-purifying with clathrin vesicles. Two additional complexes, AP-3 and AP-4, which could function independently of clathrin were later identified based on homology. All four complexes are found in mammals, while in yeast only AP-1-3 are present (Robinson, 2004). AP-1 functions in TGN to endosome traffic, which also holds for AP-3 and AP-4 (Robinson, 2004). AP-2 on the other hand has a well described role in clathrin mediated endocytosis at the plasma membrane (Traub, 2009).

Another family of conserved clathrin adaptors is comprised of the GGAs. These are small proteins with ARF-, cargo- and clathrin binding sites, implicated in *trans*-Golgi network (TGN) to endosome sorting (Boman, 2001; Robinson and Bonifacino, 2001). GGAs also bind ubiquitin and are proposed to sort ubiquitylated cargo to endosomes (Pelham, 2004; Scott et al., 2004). Association of adaptors with discrete membrane domains is assisted by the interaction with phosphoinositides (PIs), which confers additional specificity to cargo sorting. The interaction with PIs enables for example the clathrin adaptors AP-1 and AP-2 to act at different cellular compartments, despite their shared recognition of the same sorting signal and association with the same coat protein (clathrin). In turn, the PIs are spatially and temporally regulated by PI kinases and phosphatases throughout the cell (Vicinanza et al., 2008). Further fine-tuning of protein trafficking is governed by adaptor associated proteins that act as specific cargo adaptors. Internalization of mammalian G-protein coupled receptors (GPCRs) is for instance dependent on their phosphorylation and subsequent binding of ubiquitin dependent beta-arrestins. Beta-arrestins subsequently interact with both AP-2 and clathrin (Goodman et al., 1996; Shenoy et al., 2009). The epsins constitute yet another family of clathrin adaptors that are characterised by the evolutionary conserved ENTH domain. In yeast, two homologues of the mammalian epsins exist. Like beta-arrestins, the epsin proteins can bind to clathrin, ubiquitin and AP-2 to

mediate endocytosis at the cell surface (Horvath et al., 2007). An epsin-like protein has also been implicated in TGN to endosome sorting of ubiquitylated cargo, through interaction with GGAs (Horvath et al., 2007; Mills et al., 2003). The epsin and arrestin family of proteins are two examples of a growing collection of auxiliary proteins, having specific roles in protein sorting (Robinson, 2004).

3.4 Proteomics of secretory vesicles

A number of studies have aimed to identify proteins of the secretory machinery. The combination of sub-cellular fractionation with highly advanced methods for protein identification has offered detailed insights into the interplay between proteins in cellular processes (Ritter et al., 2004). In two tandem MS analyses of clathrin coated vesicles (CCVs) from rat brain, a role for CCVs in recycling of synaptic vesicle proteins could be established. In addition to some previously undescribed CCV constituents, the data also confirmed presence of known endocytic vesicle cargos such as SNAREs, AP-complexes and epsin (Blondeau et al., 2004; Girard et al., 2005). Despite the preparation of highly concentrated CCV samples, neither study could definitely distinguish between *bona fide* vesicle proteins and contaminants. This challenge was taken on by Borner *et al*., using an approach where they compared CCV samples with mock preparations from a clathrin deficient mutant. By this refinement, 63 true CCV constituents could be determined, of which 28 had not been previously described as associated with CCVs (Borner et al., 2006). Continuing to outline the secretory pathway, Gilchrist et al. conducted an extensive proteomic study of the ER and Golgi in rat liver cells, where quantitative and spatial data for a total of 1400 proteins were presented (Gilchrist et al., 2006). Out of these, 345 were found to be uncharacterised, indicating that there is still much to be unravelled in the secretory machinery. In our lab, an attempt to characterise yet another step of the secretory pathway has been made (**paper II**). Our primary aim was proteomic analysis of post-Golgi vesicles isolated from control cells and mutants lacking the cell polarity protein and tumour suppressor homologue Sro7p. *sro7* mutants have exocytic defects leading to aberrant protein secretion and accumulation of PGVs (Lehman et al., 1999; Wadskog et al., 2006). Sro7p and its paralogue Sro77p will be described in more detail in section 5. We used the temperature-sensitive secretory mutants *sec6-4* to isolate control PGVs and *sec23-1* to

isolate a mock fraction depleted of PGVs. The *sec23-1* mutant has a defect in ER to Golgi protein traffic, blocking production of PGVs at restrictive temperature (Kaiser and Schekman, 1990). Isolated velocity gradient fractions were labelled with iTRAQ tags and analysed by tandem mass spectrometry. This state-of-the-art technique allows quantification of the identified proteins (Ross et al., 2004). The analysis enabled us not only to provide the first description of the yeast PGV proteome, but also perform a quantitative comparison with protein constituents of vesicles prepared from *sro7*Δ mutants. Taken together, we could identify 107 genuine PGV proteins, including known, assumed and previously undescribed components. A subset of these was depleted in *sro7*Δ mutants (**paper II**).

4. Cell polarity

4.1 Polar growth in yeast

A yeast cell shows polarized growth during bud emergence and during the formation of a projection in response to mating pheromone. Bud formation is the result of sequential events, starting with the marking of a spatial cue already during the previous cell cycle. Haploid yeast cells grow a new bud adjacent to the previous, which is termed axial budding pattern. Diploid cells, on the other hand, display a bipolar budding pattern, with the new bud constructed near or at the opposite end of the detached daughter cell (Drubin and Nelson, 1996). The bud site selection is tightly regulated and involves Rsr1p, a member of the Ras family of GTPases. Next, the machinery required for bud formation is directed towards the selected site. This is governed by a subset of proteins including the highly conserved Rho GTPase Cdc42p and its effectors. Activated Cdc42p guides polarization of the actin cytoskeleton, thus providing directional tracks for Sec4p mediated exocytosis that in turn reinforces polarity (Iwase et al., 2006; Park et al., 1997). Preceding bud emergence, a ring is formed from septins, which are GTP-binding filament forming proteins that assemble as another downstream effect of Cdc42p activation (Iwase et al., 2006). Maintaining Cdc42p in the activated state is assisted by the signalling protein Bem1p, which binds both Cdc42p and its effector the GEF Cdc24p (Park et al., 1997). Polarization of mRNA is a process possibly contributing to localised enrichment of Cdc42p and other polarity factors (Sec4p, Sro7p) in the incipient bud. In higher eukaryotes, polarized

targeting of mRNA plays an important role in for example *Drosophila* embryonic development and in the subcellular localisation of proteins in neuronal axons (Aronov et al., 2007). In yeast, asymmetrical distribution of *ASH1* mRNA in daughter cells regulates mating type switching by localised expression of the repressor Ash1p (Long et al., 1997). As for Ash1p, mRNA targeting of the polarity determinants is dependent on the actin cytoskeleton, the *SHE* genes and the 3' untranslated region (3'-UTR) of the mRNA (Aronov et al., 2007; Long et al., 1997). The observed dependence of polarized localisation of several polarity factor mRNAs on an intact secretory machinery, stresses the complexity and further entangles the processes of cell polarity and exocytosis.

4.2 Polarity in epithelial cells

Cells that have distinct plasma membrane domains require additional mechanisms to sort the proteins destined to the different compartments. This is the case in for example the highly polarized epithelial cells, which need to discriminate proteins going to the apical or basolateral membrane. Early clues to asymmetric distribution of proteins came from studies of virus budding in epithelial cells, where it was seen that the glycoprotein envelopes of influenza virus (hemagglutinin-HA) and vesicular stomatitis virus (VSVG) are assembled at the apical or basolateral cell surface, respectively (Rodriguez-Boulan et al., 2005).

In a vertebrate epithelial cell, the apical plasma membrane domain is separated from the basolateral domains by tight junctions and adherens junctions (septate junctions in invertebrates). Neighbouring cells are linked by gap junctions in the lateral domains. Being responsible for nutrient exchange and ion transport as well as signal sensing at all interfaces throughout an organism, it is of outermost importance that channels and receptors are confined to their site of action in the membrane (Tanos and Rodriguez-Boulan, 2008). The sorting to the proper membrane domain takes place at the Golgi apparatus or the endosomal system, guided by apical or basolateral sorting signals. For routing to the apical membrane this signal can for example be glycosylphosphatidylinositol (GPI) anchors, which mediate the association with lipid microdomains called rafts in the Golgi complex (Rodriguez-Boulan et al., 2004). Several examples where N-glycans or O-glycans seem to act as apical sorting signals have also been shown. However, it is not

known exactly how glycosylation directs apical delivery (Delacour and Jacob, 2006). Basolateral sorting involves motifs commonly involved in endocytosis, namely short tyrosine motifs and dileucine or monoleucine motifs (Tanos and Rodriguez-Boulan, 2008). Adding to the connection with endocytosis is the recently shown dependence on clathrin, which was demonstrated by the depolarization of most basolateral proteins upon clathrin knock-out. Exclusion of cargo proteins from basolateral transport vesicles subsequently led to a default apical routing (Deborde et al., 2008; Tanos and Rodriguez-Boulan, 2008). The same is seen in different *cdc42* mutants, where Golgi exit of basolateral proteins in MDCK cells is abolished in an actin cytoskeleton dependent manner, concurrent with a stimulated transport of apical cargo (Musch et al., 2001).

4. 3 Cell polarity complexes

Polarization of epithelia is dependent on three protein complexes, shortly referred to as the Par-, the Crumbs- and the Scribble complexes, each including PDZ domain proteins. Together, they function in cell polarity through well conserved pathways involved in diverse functions such as embryogenesis, epithelial morphogenesis, neuronal differentiation and migration of fibroblasts (Aranda et al., 2008; Mostov et al., 2003). Of these complexes only the Par complex will be considered in the following.

4.3.1 The Par complex

Assymetric cell division in the small nematode *Caenorhabditis elegans* is a prerequisite for the differentiation of an anterior and a posterior end. A screen for embryonic lethal mutants in *C. elegans* that fail to develop endoderm identified a set of proteins affecting the partitioning of cell fate determinants and RNAs. This group of six proteins were termed the Par proteins, for 'partition defective' (Goldstein and Macara, 2007; Kemphues et al., 1988). Identification of a seventh Par protein encoding an atypical protein kinase C, which physically interacted with a subset of the previously isolated Par proteins, lead to the description of the cell polarity complex Par6/Par3(Bazooka)/aPKC (Assemat et al., 2008). Par-3 and Par-6 are PDZ domain scaffold proteins that together with aPKC constitute the core complex , which in turn associates with other regulatory proteins to perform its function (Humbert et al., 2003; Wodarz, 2002). Despite differences in spatial cues to

initiate polarization, the Par proteins have been shown to be shared downstream effectors for polarity establishment in *C. elegans*, *Drosophila* and mammalian cells (Wodarz, 2002). Playing a crucial part in the assembly of tight junctions in mammalian epithelial cells, the Par complex mediates the separation of the apical and basolateral domains (Wodarz, 2002). In *Drosophila* embryogenesis, the Par complex assembles apically in developing epithelial cells, guided by Par-6. Correct localisation of the complex is in turn assisted by Par-6 binding of activated Cdc42 (Hutterer et al., 2004). Another key function of Par-6 is to mediate the interaction of aPKC with downstream effectors (Assemat et al., 2008; Henrique and Schweisguth, 2003). Physical interaction of aPKC with proteins of both the Crumbs and the Scribble complexes enables the interplay necessary for coordinating overall cell polarity (Humbert et al., 2003).

5. The *LGL* family

Lgl proteins have a role in the cell polarization process and are well conserved from the yeast Sro7p and its paralogue Sro77p to the human Hugl-1 and Hugl2 (Klezovitch et al., 2004). In **paper III** we show that this conservation extends into the plant kingdom, by demonstrating that the *Arabidopsis* homologue AtLGL1 (At4g35560) can partly substitute for the yeast Sro7/77 proteins. The Lgl proteins belong to the WD-40 repeat family of proteins, characterised by the presence of at least four repeated stretches of about 40 amino acids often terminating in a tryptophan-aspartic acid (WD) pair. The WD motifs are predicted to fold into a circular β-propeller, with each WD repeat forming a blade of the propeller. Proteins carrying WD repeats have been implicated in mediating protein-protein interactions and have critical roles in many cellular processes (Li and Roberts, 2001).

5.1 *Lgl in* Drosophila*;* a tumour suppressor

The Lgl (lethal giant larvae) gene in *Drosophila* was the first described example where a loss of function mutation led to tumour formation in a recessive manner, thus fitting the criteria of a tumour suppressor gene (Gateff, 1978). The illustrative name comes from the originally observed phenotype of *lgl* mutants in *Drosophila*, where loss of Lgl gives rise to larvae that continue to grow instead of pupating, and eventually die at the larval stage.

Dissection of these larvae reveals overgrowth of the brain and imaginal discs, which are precursor cells of adult structures (Bilder, 2004; Wirtz-Peitz and Knoblich, 2006). The characteristic disruption in the shape of the affected tissue places Lgl into the group of neoplastic tumour suppressors, together with its functional partners Scribble and Dlg (Wirtz-Peitz and Knoblich, 2006). Loss of these gene products results in the missorting of apical proteins to the basolateral domain, including adherens junction proteins (Bilder, 2004). Cancer is a disease that to a large extent involves epithelia. In fact, 90% of human cancers are carcinomas that derive from epithelial cells (Tanos and Rodriguez-Boulan, 2008). Moreover, there is a strong correlation between the degree of epithelial disorganization and malignancy in most tumour forms, which makes understanding the mechanisms behind cell organization highly relevant (Aranda et al., 2008). The connection between cell polarity and tumorigenesis remains elusive, but studies in different systems are continuously adding pieces to the puzzle. Many tumours have a normal cell cycle, but fail to respond to arrest cues. The *lgl* dependent overgrowth of the *Drosophila* imaginal discs for instance, results from failure to coordinate tissue size with exit from the proliferative cycle (Bilder, 2004).

5.2 Lgl, a mammalian tumour suppressor?

Since the functional conservation of Lgl proteins seems well preserved the tumour suppressor function, first identified in flies, has been implicated in many human cancers (Bilder, 2004; Froldi et al., 2008; Humbert et al., 2003). Expression of human Lgl (Hugl-1) can suppress the phenotype of *Drosophila lgl* mutants, completely abolishing imaginal disc overgrowth (Grifoni et al., 2004). Lgl downregulation has been implicated in a number of human cancer forms including malignant melanoma and colorectal cancer (Kuphal et al., 2006; Schimanski et al., 2005). *Lgl1* knockout mice exhibit loss of neuroepithelial cell polarity and contract hydrocephalus due to failing cellular ability to exit from the cell cycle. The brain lesions of the mutant mice were histologically similar to primitive neurectodermal tumours in humans (Klezovitch et al., 2004). More examples indicating a critical role for Lgl in cell polarity are reported from a wide variety of organisms, ranging from frog to fish (Wirtz-Peitz and Knoblich, 2006).

5.3 Lgl in the maintenance of cell polarity

Polarization of *Drosophila* cells involves concentration of the Par6/aPKC in the apical part of the cell where it binds activated Cdc42. Lgl has no role in this process but is required to maintain Par6 at the apical side (Hutterer et al., 2004). Following activation of aPKC by Cdc42, aPKC phosphorylates Lgl on a cluster of serines in the C-terminal half of the protein. This phosphorylation results in autoinhibitory interactions between the phosphorylated region and the N-terminus (Betschinger et al., 2005). Hence, the protein becomes inactivated in the apical side of the cell, while remaining active at the basolateral side where Lgl prevents Par6 from associating with the cortex (Hutterer et al., 2004).

Given its role in maintenance of epithelial cell polarity, it is perhaps not surprising Lgl is also involved in asymmetric cell division. *Drosophila* neural precursor cells (neuroblasts) divide into two distinct daughter cells, of which one gives rise to a new neuroblast and the other forms a mother ganglion cell. The differential sorting of cell fate determinants into the basal cortex precedes budding of the mother ganglion cell from this domain. Lgl plays a pivotal role in this basal sorting, assisted by Dlg and myosins (Ohshiro et al., 2000; Peng et al., 2000). A recent study has shown that aPKC-mediated phosphorylation of Lgl leads to a cascade of events that culminates in the phosphorylation of the fate determinant Numb on one side of the plasma membrane causing its accumulation on the opposite side (Wirtz-Peitz and Knoblich, 2006).

Homologues of the *Drosophila* Lgl in both yeast and mammals have been found to interact with t-SNARES, indicating that the contribution to cell polarity by Lgl also involves a role in the exocytic machinery (Fujita et al., 1998; Lehman et al., 1999). The involvement of the yeast Lgl (Sro7/77p) in SNARE regulation will be discussed in section 6.1.4. In Madin-Darby canine kidney (MDCK) cells, Lgl (Mgl-1) interacts selectively with the basolateral t-SNARE Syntaxin-4, thereby possibly promoting a basolateral route for exocytosis (Musch et al., 2002). By analyzing chimeric Lgl proteins composed of mammalian and yeast proteins, Gangar *et al.* found evidence that the SNARE interacting

C-terminal domain is critical for the function of the proteins (Gangar et al., 2005a). The observations indicate conserved structural organization between yeast and mammals. Since neither yeast nor plants are dependent on a Par complex to establish and maintain cell polarity, the role of Lgl in targeted exocytosis is likely be a common denominator for Lgl function across species borders.

5.4 Tomosyn: Lgl homologues that regulate SNARE assembly

Sequence alignments show that tomosyn proteins are closely related to Lgl proteins (**paper III**) (Ashery et al., 2009; Hattendorf et al., 2007). Despite the overall sequence similarity, the two protein families still appear to have a different function. Tomosyn was first identified as a syntaxin-binding protein in rat brain, promoting SNARE complex formation and subsequent release of neurotransmitters (Fujita et al., 1998). Like the yeast Sro7p/77p proteins the mammalian tomosyns regulate polarized exocytosis by controlling SNARE function. However, this function is dependent on a conserved R-SNARE in the C-terminus of tomosyn that is absent in the yeast Lgl homologues. This C-terminal motif is believed to function as a surrogate SNARE, by which tomosyn regulates the assembly of the SNARE complex (Ashery et al., 2009). In agreement with this structural and functional difference between Lgl proteins and tomosyn, rat Lgl (Rgl-1), but not rat m-tomosyn, can rescue the salt sensitivity of the *sro7*Δ*sro77*Δ mutant (Kim et al., 2003).

We isolated and characterised two putative Lgl homologues from *Arabidopsis thaliana*, which we named AtLGL1 and AtLGL2 **(paper III).** Homozygous T-DNA insertion mutants in *AtLGL1* or *AtLGL2* resulted in decreased growth of lateral roots, which is consonant with a role for AtLGL1 and AtLGL2 in cell and tissue polarity (*cf* (Benkova et al., 2003; De Smet et al., 2007)). Interestingly, only AtLGL1 but not AtLGL2 was able to partially complement the yeast *sro7*Δ*sro77*Δ salt sensitive phenotype. Sequence analysis and structure modelling revealed that both proteins can fold into the two consecutive β-propellers that constitute the structure of the yeast Lgl homologue Sro7p (Hattendorf et al., 2007). However, AtLGL2 differs from AtLGL1 in carrying a full R-SNARE motif in its C-terminal. These observations suggest that ALGL2 is a plant homologue of tomosyn which

may explain its failure to substitute for the yeast Lgl homologues (**paper III**). Our report is the first to demonstrate conservation of Lgl and tomosyn function also in plants.

6. The *SRO* genes

6.1 Identification of the *SRO* genes

The yeast Lgl homologue, *SRO7*, was picked up in a screen for osmosensitive mutants in our lab. The gene was originally called *SOP1* (sodium protection) and was recognized as a weakly expressed gene encoding a protein with a molecular mass of 114.5 kDa. Sequence analysis revealed the presence of an isogene in the yeast genome, *SRO77* (S*OP2*), and a comparison of the inferred amino acid sequences showed about 50% homology between Sro7p and Sro77p. In an independent study, *SRO7/77* were simultaneously described by a group in Japan after being isolated as high copy suppressors of the growth defect in *rho3*Δ mutants, hence the name *SRO* (suppressor of rho). In both cases, homology searches pointed to the relation to the *Drosophila LGL* tumour suppressor and its mammalian counterparts (Kagami et al., 1998; Larsson et al., 1998; Matsui and Toh, 1992).

6.1.1 NaCl sensitivity of the sro7Δ mutant

Hyperosmotic stress faces yeast with the problem of water efflux, which is counteracted by production and intracellular accumulation of glycerol, the compatible solute of *S. cerevisiae.* Glycerol accumulation promotes retention of water, which helps the yeast cell to sustain its turgor and keep a physiologically adjusted environment (Blomberg and Adler, 1992). Upon NaCl stress, an additional acute problem is the influx of Na^+ ions, which has the potential of perturbing important cellular processes. As part of the osmotic stress response, signalling pathways activate transcription of genes encoding ion transporters.

The mutant that was complemented by *SRO7* in our screen proved to have a normal glycerol production. In addition, it showed no general susceptibility to osmostress, but was specifically affected in its NaCl tolerance. Deleting *SRO77* gave no obvious phenotype, while a *sro7*Δ*sro77*Δ double knockout displayed hypersensitivity to NaCl, as well as a sensitivity to high K^+ and Li^+ concentrations that was not seen with the *sro7*Δ single mutant. Still, no growth defect was brought about by sorbitol stress, indicating that the effect specifically involved ion balance. However, there was no increase in *SRO7*

transcription upon salt stress. Neither did *SRO7* overexpression confer increased salt tolerance. The severe phenotype of the double mutant further suggested that the two isogenes function in the same pathway, with Sro7p being able to substitute for Sro77p. The connection to the Lgl protein family was further established by the partial rescue of the double mutant by *Drosophila* L(2)gl expression (Larsson et al., 1998).

6.1.2 Mistargeting of Ena1p

The nature of the salt sensitivity and the finding that high external NaCl leads to an accumulation of Na^+ ions in the *sro7*Δ mutant, pointed to involvement of the sodium transport system in the observed phenotype (Larsson et al., 1998). In yeast, sodium export is governed by two Na^+-ATPases that are active at different pH. *ENA1* (*PMR2A*) is induced upon salt stress at alkaline and neutral pH, while the constitutively expressed sodium/proton antiporter Nha1p is significant only at an acidic pH (Prior et al., 1996). At high Na^+ stress, *ENA1* is up-regulated as a response to different signalling pathways, one being mediated by Ca^{2+}/calcineurin following a calcium release elicited by hyperosmotic stress (Matsumoto et al., 2002). The fact that the NaCl sensitivity was seen at near neutral pH, prompted us to look at *ENA1* transcription in the *sro7*Δ mutant. However, no difference in the transient but strong expression of *ENA1* could be seen between wild type and mutant cells. In agreement with this result, over-expression of *ENA1* in the *sro7*Δ mutant did not increase salt tolerance. However, when looking at effects on protein level, Ena1p was clearly unstable in the *sro7*Δ mutant. Immunoblots revealed that the sodium pump was degraded in mutant cells, concurrent with a strong accumulation in wild type cells (**paper I**).

To establish at what point Ena1p is diverted for degradation, double deletions of *SRO7* in combination with key genes in the secretory and endosomal pathways were constructed. Out of these, the same pattern of degradation was seen when *SRO7* was co-deleted with *END4*, mediating internalization from the PM. This result clearly indicated that Ena1p incorporation in the plasma membrane is not a prerequisite for subsequent degradation. Neither is Golgi to PM transport required for misrouting, since degradation also occurred in the *sro7sec15* mutant. This suggested that Ena1p is misrouted prior to Sec15p mediated

vesicle tethering at the PM. The stabilisation of Ena1p in an *sro7vps27* mutant indicated that the protein is routed via the endosomal system. Since stabilisation was also seen in *sro7pep4* mutants that lack the main vacuolar protease, we concluded that Ena1p is sorted via MVB pathway to the vacuole for degradation. We were able to confirm this by microscopic studies, where GFP-tagged Ena1p could be detected in the vacuole of the *sro7*Δ mutant. However, this misrouting was conditional and only seen under salt stress and not when expressing *ENA1* from an inducible promoter under non-stress conditions. To establish if Ena1p become mis-routed in salt stressed *sro7*Δ mutants due to a defect in the late (post-Golgi) secretory pathway, we isolated secretory vesicles by velocity gradient centrifugation of membrane fractions from *sro7*Δ mutants and the late secretory mutant *sec6-4*. While seeing a peak of HA-tagged Ena1p coincident with a peak of the post-Golgi vesicle markers Snc1p/2p in the *sec6-4* derived fractions, we detected no Ena1p in the corresponding *sro7*Δ fractions. This indicated that Ena1p sorting into post-Golgi vesicles requires the presence of Sro7p (**paper I**).

Aiming to determine if the sorting defect is Ena1p-specific, we examined the fate of other GFP-tagged membrane proteins in wild-type and *sro7*Δ strains. However, neither the sodium/proton antiporter Nha1p, the general amino acid permease Gap1p, nor the polar osmosensor Sho1p showed defective localisation in salt stressed *sro7*Δ mutants (**paper I**). Our proteomic analysis allowed for a more thorough search for secretion defective candidates (summarized in section 3.3), and yielded a subset of proteins that are depleted in *sro7*Δ derived post-Golgi vesicles. The mechanism behind their partial exclusion from these vesicles, remains to be determined. We could also conclude that under the experimental procedures used in this analysis, Ena1p was not fully excluded from the vesicles in the *sro7*Δ mutant (**paper II**).

6.1.3 Suppression of rho3 and interaction with myosins

The *Drosophila* and human Lgl proteins have been shown to interact with nonmuscle myosin II in larger complexes, thus providing a link to the cytoskeletal network (Strand et al., 1994; Strand et al., 1995). In yeast, the type II myosin is encoded by *MYO1*, and localizes primarily to the contractile ring at the bud neck. As in fly and human, Sro7p

physically interacts with Myo1p. *MYO1* deletion causes defects in mother-daughter cell separation, which is overcome by a co-deletion of *SRO7* and *SRO77*. In turn, deleting *MYO1* suppresses the slow growth displayed by the *sro7*Δ*sro77*Δ double mutant (Kagami et al., 1998).

The type V myosin Myo2p acts in conjunction with Myo4p as a motor protein in the delivery of secretory vesicles, organelles and mRNA to the bud. The myosin dependent transport occurs along actin tracks that are polarized and nucleated by Rho-activated formins (Pruyne et al., 2004). Overexpression of *SRO7* rescues *myo2-66* mutants, which have a reduced Myo2p activity. Also here the reverse is true; *MYO2* overexpression rescues the *sro7*Δ*sro77*Δ double mutant (Kagami et al., 1998). Furthermore, a physical interaction has also been established between Myo2p and Sro7p (Gangar et al., 2005b). Suppression of the *rho3* mutant by *SRO7* overexpression was suggested to result from the role of Sro7p in polarized secretion, via its connection to myosins (Kagami et al., 1998). Rho3p is required for normal actin polarity and has in addition been shown to have a Myo2p dependent role in the transport, docking and fusion of secretory vesicles (Adamo et al., 1999). The observed direct interaction with Myo2p has been proposed to be important for the transport of secretory vesicles or for a polarization of Sro7p itself (Gangar et al., 2005b).

6.1.4 Connections with the exocytic machinery

As described in section 5.4, tomosyn was isolated based on its ability to modulate SNARE complex formation by binding the Q-SNARE syntaxin. A similar function has been proposed for the Sro7/77 proteins, as they were found to bind the yeast t-SNARE Sec9p both *in vivo* and *in vitro* (Lehman et al., 1999). In agreement with this finding, *sro7*Δ*sro77*Δ mutants have a strong secretion defect at restrictive cold temperature (Kagami et al., 1998). Further supporting these observations, we found that *sro7*Δ mutants exposed to high salinity shares the characteristic late exocytic defect of *sec9* mutants in accumulating post-Golgi vesicles. The secretory defect was accompanied by a decreased cell surface delivery of the endo-glucanase Bgl2p but had no effect on invertase secretion, indicating a cargo specific effect of Sro7p loss (**paper I**). The dissociation of actin cables and concomitant dispersal of actin patches that was reported as a feature of *sro7*Δ*sro77*Δ

cold-sensitivity (Kagami et al., 1998), were later proposed to be a secondary effect. This conclusion was based on the observation that vesicle accumulation precedes the actin disorder, which instead probably results from an impaired Sro7p/77p-dependent transport of polarity markers to the cell surface (Lehman et al., 1999).

A bridging role of Sro7p between the vesicle docking and fusion machinery is further supported by the suppression of several late *sec* mutants by *SRO7* over-expression (Lehman et al., 1999). The reported high copy suppression of temperature sensitive alleles of genes encoding the exocyst components Sec3p, Sec8p, Sec10p and Sec15p, was later supported by our demonstration of synthetic effects when *SRO7* was deleted in the various *sec* mutant backgrounds (**paper I**). In addition, Sro7p was found to bind yet another protein, the exocyst subunit Exo84p. Binding of Exo84p was proposed to precede Sro7p promoted SNARE complex formation, and deleting *SRO7* or *SRO77* in an *exo84* mutant background, severely aggravated the secretion defect. Genetic interactions in the same study placed Sro7p downstream of the exocyst and the regulating GTPases (Zhang et al., 2005).

Further support of the involvement of Sro7p with the exocyst came from the identification of Sro7p as an effector of the Rab GTPase Sec4p, which mediates Myo2p dependent movement of secretory vesicles and assembly of the exocyst complex. Sro7p was found to co-immunoprecipitate with Sec4p, and specifically interact with the GTP-bound form of the protein. Moreover, Sro7p, Sec9p and Sec4p were reported to form a ternary complex, suggesting that Sro7p mediates a Rab dependent regulation of SNARE function (Grosshans et al., 2006; Novick et al., 2006b). In agreement with these findings, overexpression of *SRO7* was recently shown to increase SNARE complex formation supporting a positive role for Sro7p in SNARE assembly (Williams and Novick, 2009).

6.2 The crystal structure of Sro7p

Recently, the crystal structure of Sro7p was solved, providing substantial insights to its function in late exocytosis (Hattendorf et al., 2007). The structure revealed 14 WD-40 repeats that were more or less evenly distributed throughout the sequence. The repeats fold

into two consecutive seven bladed β-propeller domains, with each WD repeat forming a blade. The structure topologically resembles the *C. elegans* actin binding protein, Aip1 (Voegtli et al., 2003). The N-terminal and C-terminal propellers form together an open clamshell-shaped structure that provides a multitude of protein binding surfaces oriented in fixed angles relative each other. The structural studies also revealed that a part (60 amino acids) of the C-terminal tail folds back to bind the N-terminal propeller. Given the structure of Sro7p, Hattendorf *et al.* went on to study how Sro7p binds Sec9p (Hattendorf et al., 2007). They found that Sro7p associates with the amino-terminal of Sec9p via conserved surface residues in the C-terminal propeller, while the interaction with the Sec9p Q-SNARE domain is weaker. The C-terminal tail of Sro7p appears to have a key function in the interaction, since Sro7p was found to bind Sec9p with a three times higher affinity (0.8 μM) when the tail was deleted. In fact, *in vitro* binding of the Sec9p SNARE motif was only seen in absence of the tail. Moreover, SNARE complex formation was delayed when a Sro7p-version lacking the tail was expressed. Taken together, these results indicated that Sro7p binding of Sec9p prevents SNARE complex formation. A model was proposed suggesting that the Sro7p tail masks a binding site for the Sec9p Q-SNARE domain. Relieving the binding site promotes interaction with the Sec9p Q-SNARE domain, thus preventing SNARE complex formation with Sso1/2p and Snc1/2p. An unknown factor subsequently causes an autoinhibitory rebinding of the tail, thus releasing the Sec9p SNARE domain to take part in complex formation. Exo84p and Sec4p were suggested as candidates for such a factor, based on their reported interaction with Sro7p (see section 6.1.4). In addition to a role in the proposed regulation of the fusion step, Sro7p was suggested as a candidate for mediating the localisation of Sec9p to the plasma membrane, since Sec9p lacks lipid anchors and transmembrane domains (Hattendorf et al., 2007).

A predicted α-helix at the C-terminal was not included in the crystal structure due to protease sensitivity. The terminal heptad repeat sequence has been predicted to form at best a degenerate R-SNARE motif. The mammalian Lgl-like protein tomosyn, on the other hand, has a full R-SNARE in its C-terminal. This coiled-coil motif is essential for the function of tomosyn; it is used to substitute for synaptobrevin in the formation of a SNARE-complex with syntaxin and SNAP-25. According to alignment with Sro7p, tomosyn is also predicted to fold into two consecutive β-propellers (Hattendorf et al.,

2007). These similarities raised the proposal that Sro7p and Sro77p are tomosyn orthologs (Fasshauer and Jahn, 2007). Contradicting this suggestion is the failure of rat tomosyn - but not rat Lgl - to partially complement the *sro7Δsro77Δ* salt sensitivity phenotype (Kim et al., 2003). Our study of the two *Arabidopsis* Lgl/tomosyn homologues gave similar results; the predicted tomosyn-like candidate, AtLGL2, that carries a full C-terminal R-SNARE motif could not complement the growth defect of *sro7Δsro77Δ* mutants (**paper III**). We suggested that tomosyn-like R-SNAREs might form unproductive SNARE complexes in yeast.

6.3 Localisation of Sro7p/77p

Sro7p has in previous studies been shown to localize to the cell periphery and partly associate with the plasma membrane (Larsson et al., 1998; Lehman et al., 1999), consistent with the localisation reported for *Drosophila* and human Lgl (Strand et al., 1994; Strand et al., 1995). However, in these studies *SRO7* was over-expressed from multi-copy plasmids. When we used the regulatable *MET25* promoter to adjust *SRO7* expression closer to native conditions, we could see a clearly polar distribution of the protein. This localisation followed the sites for polar exocytosis throughout the cell cycle, consistent with the established role for Sro7p in exocytosis (Fig. 2). As already mentioned in section 4.1, *SRO7/77* mRNA has been observed to be transported to the incipient bud, assisted by the myosin Myo4p and the myosin/mRNA binding protein She3p (Aronov et al., 2007). This asymmetric delivery of mRNA, which is strictly dependent on the 3´-untranslated region of the m-RNA molecule, is believed to ensure a high concentration of Sro7p/77p at the site of active exocytosis. However, polar m-RNA distribution can not fully account for the localisation of Sro7p/77p. In our hands, the polar localisation of Sro7p was found to be independent of the 3'-untranslated region (Forsmark A., unpublished results). A concurrent transport by the secretory machinery probably targets the translated protein to the active sites, although neither Sro7p nor Sro77p has been detected in post-Golgi vesicles (Lehman et al., 1999). This might be due the preparation method used which is likely to lead to loss of proteins weakly associated with the vesicles, as discussed in paper II.

The faint cytosolic distribution that is seen concurrent with the asymmetric Sro7p localisation is possibly partly contributed by the Golgi/endosome association that we have

proposed to govern Ena1p sorting into post-Golgi vesicles (**paper I**). For Sro77p this localisation was more compellingly confirmed for a fraction of the GFP-tagged protein that co-localized with the Golgi marker Kex2p in sucrose density gradients (A. Forsmark, unpublished results).

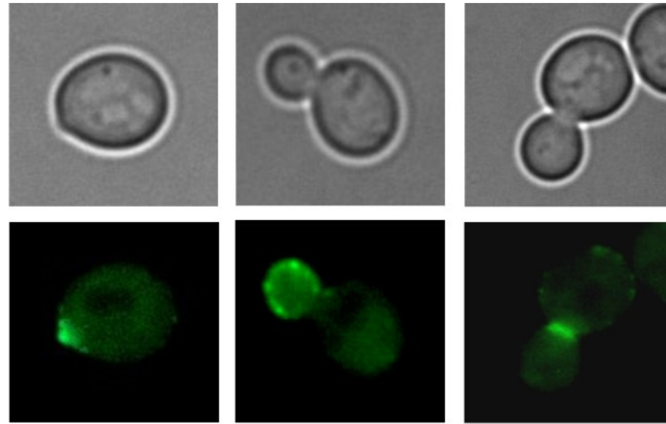

Figure 2. Sro7p/77p localises to sites of active exocytosis throughout the cell cycle

6.4 Suppressors of the *sro7*Δ phenotype

6.4.1 Sro77p

The aggravated phenotype of the *sro7*Δ*sro77*Δ mutant compared to the *sro7*Δ single mutant and the apparent lack of phenotype of the *sro77*Δ mutant, indicate that the two Lgl homologues have different but overlapping functions in yeast. Sro7p seems to be capable of fully substituting for the loss of Sro77p, while Sro77p only partially rescues the *sro7*Δ phenotype. The effect can partly be explained by differences in expression, since overexpressing *SRO77* from a multi-copy plasmid actually does increase the salt tolerance in *sro7*Δ. However, the suppression is not complete, supporting a specific function for Sro7p that can not be performed by Sro77p (Wadskog, 2003).

6.4.2 Calcium

The notion that the salt sensitivity of *sro7*Δ varied with the composition of the growth medium prompted us to look at the effect of calcium on NaCl tolerance. A decrease in salt sensitivity of *sro7*Δ mutants could be observed when adding calcium to a normally calcium poor medium, like CBS. Conversely, addition of the calcium chelator EGTA to a calcium rich medium, like standard YNB, impaired growth of *sro7*Δ cells in high salt. Since no calcium-induced increase of salt tolerance was detected in wild type cells, the requirement for calcium seemed to arise in the absence of Sro7p. When deleting either of two genes governing calcium uptake in the *sro7*Δ background, the cells became hypersensitive to NaCl in low calcium media, emphasizing the dependence on external calcium (Wadskog, 2003).

Expression of *ENA1* from a Ca^{2+}-independent promoter did not affect the calcium mediated suppression of the *sro7*Δ salt sensitivity phenotype, demonstrating that the calcium effect is not linked to an upregulated Ca^{2+}/calcineurin dependent transcription of *ENA1*. However, at high calcium Ena1p was not degraded in salt stressed *sro7*Δ mutants but remained as stable as in wild type cells (Wadskog, 2003).

Given the well established role of calcium in SNARE complex formation in e.g. the fusion of exocytic vesicles with the presynaptic membrane (Jena, 2009; Rettig and Neher, 2002), makes it appealing to suggest a similar function for calcium in yeast exocytosis. However, the observation that calcium supplementation does not suppress the salt sensitivity of the *sro7*Δ*sro77*Δ double mutant (A. Forsmark unpublished results) has evoked an alternative explanation. We hypothesize that the suppression might derive from a calcium-dependent substitution of Sro7p function by its paralogue, Sro77p. At high salt concentrations there might be a competition between calcium and sodium uptake for similar transport sites, thus preventing efficient calcium uptake (Philipson and Nicoll, 2000). This could explain the puzzling fact that mis-sorting of Ena1p in *sro7*Δ mutants only occurs in high salt media (**paper I**). However, experimental support for this explanation is yet to be produced.

6.4.3 RSN1

In a multicopy suppressor screen using a pHR81 based yeast-genomic library (Nehlin et al., 1989), two genes apart from *SRO7* were found to strongly suppress *sro7*Δ salt

sensitivity. A BLAST search identified one of them as the 2862 bp hypothetical open reading frame (ORF), YMR266W, encoding a putative membrane associated protein. The other suppressor gene was identified as the 1762 bp ORF YGR068C encoding another unknown protein. Since neither of the genes had previously been characterised, they were named *RSN 1* and *RSN2* (rescue of sro7 at high NaCl) (**paper I**).

Further studies of the *RSN1* (YMR266W) suppression showed that overexpression led to Ena1p stabilization and recovery at the cell surface. Consequently, *sro7*Δ cells transformed with a multicopy *RSN1* plasmid had near wild type growth on solid high salt media. The possibility of *RSN1* encodes a protein conferring sodium protection was dismissed by failure to rescue the highly salt sensitive *ena1*Δ mutant by multicopy *RSN1* expression. Further, deletion of *RSN1* in wild type cells caused no obvious phenotype. Confocal microscopy showed that Rsn1p is localized at the cell periphery, primarily concentrated at the bud neck. A membrane association is in agreement with the presence of 11 or 12 predicted transmembrane regions in Rsn1p, and membership in the yeast facilitator family. Accordingly, Rsn1 was found to be strongly associated with membranes in subcellular fractionation experiments (**paper I**).

A possible, but speculative explanation of the Rsn1p mediated suppression was raised by analogy with the *AST1* mediated multicopy suppression of a *pma1-7* mutant. This mutant plasma membrane ATPase, Pma1-7p, is mistargeted to the vacuole and exhibits an impaired ability to associate with lipid rafts (Bagnat et al., 2001). Lipid rafts are platforms of cholesterol and sphingolipids that are thought to promote polarized delivery of proteins and lipids to the plasma membrane (Simons and Ikonen, 1997). Overexpression of *AST1* retargets the mutant Pma1p to the cell surface. The association with lipid rafts is restored, which is proposed to be a consequence of Ast1p induced Pma1p aggregation into clusters. Ast1p itself is a peripheral membrane protein, directly interacting with Pma1p (Bagnat et al., 2001). With Ena1p belonging to the same class of ATPases as Pma1p, it is yet to be established if Rsn1p might act in a similar manner as Ast1p to retarget Ena1p. So far, we have made attempts in deleting genes encoding proteins in the sphingolipid or ergosterol biosynthetic pathway (Lees et al., 1995; Oh et al., 1997). However, neither deletions of *ERG6* nor *ELO3* confer salt sensitivity, which would be expected if Ena1p partitioning into lipid raft was essential (A. Forsmark, unpublished results).

6.4.4 RSN2/ART5

The second gene that was identified in the multicopy suppressor screen was given the preliminary name *RSN2* (YGR068C). Like for *RSN1*, microscopy showed that overexpression of *RSN2* lead to recovery of Ena1p at the cell surface in salt stressed *sro7*Δ mutants. Consonant with this observation, overexpression of *RSN2* restored almost wild type NaCl tolerance of the mutants. Surprisingly, deleting *RSN2* had the same effect (**paper IV**). Rsn2p has been found to interact with Sft2p (Uetz et al., 2000), which is a non-essential membrane protein thought to promote fusion events in late Golgi (Conchon et al., 1999). However, the *sro7*Δ*sft2*Δ double mutant has the same phenotype as *sro7*Δ (A. Forsmark, unpublished).

6.4.4.1 The role of RSN2/ART5 in Ena1p ubiquitylation

Another well established interacting partner of Rsn2p (YGR068C) is the ubiquitin ligase Rsp5p (Belgareh-Touze et al., 2008), which lead us to look at the role of Rsn2p in the ubiquitylation of Ena1p (the process of ubiquitylation is covered in more detail in section 8). We had previously established the involvement of ubiquitylation in Ena1p MVB sorting. In high salt, Ena1p ends up in the vacuolar limiting membrane in a *sro7*Δ*npi1*Δ mutant (**paper I**), which exhibits low *RSP5* expression (Hein et al., 1995). During the course of our study YGR068C became established as an arrestin-like ubiquitin ligase adaptor involved in endocytosis and grouped into the *ART* family of proteins. YGR068C (*RSN2*) was given the name *ART5* (Lin et al., 2008) and will be referrer to by this name in the following. To verify that Ena1p is indeed ubiquitylated, Ena1-GFP was immunoprecipitated in lysates prepared from salt stressed *ena1*Δ and *sro7*Δ*ena1*Δ cells co-transformed with plamids expressing Ena1p-GFP and ubiquitin. Blots probed with anti-GFP or anti-Ub showed a ubiquitination smear larger than the fusion protein in salt stressed *sro7*Δ mutants. To establish the role of Rsp5p in Ena1p ubiquitylation a similar experiment was carried out in the *sro7*Δ*rsp5-1* strain, having a temperature sensitive allele of *RSP5* (Dunn and Hicke, 2001) (**paper IV**). Since the high molecular weight ubiquitin smear almost completely disapperaed at restrictive temperature, we concluded that Rsp5p is the responsible ligase mediating Ena1p ubiquitylation. Inactivation of Rsp5 also retargeted Ena1p to the cell surface as shown by fluorescence microscopy. In the subsequent work the

possible role of *ART5* was examined. Here, we noted the same disappearance of ubiquitylated Ena1p species in *sro7*Δ*art5*Δ mutans as seen in the *rsp5-1Δ sro7*Δ background at restrictive conditions. Microscopic studies could also confirm the same re-localisation of Ena1p to the plasma membrane and drop test showed an almost complete suppression of the salt sensitivity. This role of Art5p in Rsp5p-mediated Ena1p ubiquitylation appears to be specific, since no similar recovery of *sro7*Δ salt tolerance could be seen in double deletions with other genes encoding established Rsp5p adaptors (**paper IV**).

7. Quality control and regulation

7.1 Protein quality control in the ER

Proteins destined for the secretory route are synthesized at the membrane of the endoplasmic reticulum (ER) and translocated into the ER. Incorrectly folded or in other ways aberrant proteins are recognized and retained in the ER and eventually routed for degradation. The ER harbours different groups of chaperones mediating correct folding of proteins prior to their release from the ER. Prolonged protein-chaperone interaction due to mis-folding, reduces availability of free chaperones. This in turn triggers the unfolded protein response (UPR), which attenuates translation and induces transcription of genes involved in the processing of aberrant proteins. Proteins that fail to be repaired are exported to the cytosol by the process of ER-associated degradation (ERAD). Here they are degraded by the ubiquitin-proteasome system, after recognition by ER based ubiquitin ligases (Meusser et al., 2005; Nishikawa et al., 2005).

7.2 Quality control at the Golgi

An accumulating number of reports strengthens the existence of a post-ER quality control system promoting routing of non-native Golgi proteins to the endosomal/vacuolar pathway (Arvan et al., 2002). The Pma1-7p mutant that fails to associate with lipid rafts (see section 6.4.3) is an established substrate for Golgi-mediated ubiquitylation and degradation (Chang and Fink, 1995; Pizzirusso and Chang, 2004). The signal underlying the selection of ubquitylation targets at Golgi has been examined for substrates of the ubiquitin ligase Tul1p. This Golgi resident ubiquitin ligase is suggested to recognize polar trans-membrane

domains (TMDs) that become exposed to the lipid bilayer in mis-folded or unassembled proteins (Reggiori and Pelham, 2002). The same recognition of hydrophilic TMD residues promotes Bsd2p mediated quality control at Golgi. Bsd2p assist in recruiting the ubiquitin ligase Rsp5p to selected proteins to promote their marking by ubiquitin and subsequent degradadation in the vacuole (Hettema et al., 2004; Stimpson et al., 2006). Proteins failing to localise correctly to the plasma membrane has also been suggested to be marked for degradation (Arvan et al., 2002). It is plausible that Ena1p accumulates in the Golgi or endosomes when not efficiently sorted into exocytic vesicles, as shown for *sro7*Δ mutants at high salt (**paper I, paper II**). This might promote recognition by a quality control system relieving the Golgi/endosomes from a too high load of the protein by targeting the transporter to the vacuole for degradation. Data in **paper IV** suggests that Art5p is a selecting component of this control. It remains, however, to establish what sorting cues could trigger this regulation.

7.3 Endocytosis

Endocytosis is a critical process for control of the protein and lipid composition of the membrane. It governs regulation and recycling of signalling receptors, transporters and channels (Dupre et al., 2004). The yeast endocytic pathway is reminiscent of the mammalian system, following the same stages of internalization and transport to the vacuole/lysosome via early and late endosomes. In yeast, studies of endocytosis have mainly focused on two groups of proteins, one being the G-protein coupled receptors, which are characterised by their seven trans-membrane spanning segments. The other class is represented by a number of transporters belonging to the multi-facilitator family. The first group, exemplified by Ste2p and Ste3p, is subjected to ligand-induced endocytosis upon binding of the mating pheromone. The latter class is tightly regulated in response to environmental changes (Rotin et al., 2000). Studies of Ste6p, which is responsible for mating pheromone secretion, provided the first link between ubiquitylation and endocytosis. Endocytic mutants revealed that Ste6p accumulated in a ubiquitylated form at the plasma membrane (Kolling and Hollenberg, 1994). Since then, ubiquitin has been identified as a key player in the endocytic pathway, promoting the entry of endocytic cargo into vesicles (Hicke and Dunn, 2003).

8. Ubiquitylation

8.1 Steps in ubiquitylation by E1, E2, E3 enzymes

Ubiquitin is an evolutionary conserved 76 amino acid protein found in all eukaryotes. In most cases, ubiquitin is linked to a lysine residue of the target protein by the sequential action of different enzymes. First, ubiquitin is activated in an ATP dependent manner by a ubiquitin activating enzyme termed E1. Second, the ubiquitin is transferred to a cysteine residue of the ubiquitin conjugating enzyme, E2. The final transfer of ubiquitin to the target protein is mediated by E3, a ubiquitin ligase. Most ubiquitin ligases sort into two major groups, constituted by the HECT domain and RING finger E3s (Dupre et al., 2004; Morvan et al., 2004). RING-finger E3 ligases function as scaffolds, promoting contact between the substrate and E2. HECT domain E3s, on the other hand, are catalytically active in passing the ubiquitin from E2 to the substrate (Belgareh-Touze et al., 2008).

A prominent role in the ubiquitylation of plasma membrane proteins is played by ubiquitin ligases belonging to the HECT-domain Nedd4/Rsp5p family. They are characterised by carrying an N-terminal, often Ca^{2+} -dependent, lipid/protein binding domain (C2), two to four WW domains and a catalytic HECT-domain in their C-terminus. In *S. cerevisiae*, the only Nedd4 homologue is Rsp5p, which is implicated in the ubiquitylation and internalization of most plasma membrane proteins (Dunn and Hicke, 2001; Dunn et al., 2004; Rotin et al., 2000). In addition, this essential enzyme is involved in the heat shock response, in the ubiquitylation of arrested RNA polymerase II and in directing intracellular traffic of membrane proteins between other compartments (Belgareh-Touze et al., 2008).

8.2 Modes of ubiquitylation

The formation of an isopeptide bond between the ubiquitin C-terminus and the aminogroup of a lysine in the target protein completes ubiquitylation. In turn, ubiquitin has seven lysine residues and can itself be ubiquitylated, thus enabling the formation of polyubiquitin chains. The length and configuration of the chain is significant to the differential fates of ubiquitylated proteins. At least eight types of ubiquitin chains have been identified. Linkage via lysine 48 (K48) forms chains that signal degradation by the proteasome, while non-proteolytic sorting mostly involves Lys63-linked (K63) ubiquitin chains (d'Azzo et al.,

2005; Komander et al., 2009). The addition of one ubiquitin moiety at a single lysine (monoubiquitylation) or multiple lysines (multiubiquitylation) is enough to trigger internalisation and targeting of ion channels or receptors to the endosomal pathway. Endocytosed proteins can either be recycled to the plasma membrane, or passed to the lysosome/vacuole for subsequent degradation (d'Azzo et al., 2005). Monoubiquitylation can also work as a signal in targeting newly synthesized proteins from Golgi to the plasma membrane or lysosome/vacuole (Bonifacino and Traub, 2003).

8.3 Deubiquitylating enzymes (DUBs)

Ubiquitylation being a reversible process is important for subsequent sorting steps, as well as a continuous availability of free ubiquitin. Enzymes responsible for the processing of ubiquitylated proteins are collectively termed deubiquitylating enzymes (DUBs) (Amerik and Hochstrasser, 2004; Wilkinson, 2009). The specificity of DUBs depends on their ability to recognize different types of polyubiquitin chains. However, their specificity is probably dependent on different scaffolds and substrate adaptors, as *in vitro* assays largely abolish substrate recognition (Wilkinson, 2009). In yeast, the DUB Doa4p (Ubp4) has been shown to play a crucial role in ubiquitin homeostasis by rescuing ubiquitin from proteolytic degradation along with the substrate (Swaminathan et al., 1999). In a *doa4*Δ mutant, Ste6p was shown to be excluded from the MVB pathway and accumulate in the vacuolar delimiting membrane, which was suggested to result from insufficient ubiquitylation. This, in turn, impaired recycling of Ste6p to the plasma membrane (Losko et al., 2001). Later, however, Doa4p was shown to play a direct role in MVB sorting that was not connected to ubiquitin levels. Deubiquitylation of the general amino acid permease Gap1p and carboxypeptidase S (Cps1) by Doa4p was found to be a prerequisite for sorting into MVBs (Nikko and Andre, 2007).

8.4 E3 adaptors in yeast

Rsp5p binds short PY motifs via its WW domains. With few exceptions, though, Rsp5p substrates lack PY motifs, which led to the prediction that accessory proteins harbouring a PY motif might act as adaptors to Rsp5p. Indeed, there is now a growing number of established Rsp5p adaptors (Belgareh-Touze et al., 2008; Shearwin-Whyatt et al., 2006).

Bsd2p was first identified by its role in sorting the manganese transporter Smf1p to the vacuole (Liu and Culotta, 1999). Later, Bsd2p was found to mediate alternative ubiquitylation of carboxypeptidase S (Cps1) and polyphosphatase (Phm5), which polar trans-membrane domains are recognised by the ubiquitin ligase Tul1p. Via its PY motif, Bsd2p was demonstrated to act as an Rsp5p specific adaptor for Cps1p and Phm5p (Hettema et al., 2004). The previously established role in Smf1p regulation was further shown to require two additional PY proteins, Tre1p and Tre2p (Stimpson et al., 2006; Sullivan et al., 2007). A similar role was described for Bul1p and Bul2p in Gap1p regulation (Soetens et al., 2001) and in sorting of Pma1-7p to the endosomal/vacuolar pathway (Pizzirusso and Chang, 2004). Overexpression of Bul1p/2p caused sorting of Gap1p to the vacuole. Conversely, deletion of both genes promoted targeting of Gap1p to the cell surface (Helliwell et al., 2001). In addition, a protein encoded by *LST4* has been implicated in the intracellular trafficking of Gap1p, either promoting retrieval of the permease back to Golgi or possibly blocking vacuolar sorting (Helliwell et al., 2001; Roberg et al., 1997). Recently, two redundant proteins, Ear1p and Ssh4p, were found to mediate vacuolar targeting of multiple cargoes from both Golgi and the plasma membrane. Ear1p carries a PY motif and is itself ubiquitylated by Rsp5p and sorted to the vacuole (Leon et al., 2008). This is in agreement with the finding that ubiquitylation of arrestins, mediating ubiquitin-dependent down-regulation of GPCRs, is a prerequisite for internalization of the receptors (Shenoy and Lefkowitz, 2003). We have similarly established that the adaptor protein Art5p as being a substrate for Rsp5p. However, Art5p is a cytosolic protein (**paper IV**) that lacks trans-membrane regions and is not sorted to the vacuole for disposal, but possibly degraded in the cytosol. Interestingly, Ear1p overexpression resulted in accumulation of Rsp5p at endosomes, possibly leading to impairment of Rsp5p function. This suggests a need for tight regulation of Rsp5p adaptors not to cause unnecessary sequestration of Rsp5p, preventing it from fulfilling its many other functions (Leon and Haguenauer-Tsapis, 2009). Consistent with these observations, we found that both deletion and overexpression of *ART5* promoted the same effect; restoration of salt tolerance of the *sro7*Δ mutant. We suggest that the suppression of the *sro7*Δ phenotype following *ART5* overexpression is indeed a result from perturbed steady-state levels of the encoded protein (**paper IV**).

8.4.1 The ART family of E3 adaptors

The *ART* family of proteins (for arrestin-related trafficking adaptors) were classified by their resemblance to mammalian arrestins (see section 3.3) and the presence of several PY motifs primarily in the N-terminal portion. In contrast to the Golgi-based Bsd2p, Bul1p/2p and Ear1p/Ssh4p, the ARTs in agreement with mammalian arrestins, were implicated in endocytosis at the plasma membrane. They could also be attributed to confer cargo specificity in endocytic sorting. Deletion of *ART1* (*CVS7*), for instance, was found to affect internalization of the arginine transporter Can1p and the methionine transporter Mup1p, with the endocytosis of several other transporters being unaffected (Lin et al., 2008). Similarly, Art2p (Ecm21p) or Art8p (Csr2p) were shown to mediate internalization of the transporter Smf1p at the plasma membrane, where Bsd2p has no established function (Nikko et al., 2008). The action of Art1p and Art2p are dependent on their PY elements which recognize Rsp5p WW-domains. Consequently, all but two Art proteins (Art5p and Art9p) were found to co-purify with Rsp5p (Lin et al., 2008). However, Art5p has previously been found interact with Rsp5p (Gupta et al., 2007). Localisation studies show Art1p to localize to the Golgi under non-stress conditions, while being enriched at the plasma membrane upon stress or shifting nutrient availability. Mutations in the two PY motifs caused Art1p to be diffusely localised to the cytosol. Art5p was observed in the cytosol, nucleus and peripheral punctae under non-stress conditions (Lin et al., 2008). This localisation of Art5p was confirmed in our studies, where we also could see a cytoplasmic dispersal upon inactivation of Rsp5p, or proline- to alanine substitution of the canonical PY motif in Art5p (**paper IV**). Interestingly, our studies demonstrate the involvement of Art5p in ubiquitylation of the sodium transporter Ena1p at Golgi or endosomes. It is yet to be established if Art5p shares the additional role in endocytosis that is proposed for the Art protein family.

8.5 Regulation by ubiquitylation

Ubiquitylation has been established as a key factor in the turn-over and regulation of a growing number of membrane proteins in yeast, examples being the ferrichrome transporter Arn1p (Kim et al., 2007), low affinity phosphate transporters Ph87p and

Pho91p (Estrella et al., 2008), the siderophore transporter Sit1p (Erpapazoglou et al., 2008), as well as the tryptophan permease Tat2p (Beck et al., 1999).

The thorough studies of the general amino acid permease Gap1p provide the framework for the mechanistics behind ubiquitin-dependent intracellular traffic. A shift from a poor to a rich nitrogen medium triggers Rsp5p dependent ubiquitylation and internalization of Gap1p at the cell surface (Springael and Andre, 1998; Springael et al., 1999). Gap1p is mono-ubiquitylated at two lysine residues (K9, K16) when grown in the poor nitrogen source proline, while a shift to rich NH_4^+ medium results in the formation of short K63 linked polyubiquitin chains, mediating its rapid and efficient internalization. Accordingly, mutational change of both lysines stabilises Gap1p at the cell surface (Soetens et al., 2001; Springael et al., 1999). Stimulated endocytosis by short K63 linked ubiquitin chains was also shown for the uracil permease Fur4p (Galan and Haguenauer-Tsapis, 1997). The shift to a rich nitrogen source has also been proposed to result in Rsp5p/Bul1p/2p dependent ubiquitylation and vacuolar targeting of newly synthesized Gap1p via the endosomal pathway (Helliwell et al., 2001; Soetens et al., 2001). Again, the same direct Rsp5p-dependent endosomal/vacuolar sorting is seen for Fur4p at high levels of intracellular uracil (Blondel et al., 2004). It has recently been shown, however, that neither K63-linked polyubiquitin chains, nor the Gga ubiquitin binding adaptors (see section 2.4) are a prerequisite for Golgi to endosome traffic of Gap1p, albeit crucial for its sorting into the MVB pathway (see section 2.4). Likewise, the K63 linked polyubiquitylation at the plasma membrane is proposed only to influence MVB sorting, whereas a single ubiquitin is sufficient for internalization of Gap1p (Lauwers et al., 2009).

Depletion of free ubiquitin in a *doa4*Δ mutant prevents the subsequent sorting of Fur4p into MVBs (see section 2.4), whereby it accumulates in the delimiting membrane of the vacuole (Blondel et al., 2004). Gap1p has been proposed to cycle between the Golgi and endosomes, thus constituting a pool of permease that can be redirected to the plasma membrane if external nitrogen becomes scarce (Helliwell et al., 2001). In the endosomal pathway, the sorting into MVBs and the subsequent vacuolar degradation depends on K63-linked Ub chains (Lauwers et al., 2009). Consequently, a block in MVB sorting as occurs in so called *class E* mutants, results in efficient retrieval of Gap1p to the cell surface (Risinger AL, 2008; Rubio-Texeira and Kaiser, 2006).

9. Concluding remarks

Targeted delivery of proteins to the cell surface is of outermost importance for cell polarization and proper cell physiology. Loss of cell polarity in metazoans can lead to disrupted morphogenesis, uncontrolled cell division and even tumour-like growth (reviewed in (Klezovitch et al., 2004; Wirtz-Peitz and Knoblich, 2006)). The Lgl-family of proteins has been implicated in maintenance of cell polarity and control of cell division from fly to human. Despite little sequence homology, Lgl-proteins seem to have a basic cellular function that is well conserved over species borders. Several important insights into the molecular role of Lgl proteins come from studies of the *S. cerevisiae* homologues Sro7p and Sro77p. Ectopic expression of *Drosophila*, human or rat Lgl-homologues in yeast have been shown to complement loss of Sro7/77p, making functional studies in yeast appealing and significant. Recently, we also identified two plant Lgl homologues, the *A. thaliana* AtLGL1 and AtLGL2 (**paper III**). The plant mutant phenotype and the observed complementation of the yeast *sro7*Δ*sro77*Δ defect by one of the plant homologues (AtLGL1), further stresses the view that Lgl proteins represent an ancestral and therefore fundamental regulatory function in targeted protein secretion. The aim of this thesis has been to further contribute to the understanding of the conserved Lgl function in protein traffic by studies in the experimentally amenable *S. cerevisiae* system.

In yeast, Sro7p has an established role in late secretion being a binding partner of the yeast t-SNARE Sec9p, the exocyst subunit Exo84, the Rab GTPase Sec4p and the type-V myosin, Myo2p (reviewed in (Brennwald and Rossi, 2007a; Novick et al., 2006a)). Consistent with a role of Sro7p as a regulatory link between vesicle tethering and SNARE mediated fusion, deletion of *SRO7* aggravates defects caused by mutations in the exocyst components (**paper I**). In paper I we also made observations indicating that Sro7p has a second role, involved in sorting of protein(s) into post-Golgi vesicles. In *sro7*Δ mutants exposed to salt stress, *ENA1* expression is induced in a normal way, although the synthesized protein is not delivered to the plasma membrane but mis-targeted to the endosomal/vacuolar pathway. We also noted that *sro7*Δ mutants, in contrast to other late acting sec mutants, do not sort Ena1p into post-Golgi vesicles. Although this effect was less distinct under other experimental conditions (**paper III**), the results indicate the sorting defect is not due to secondary consequences of a

late block in secretion. What role Sro7p may play in protein sorting at Golgi/early endosomes is entirely open to speculation. To reach the plasma membrane Ena1p probably interacts with receptor(s) that facilitate selective inclusion into the forming secretory vesicles. Cargo receptors recognize in turn distinct coat proteins that aid in cargo selection and deform the membrane into buds, eventually leading to the release of transiently coated vesicles (Bonifacino and Glick, 2004). The capacity of Sro7p to serve as a scaffold-protein makes it suitable for participating in protein complex formation. However, little is known about formation of post-Golgi secretory vesicles and there are no reported protein-protein interactions to support a role of Sro7p in this process. Nevertheless, a discriminatory role for Sro7p in protein secretion was further corroborated by our proteomics analysis of post-Golgi vesicles **(paper II).** Of the 107 *bona fide* vesicle constituents identified in *sec6-4* control cells, some 20 were significantly depleted from *sro7*Δ derived vesicles, and therefore possible candidates for mis-sorting. Further analysis of one of these, the glycerol/proton symporter Stl1p showed a decreased plasma membrane- and increased vacuolar localisation in *sro7*Δ mutants, supporting a defective sorting into post-Golgi vesicles. Identifying the "complete (or nearly complete) proteome" of post-Golgi secretory vesicles in an important model organism for membrane trafficking should be of significant general interest. The use of this technique to examine the effect of the polarity protein Sro7p on sorting into the vesicle compartment, illustrates that the analysis can be used to study effects of particular factors on the composition of the proteome.

Upon exclusion from its normal pathway, Ena1p is marked with ubiquitin by the Rsp5p ubiquitin ligase (**paper IV**) and sent for destruction via the MVB pathway. When ubiquitylation is prevented in *rsp5-1* mutants, Ena1p is rerouted to the cell surface, showing that ubiquitylation is the determining sorting signal. We have also demonstrated that ubiquitylation is dependent on a putative adaptor protein, Art5p. Ena1p lacks the PY element(s) that serves as substrate recognition motifs for Rsp5p. Art5p, on the other hand, possesses PY elements and is shown to interact physically with Rsp5p. Although we have not been able to demonstrate that Art5p interacts with Ena1p, Art5p is strictly required for Ena1p ubiquitylation and can not be substituted by the other 8 members of the ART family of proteins, or other known Rsp5p adaptor proteins. Art5p is also shown to interact with

Sft2p a Golgi localized protein (Conchon et al., 1999) and may be part of a Golgi localized quality-control system that recruits accumulated Ena1p to Rsp5p for ubiquitylation and disposal via the MVB pathway. Taken together, our observations support a model for Ena1p trafficking that in response to external sodium levels depends on Sro7p, Rsp5p and Art5p. The different decisive steps correspond well with the sites for regulation of the trafficking of the well-studied general amino acid permease Gap1p (section 8.5), which is used as a comparative model in Fig. 3.

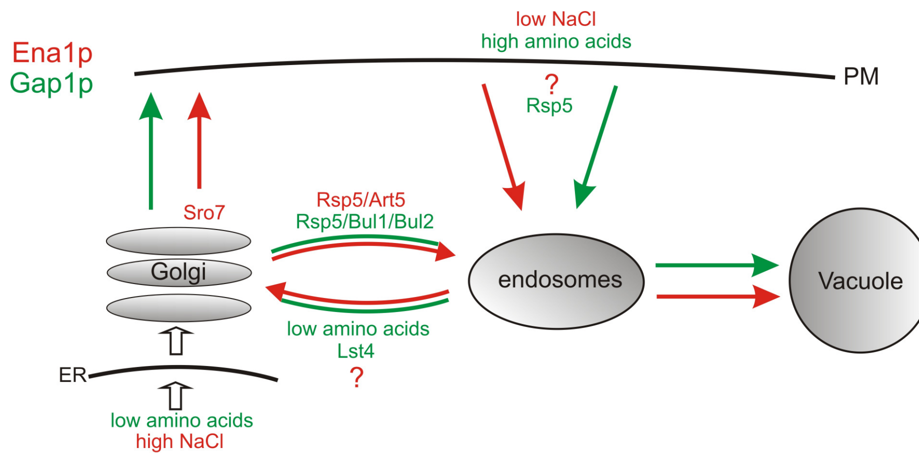

Fig 3. Levels of regulation in the intracellular trafficking of the sodium ATPase Ena1p and the general amino acid permease Gap1p, depicting proteins that are established or suggested to be involved at each step. Text and arrows referring to Gap1p are green, while those concerning Ena1p are marked red. Adapted from Helliwell et al. 2001 (Helliwell et al., 2001).

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