Functional role of the Mso1p-Sec1p complex in membrane fusion regulation

Marion Weber-Boyvat

Institute of Biotechnology and
Department of Biological and Environmental Sciences
Division of Genetics
Faculty of Bioscience and
Viikki Graduate School in Biosciences
University of Helsinki

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Supervised by:
Docent Dr. Jussi Jäntti
Institute of Biotechnology
University of Helsinki, Finland

Reviewed by:
Docent Dr. Peter Richard
VTT Biotechnology and Food Research,
VTT, Finland

and

Docent Dr. Vesa Olkkonen
Minerva Foundation Institute for Medical Research
Finland

Opponent:
Professor Anne Spang
Growth and Development Biozentrum
University of Basel, Switzerland

Cover figure (left to right): Localisation of the Mso1p-Sec4p, Mso1p-Sec1p and Sec1p-Sso1p Bimolecular Fluorescence Complementation interaction sites in *Saccharomyces cerevisiae.*

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their roman numerals.

I. Knop M, Miller KJ, Mazza M, Feng D, **Weber M,** Keränen S, Jäntti J. (2005), Molecular interactions position Mso1p, a novel PTB domain homologue, in the interface of the Exocyst complex and the exocytic SNARE machinery in yeast. Mol. Biol. Cell. 16:4543-56.

II. **Weber M**, Chernov K, Turakainen H, Wohlfahrt G, Pajunen M, Savilahti H and Jäntti J. (2010), Mso1p regulates membrane fusion through interactions with the putative N-peptide-binding area in Sec1p domain 1. Mol. Biol. Cell. 21(8):1362-74.

III. **Weber-Boyvat M**, Aro N, Chernov KG, Nyman T, Jäntti J. (2010), Sec1p and Mso1p C-terminal tails co-operate with the SNAREs and Sec4p in polarized exocytosis. Accepted at Mol. Biol. Cell.

IV. **Weber-Boyvat M**[*], Zhao H[*], Aro N, Peränen J, Lappalainen P, Jäntti J. A novel layer of regulation in SNARE mediated exocytic membrane fusion revealed by Mso1p membrane interactions. Manuscript, [*] Authors contributed equally.

ABBREVATIONS

BiFC	Bimolecular Fluorescence Complementation
Ca^{2+}	calcium ion
C-terminus	the end of a protein with a free carboxyl group
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
GTPase	GTP phosphatase
kb	kilo basepair
kDa	kilo Dalton
N-terminus	the end of the protein with a free amino group
MBP	maltose binding protein
PCR	polymerase chain reaction
PI(3)P	Phosphatidylinositol 3-phosphate
$PI(3,5)P_2$	Phosphatidylinositol (3,5)-bisphosphate
PI(4)P	Phosphatidylinositol 4-phosphate
$PI(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
PIP	Phosphatidylinositol phosphate
PIP_2	Phosphatidylinositol bisphosphate
PTB domain	phosphotyrosine binding domain
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SM	Sec1/Munc18
TGN	trans Golgi network

Amino acid abbreviations

single letter code	three letter code	amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

ABSTRACT

Sec1/Munc18 (SM) protein family members are evolutionary conserved proteins. They perform an essential, albeit poorly understood function in SNARE complex formation in membrane fusion. In addition to the SNARE complex components, only a few SM protein binding proteins are known. Typically, their binding modes to SM proteins and their contribution to the membrane fusion regulation is poorly characterised. We identified Mso1p as a novel Sec1p interacting partner. It was shown that Mso1p and Sec1p interact at sites of polarised secretion and that this localisation is dependent on the Rab GTPase Sec4p and its GEF Sec2p. Using targeted mutagenesis and N- and C-terminal deletants, it was discovered that the interaction between an N-terminal peptide of Mso1p and the putative Syntaxin N-peptide binding area in Sec1p domain 1 is important for membrane fusion regulation. The yeast Syntaxin homologues Sso1p and Sso2p lack the N-terminal peptide. Our results show that in addition to binding to the putative N-peptide binding area in Sec1p, Mso1p can interact with Sso1p and Sso2p. This result suggests that Mso1p can mimic the N-peptide binding to facilitate membrane fusion. In addition to Mso1p, a novel role in membrane fusion regulation was revealed for the Sec1p C-terminal tail, which is missing in its mammalian homologues. Deletion of the Sec1p-tail results in temperature sensitive growth and reduced sporulation. Using *in vivo* and *in vitro* experiments, it was shown that the Sec1p-tail mediates SNARE complex binding and assembly. These results propose a regulatory role for the Sec1p-tail in SNARE complex formation.

Furthermore, two novel interaction partners for Mso1p, the Rab GTPase Sec4p and plasma membrane phospholipids, were identified. The Sec4p link was identified using Bimolecular Fluorescence Complementation assays with Mso1p and the non-SNARE binding Sec1p(1-657). The assay revealed that Mso1p can target Sec1p(1-657) to sites of secretion. This effect is mediated via the Mso1p C-terminus, which previously has been genetically linked to Sec4p. These results and *in vitro* binding experiments suggest that Mso1p acts in cooperation with the GTP-bound form of Sec4p on vesicle-like structures prior to membrane fusion. Mso1p shares homology with the PIP_2 binding domain of the mammalian Munc18 binding Mint proteins. It was shown both *in vivo* and *in vitro* that Mso1p is a phospholipid inserting protein and that this insertion is mediated by the conserved Mso1p amino terminus. *In vivo,* the Mso1p phospholipid binding is needed for sporulation and Mso1p-Sec1p localisation at the sites of secretion at the plasma membrane. The results reveal a novel layer of membrane fusion regulation in exocytosis and propose a coordinating role for Mso1p in connection with membrane lipids, Sec1p, Sec4p and SNARE complexes in this process.

REVIEW OF THE LITERATURE

1. The secretory pathway

Eukaryotic cells contain intracellular compartments that display specific lipid and protein compositions and carry out specialised functions. To maintain this intracellular organization eukaryotic cells require molecular mechanisms that ensure correct targeting and delivery of proteins to their functional location. These mechanisms are essential e.g. for neurotransmission and cell polarity generation and maintenance. Intracellular compartments that mediate transport of lipids and proteins from their site of synthesis, the endoplasmic reticulum membrane, to the cell surface plasma membrane, constitute the secretory pathway (Palade, 1975; Novick *et al.*, 1981; Bonifacino and Glick, 2004). The secretory pathway is a highly dynamic membrane system that involves a vast array of regulatory molecules in order to maintain a balance between protein and membrane biosynthesis, their transport and constant recycling at the plasma membrane. It has been estimated that about 30% of the synthesised proteins are targeted via this pathway. Newly synthesised proteins enter the secretory pathway via the endoplasmic reticulum. From there they are subsequently transported along actin cables or microtubules to the Golgi apparatus, where they are sorted for further transport to the vacuole or plasma membrane (Harter and Wieland, 1996, Figure 1).

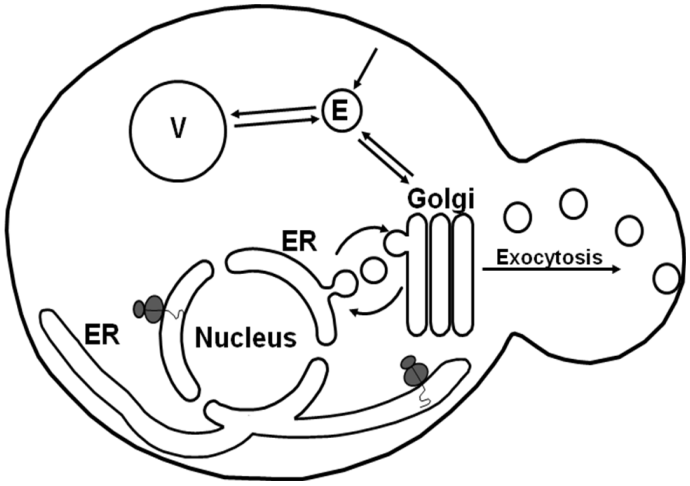

Figure 1. Schematic presentation of the yeast secretory pathway and the pathway to the endocytic compartment. After synthesis, proteins are translocated to the ER, followed by further transport via vesicles to the Golgi, endosome (E), vacuole (V) and plasma membrane. From the plasma membrane proteins and lipids can be recycled through endocytosis.

Different subcompartments within this pathway communicate with each other by membrane bound transport vesicles. Homologous proteins from yeasts to mammalian cells regulate intracellular membrane fusion events through well conserved functions (Jahn *et al.*, 2003; Hsu *et al.*, 2004). Transport between different compartments is maintained tightly in phase with the cell division cycle program and possesses a capacity to rapidly respond to intra- and extracellular signals. Recycling of proteins and lipids for later reuse from the plasma membrane is mediated by endocytosis (Mukherjee *et al.*, 1997). Thereby exocytosis and endocytosis create a circular network allowing constant re-usage of regulatory proteins and lipids.

The secretory pathway has been extensively studied due to its implications in medicine and biotechnology. Several diseases in cell growth and neurotransmission have been linked to defects in secretion (Olkkonen and Ikonen, 2000). For example, defects in protein sorting can cause mucolipidosis II, which is characterised by an accumulation of undegraded proteins due to a missorting of lysosomal proteins. Defects in the vesicle recognition and docking machinery have been shown to be the cause for choroideremia and X-linked nonspecific retardation (D'Adamo et al., 1998). Furthermore the secretory pathway, especially exocytosis, is essential for neurotransmitter release. Proteins involved in this process have been linked to the development of Alzheimer's disease (Borg *et al.*, 1996). In the future detailed knowledge of these proteins could provide possible targets for the treatment of this neurodegenerative disease.

Additionally, the secretory pathway has been studied for applications in protein and enzyme production. *Saccharomyces cerevisiae* has been a potent host, as a variety of stable vectors, efficient promoters and mutant strains can be employed to maximize the production and secretion of a desired protein (Gellissen et al., 1992). It is currently used for example for the production of therapeutic human insulin and β-endorphin.

2. Exocytosis: the last step of secretion

Exocytosis is the final step of secretion (Figure 1 and 2). In yeast *Saccharomyces cerevisiae* exocytosis is initiated by the contact of transport vesicles with the plasma membrane associated protein complex, the Exocyst. Vesicle docking with the plasma membrane leads to a cascade where vesicle and plasma membrane anchored v- and t-

SNARE proteins pair with each other and fold together into a highly alpha-helical protein complex (the SNARE complex). Formation of these SNARE complexes is thought to provide sufficient force to bring transport vesicle and plasma membrane phospholipid bilayers close enough for membrane fusion (Jahn *et al.*, 2003). Several accessory molecules, implicated in SNARE complex formation, have been discovered. Well described SNARE complex regulators are the Sec1/Munc18 family (SM) proteins. Furthermore, Synaptotagmin, Complexin, the V_o component of vacuolar-ATPase etc. have been shown to be involved in SNARE complex formation (Becherer and Rettig, 2006; Wada *et al.*, 2008).

There are two different modes of exocytosis: the regulated and constitutive mode.

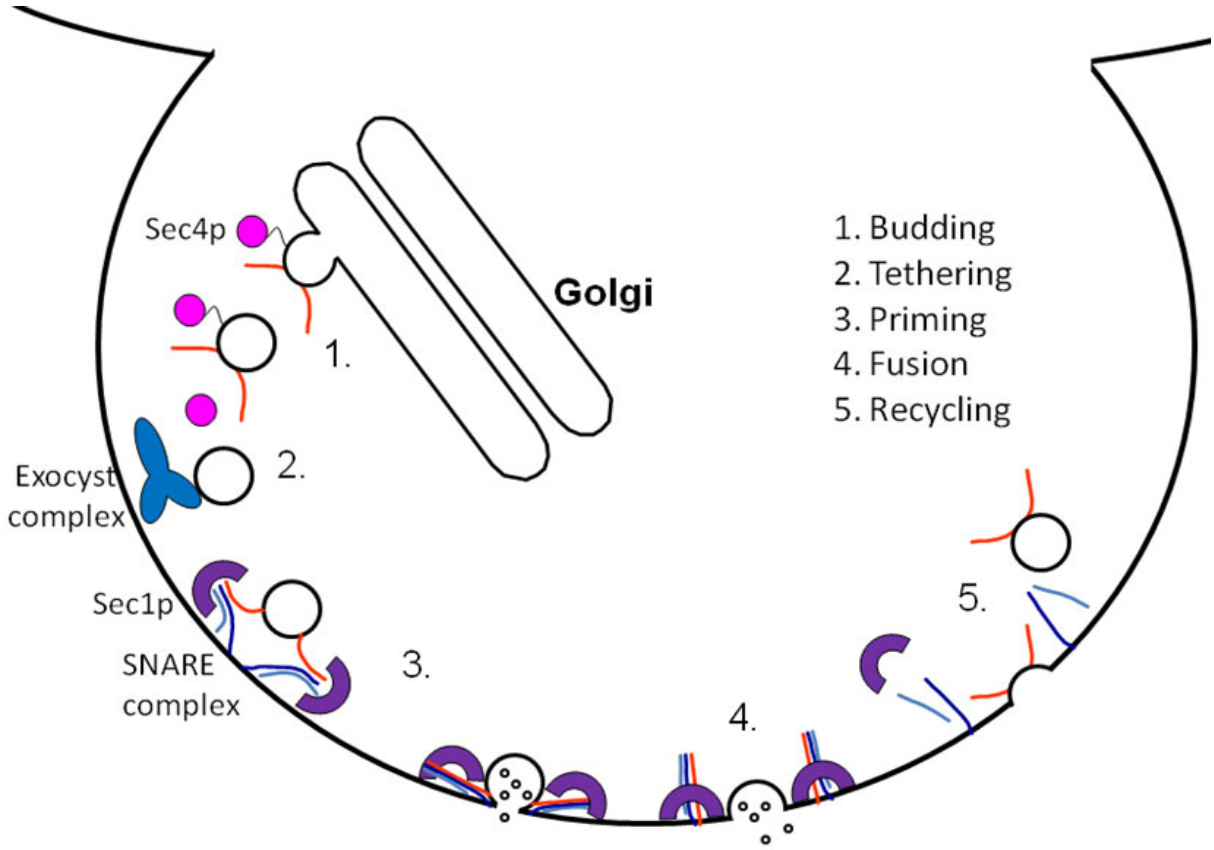

Figure 2. Schematic presentation of the yeast Exocytosis. Exocytosis, the fusion of vesicles at the plasma membrane, is subdivided into: 1. budding and transport of the vesicle from the Golgi apparatus mediated by Sec4p (pink), 2. tethering of the vesicle at the plasma membrane mediated by the Exocyst complex (blue), 3. priming of the SNARE complex (Snc1/2p in red, Sso1/2p in dark blue, Sec9p in light blue) mediated by Sec1p (purple), 4. fusion of the vesicle with the plasma membrane and 5. recycling of the vesicle.

Constitutive exocytosis describes the constant flow of vesicles from the trans-Golgi network (TGN) to the plasma membrane (Griffiths and Simons, 1986; Burgess and Kelly, 1987). On the other hand, regulated exocytosis is the triggered fusion of vesicles with the plasma membrane upon a stimulus. A well studied example of regulated exocytosis is neurotransmission, which is triggered by Ca^{2+} in neuronal cells. Several proteins working as Ca^{2+} sensors in neurotransmission have been identified (Decamilli and Jahn, 1990; Burgoyne and Morgan, 1993; Martens 2010). In yeast, a form of regulated exocytosis occurs during spore formation, where four daughter cells (spores) are formed within the mother cell. In this process, after meiosis II the four daughter cell nuclear lobes are surrounded by a *de novo* formed membrane, the prospore membrane, which is initiated at the spindle pole bodies (yeast homologues of the centrosome). This membrane elongates around the nuclei until closure can occur at the completion of meiosis (Moreno-Borchart and Knop, 2003). Even though prospore membrane formation requires essentially the same molecular machinery as constitutive secretion in yeast, it appears to be more tightly regulated as its formation must take place in phase with the meiotic divisions. Due to a lack of identified prospore membrane formation regulating proteins, its precise temporal and spatial regulation is unknown.

3. Vesicle targeting and tethering

3.1. The Rab GTPase Sec4p

Small GTP-binding proteins of the Rab-family are central regulators of cell polarity (Zerial and McBride, 2001). They possess the ability to switch between an active GTP- and inactive GDP- bound form. The cycle between these two forms is regulated by the guanine nucleotide exchange factor (GEF) and the GTPase activating protein (GAP). Furthermore, the GDP dissociation inhibitors (GDI) are needed to extract the GDP Rab from the membrane to allow them to recycle to the cytosol (Armstrong, 2000).

In yeast exocytosis, the GTPases Sec4p, Rho1p, Rho3p and Cdc42p have been implicated in vesicle targeting, tethering and membrane fusion (Guo *et al.*, 2001; Brennwald and Rossi, 2007; Wu *et al.*, 2008). The Rab GTPase Sec4p acts as an upstream regulator of SNARE mediated membrane fusion. It is needed for SNARE complex formation and fusion of vesicles with the plasma membrane. The guanine nucleotide-binding state of Sec4p is

regulated by several proteins including the guanine nucleotide exchange factor (GEF) Sec2p (Walch-Solimena *et al.*, 1997), the GTPase activating proteins (GAP) Gyl1p (Tarassov *et al.*, 2008), Gyp1p (Du *et al.*, 1998), Mdr1p (Albert and Gallwitz, 1999) and Msb4p (Albert and Gallwitz, 2000), the GDP dissociation inhibitor (GDI) Gdi1p (Collins *et al.*, 1997), and the Guanine nucleotide dissociation stimulator Dss4p (Collins *et al.*, 1997). It has been proposed that GTP-Sec4p is bound to the secretory vesicle and that GTP hydrolysis is required for its downstream signal transmission (Walworth *et al.*, 1989; Walworth *et al.*, 1992). GTP-Sec4p has been shown to associate with Sec15p on secretory vesicles. This interaction has been proposed to lead to the cascade of Exocyst complex (see 3.2.) formation at the site of secretion marked by Sec3p (Guo *et al.*, 1999). Another effector of Sec4p is the plasma membrane bound t-SNARE Sec9p, indicating an additional regulatory mechanism at the level of SNARE complex formation (Brennwald *et al.*, 1994). Yet, the mechanism how Sec4p regulates SNARE complex formation is unknown.

3.2. The Exocyst

The Exocyst complex is indispensable for polarised secretion and cell polarity generation from yeast to mammals. It is composed of eight subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (TerBush *et al.*, 1996; Guo *et al.*, 2000; Lipschutz and Mostov, 2002). Structural data of several of the subcomponents show that the subcomponents have a highly helical composition, aligning to form rod-like structures (Croteau *et al.*, 2009). Based on the identification of the amino acids important for interactions between the Exocyst components, a model has been suggested where the rod-like Exocyst subunits align side by side to form the Exocyst complex (Munson and Novick, 2006). This complex has been proposed to act as a molecular device that mediates the initial recognition and docking of the transport vesicle at the plasma membrane (Guo and Novick, 2004). In neuronal cells the Exocyst complex is not required for neurotransmitter release of the docked vesicles, yet the Exocyst has been shown to be essential for neurite outgrowth and generation of synapses (Murthy *et al.*, 2003).

Table 1. Summary of the molecular interactions of yeast Exocyst subunits with GTPases and PIPs. The structure or partial structure of six Exocyst subunits is known. All the yeast Exocyst subunits have one or several homologues in mammals.

Exocyst subunit	PIP binding	GTPase binding	known structure	mammalian homologue (isoforms)
Sec3	+	+	+	EXO C1 (1, 2)
Sec5			+	EXO C2
Sec6			+	EXO C3 (1, 2)
Sec8				EXO C4
Sec10				EXO C5 (1-3)
Sec15		+	+	EXO C6 (1-3)
Exo70	+	+	+	EXO C7 (1-6)
Exo85			+	EXO C8

The Exocyst subunit Sec15p has been shown to interact with the GTP-bound form of Sec4p on secretory vesicles (Guo *et al.*, 1999, Table 1). This interaction with Sec4p and the interactions with other upstream regulators, i.e. the actin cytoskeleton, the GTPase Cdc42p and the polarity establishment machinery component Bem1p, determine the localisation of Sec15p and subsequently the localisation and assembly of other Exocyst subunits (Zajac *et al.*, 2005; France *et al.*, 2006).

While one set of Exocyst subunits (Sec5p, Sec6p, Sec8p, Sec10p, Sec15p and Exo84p) seems to reside on the vesicle along with Sec4p (Guo *et al.*, 1999), another set of Exocyst subunits (Exo70p and Sec3p) shows a more stable localisation at the plasma membrane. Both Exo70p and Sec3p have been shown to localise there independently on the actin cytoskeleton (Boyd *et al.*, 2004). This result proposed a model where Exo70p and Sec3p function as landmarks for secretion (Wiederkehr *et al.*, 2003; Boyd *et al.*, 2004).

In support of this localisation, Exo70p and Sec3p have been shown to interact with plasma membrane PI(4,5)P_2 and GTPases (Table 1). The simultaneous interaction of Sec3p with PI(4,5)P_2 and the GTPase

Rho1p is needed for the localisation of Sec3p upon actin cytoskeleton disruption (Baek *et al.*, 2010; Yamashita *et al.*, 2010). At the same time, the interaction with the GTPase Cdc42p is needed for the initial targeting of Sec3p (Zhang *et al.*, 2001).

Exo70p interacts with the GTPase Rho3p and PI(4,5)P_2. Deletion of the Rho3p interaction site in Exo70p results in loss of localisation after actin de-polymerization (Hutagalung *et al.*, 2009). At the same time, abolishment of the PI(4,5)P_2 binding, in combination with mutations in Sec3p eliminating the PI(4,5)P_2 and Rho1p binding, causes a loss of localisation of the Exocyst (He *et al.*, 2007; Baek *et al.*, 2010). Taken together, it has been suggested that Sec3p and Exo70p work in concert in Exocyst assembly at the plasma membrane (He *et al.*, 2007).

4. Vesicle priming and fusion

4.1. The SM proteins

The Sec1/Munc18 (SM) protein family members are evolutionary conserved proteins that perform an essential function in SNARE complex regulation in membrane fusion (Gallwitz and Jahn, 2003; Kauppi *et al.*, 2004; Toonen and Verhage, 2007).

Yeast possesses four SM-family proteins (Table 2). Sly1p is needed for vesicle fusion between the endoplasmic reticulum and the Golgi complex (Ossig *et al.*, 1991; Li *et al.*, 2005), Vps33p mediates transport to the endosome and vacuole (Subramanian *et al.*, 2004), Vps45p mediates transport from the Golgi complex to the vacuole (Cowles *et al.*, 1994; Piper *et al.*, 1994), and Sec1p mediates vesicle fusion at the plasma membrane (Carr *et al.*, 1999). The mammalian homologue of Sec1p is Munc18.

4.1.1. The Structure of SM proteins

The structures of yeast Sly1p (Bracher and Weissenhorn, 2001; Bracher and Weissenhorn, 2002), rat Munc18-1 (Burkhardt *et al.*, 2008) and Munc18c (Hu *et al.*, 2007) have been solved. The three homologues show a very similar arch-like shaped structure composed of three domains (Figure 3). The structures reveal that the multi-domain protein folds together from the amino- and carboxy-terminus to form domain 2. It has been proposed that SM proteins can clasp the SNARE complex and thereby promote zipping up of the SNARE complex (Sudhof and Rothman, 2009).

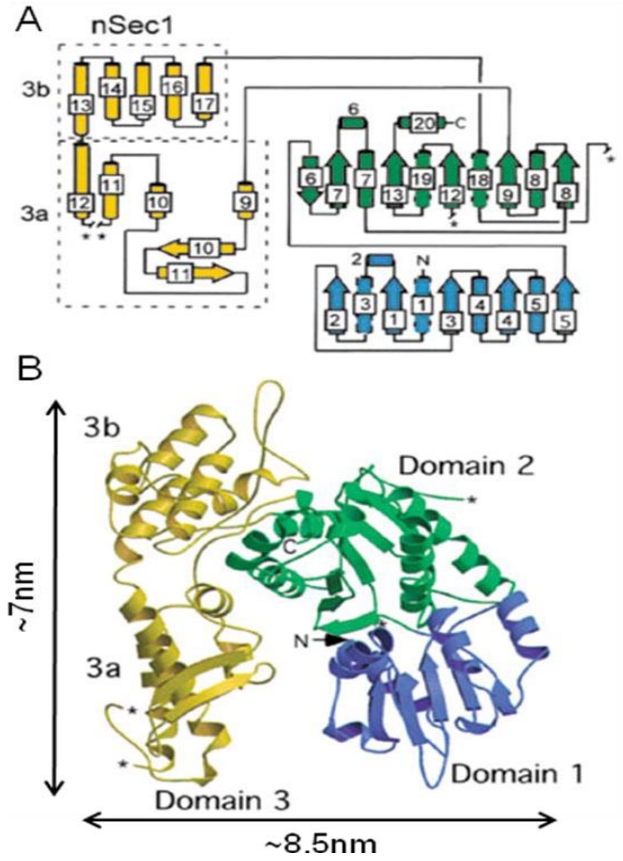

Figure 3. Crystal structure of rat Munc18-1 (modified from Misura *et al.*, 2000). A. Topology diagram of rMunc18-1. α-helices are shown as cylinders and β-strands as arrows. B. Ribbon presentation of rMunc18-1. Domain 1 is shown in blue, domain 2 in green and domain 3 in yellow.

4.1.2. SM protein binding modes to SNARE proteins

SM proteins can employ three apparently different binding modes with their interaction partners of the SNARE family proteins (Toonen and Verhage, 2007; Carr and Rizo, 2010).

First, several SM proteins have been shown to interact with their cognate SNARE complexes through binding to an N-terminal peptide in the Syntaxin homologues. (Dulubova *et al.*, 2003, Figure 4 and Table 2). The N-peptide binding mode has been first described for the yeast SM protein Sly1p. Sly1p has been shown to bind to the very N-terminal peptide of Sed5p via its SNARE N-peptide binding site in domain 1. It has been proposed that this binding mode allows Sed5p to be in the open conformation available for SNARE complex formation (Bracher and Weissenhorn, 2002; Peng and Gallwitz, 2002; Yamaguchi *et al.*, 2002; Arac *et al.*, 2005). Later, the N-peptide binding mode has been shown for the interaction between the yeast SM protein Vps45p and Tlg2p (Dulubova *et al.*, 2002; Carpp *et al.*, 2006), and for the

Table 2. The N-peptide binding mode between SM proteins and their cognate Syntaxin homologues in different pathways in yeast and mammalian cells.

S. cerevisiae				*M. musculus*			
SM protein	Syntaxin	N-peptide	Pathway	SM protein	Syntaxin	N-peptide	Pathway
Sly1	Sed5 Ufe1	Yes	ER-Golgi	mSly1	Syx5 Syx18	Yes	ER-Golgi
Vps45	Tlg2	Yes	TGN-vacuole	mVps45	Syx16	Yes	TGN-endosomes
Vps33	Vam3	No	vacuole	Vps33a Vps33b	? ?	No	endosomes
Sec1	Sso1/2	No	exocytosis	Munc18-1 Munc18-2 Munc18-c	Syx1,2,3 Syx1,2,3 Syx2,4	Yes Yes Yes	Reg. exocytosis Con. exocytosis Glut4 exocytosis

mammalian SM protein Munc18c and Syntaxin4 (Hu *et al.*, 2007).

Second, SM proteins have been shown to interact with the assembled ternary SNARE complex (Figure 4). This mode seems to be the predominant form for the yeast SM protein Sec1p and might be mediating the zipping up of the SNARE complex during membrane fusion regulation (Carr *et al.*, 1999; Scott *et al.*, 2004; Togneri *et al.*, 2006; Xu *et al.*, 2010).

Third, the mammalian SM protein Munc18-1 has been shown to bind to Syntaxin1 that is in a closed conformation (Misura *et al.*, 2000; Latham and Meunier, 2007, Figure 4). This Munc18-1-Syntaxin1 association has been proposed to maintain Syntaxin1 in a closed conformation and inhibit Syntaxin1 from entering the SNARE complex (Misura *et al.*, 2000).

However, it has become evident that the described binding modes are not exclusive. The SM protein Sly1p binds to Sed5p in the N-peptide binding mode, but it also binds to assembled SNARE complexes (Peng and Gallwitz, 2002). Moreover, Vps45p has been shown to bind to Tgl2p in a closed and open conformation (Furgason *et al.*, 2009). The mammalian SM protein Munc18 has been shown to interact with Syntaxin1 in a closed and open conformation, and with the assembled SNARE complex (Misura *et al.*, 2000; Toonen and Verhage, 2007; Dulubova *et al.*, 2007; Khvotchev

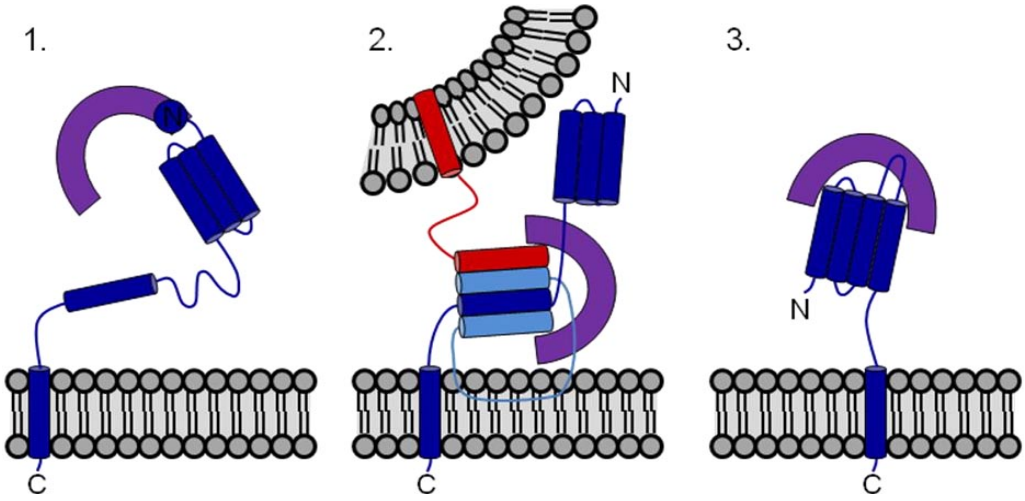

Figure 4. The different interaction modes of SM proteins (purple) with Syntaxin homologues (dark blue). SM protein binding to 1. Syntaxin N-peptide, 2. assembled SNARE complex, and 3. Syntaxin in the closed conformation.

et al., 2007; Burkhardt *et al.*, 2008). Additionally, it was shown that Munc18-1 possesses different affinities for the sole Syntaxin1, Synaptobrevin and the SNARE complex. For accomplishing this, Munc18-1 utilises the different binding modes, suggesting a dynamic switch between these different binding modes during regulation of the SNARE complex formation (Xu *et al.*, 2010). It is evident that SM proteins can apply a variety of binding modes to SNARE components. However, the spatial and temporal regulations of the transitions between these different binding modes still need to be discovered.

4.1.3. Non-SNARE interaction partners of SM proteins

Several non-SNARE SM binding proteins are known. These proteins are potential modifiers of SM protein affinity to certain SNARE complex configurations. In yeast, Vac1p, Ivy1p and Mso1p have been identified as SM binding proteins participating in different steps of the secretory pathway.

Vac1p binds to the SM protein Vps45p. Deletion of *VAC1* has been shown to cause a reduction in cell growth and defects in vacuole segregation. It was proposed that Vac1p is required for proper vacuole maintenance (Weisman and Wickner,

1992). Ivy1p was characterised as a protein binding to the SM protein Vps33p. It was shown that deletion of *IVY1* does not cause any recognizable phenotype, yet overexpression of *IVY1* causes vacuole defragmentation (Lazar *et al.*, 2002). Mso1p was identified as a multicopy suppressor for the *sec1-1* temperature-sensitive mutant. It was further shown to interact with Sec1p and to be involved in exocytosis (Aalto *et al.*, 1997). Mso1p is a non-essential gene. Yet, its deletion leads to vesicle accumulation at the site of cell growth in vegetatively grown yeast cells and a block in the *de novo* plasma membrane generation during sporulation (Jantti *et al.*, 2002).

While in yeast there is only one known non-SNARE protein interacting with Sec1p, in mammalian cells there are four Munc18 interacting proteins: Mint1, Mint2, Doc2 and Granuphilin/Slp4.

Mint1 and Mint2 have been shown to bind to PIP_2 and Munc18. They can exist in a complex with Syntaxin1 and Munc18 (Okamoto and Sudhof, 1997), as well as compete with Syntaxin1 for Munc18 binding (Becherer and Rettig, 2006). Furthermore, Mint1 interacts with the β-amyloid precursor protein (APP) that is centrally involved in the generation of the senile plaques and neurofibrillary structures in patients with Alzheimer's disease (Borg *et al.*, 1996; Thinakaran and Koo, 2008; Suzuki and Nakaya, 2008). Association of Mints with APP is mediated by the phosphotyrosine binding (PTB) domain and this interaction has been shown to affect the level of neurotransmission, and distribution and turnover of APP (King and Turner, 2004).

Doc2, a Ca^{2+} sensing protein involved in neurotransmitter release, has been shown to bind to Munc18 and Munc13 (Becherer and Rettig, 2006). The Doc2 binding site in Munc18 coincides with the Syntaxin4 binding site. It has been shown that these two proteins compete for Munc18 binding and that Syntaxin4 can displace Munc18 from Doc2 (Ke *et al.*, 2007). The interactions between these proteins are further regulated by phosphorylation of Munc18, which causes a switch from Syntaxin4 binding to interaction with Doc2 (Jewell *et al.*, 2008).

Granuphilin belongs to the family of synaptotagmin-like proteins. It is centrally involved in insulin release from pancreatic β-cells, as its overexpression causes a profound reduction of stimulus induced secretion in these cells (Coppola *et al.*, 2002). It has been shown to interact simultaneously with Munc18 and Syntaxin1 in the closed conformation, making it a potential regulator for SM protein and SNARE complex function (Becherer and Rettig, 2006).

4.2. The SNARE proteins

SNARE family proteins are essential components for membrane fusion (Aalto *et al.*, 1993; Jahn and Scheller, 2006). All SNARE proteins share a characteristic α-helical region with heptad repeats named the SNARE motive. These SNARE motives from cognate SNARE proteins interact with each other and form a highly dense helix bundle, named the SNARE complex (Sutton *et al.*, 1998; Strop *et al.*, 2008, Figure 5). The formation and zipping up of the SNARE complex from the N- to the C-terminus of the SNARE proteins is thought to provide sufficient force to mediate membrane fusion (Matos *et al.*, 2003; Walter *et al.*, 2010). *In vitro* experiments suggest that even one SNARE complex is enough to promote vesicle fusion underlining the importance of the SNARE proteins for membrane fusion (van den Bogaart *et al.*, 2010).

According to the amino acid located in the central layer of the SNARE motive SNARE proteins have been divided into Q (Glutamine) and R (Arginine) subfamilies (Fasshauer *et al.*, 1998). Alternatively, SNAREs have also been classified according to their location as v- (vesicle) and t- (target membrane) SNARES (Jahn and Scheller, 2006). A SNARE complex is formed from three Q and one R SNARE motive (Jahn and Scheller, 2006).

In yeast, the three Q SNARE motives are provided by the plasma membrane bound t-SNARE proteins Sso1/2p and Sec9p. Sso1p and Sso2p are the yeast homologues of the mammalian Syntaxin1 (Aalto *et al.*, 1993). In addition to the SNARE motive, they possess an N-terminal domain (Habc) and a C-terminal transmembrane domain, which anchors them to the plasma membrane. The Habc domain, which is composed of three short helixes, mediates the closed conformation of Syntaxin homologues (Munson *et al.*, 2000). In yeast, mutations in Sso1p destabilizing the closed conformation lead to a faster SNARE complex formation, yet deletion of the whole Habc domain in Sso1p causes lethality (Munson *et al.*, 2000). It has been proposed that the open conformation of Syntaxin homologues is needed for SNARE complex formation and that the closed conformation can regulate the speed of SNARE complex formation (Macdonald *et al.*, 2010).

Sec9p, another t-SNARE mediating yeast exocytosis, is the homologue of mammalian SNAP25. Sec9p possesses two SNARE motives, which are joined by a palmitoylated linker (Jahn and Scheller, 2006). Both of these SNARE motives provide one Q residue in the central layer of the SNARE complex.

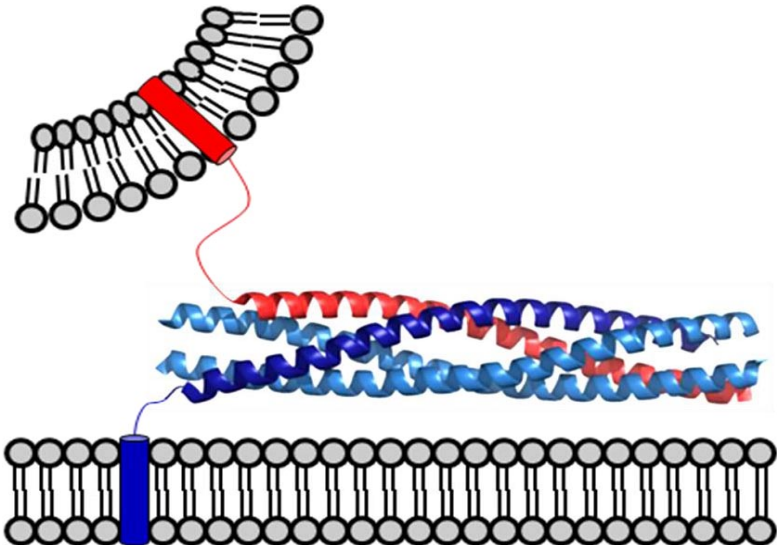

Figure 5. Model of the yeast SNARE complex (Structure from Munson *et al.*, 2000). The t-SNAREs Sso1/2p and Sec9p are shown in dark blue and light blue, respectively. The v-SNARE Snc1/2p is shown in red. Trans-membrane helixes are represented as cylinders.

The one R SNARE motive for the exocytic SNARE complex formation is provided by the v-SNARE Snc1/2p. Snc1p and Snc2p are the yeast homologues of mammalian Synaptobrevin/VAMP (Protopopov *et al.*, 1993). Similarly to Sso1/2p, Snc1/2p possess a C-terminal transmembrane domain anchoring Snc1/2p to the vesicular membrane (Jahn and Scheller, 2006).

During SNARE complex formation, Snc1/2p is vesicle anchored, while Sso1/2p and Sec9p are plasma membrane bound. This conformation of the SNAREs is called *trans*. After fusion of the vesicle with the plasma membrane all SNARE proteins reside at the same membrane, called the *cis*-conformation. The *cis*-SNARE complex can be disassembled by Sec18p/NSF and Sec17p/SNAP, allowing the components to recycle and get available for new fusion events (Wickner and Schekman, 2008).

Besides the SM proteins there are few other proteins known to modulate the SNARE complex function in mammalian cells. Synaptotagmin, a Ca^{2+} binding protein, is anchored on the vesicle and has been shown to bind to the SNARE complex and to Syntaxin and SNAP25. The binding of Synaptotagmin to Syntaxin and SNAP25 has been implicated in the block of complete assembly of the SNARE complex before Ca^{2+} influx (Gerst, 2003; Becherer and Rettig, 2006). So far, the precise mechanism of Synaptotagmin function in neurotransmitter release is unknown, yet it has been shown that Ca^{2+} binding increases its membrane affinity (Gerst, 2003). This result indicates a potential regulatory switch from SNARE

complex to membrane binding of Synaptotagmin after the Ca^{2+} influx, which would allow complete SNARE complex assembly and membrane fusion. The priming factor Munc13 binds to Syntaxin1 and the membrane anchored SNARE complex (Guan *et al.*, 2008). Munc13 has been shown to be capable of replacing Syntaxin1 from Munc18, thereby allowing Syntaxin1 to open up and making it available for SNARE complex formation (Becherer and Rettig, 2006). Complexin is a SNARE complex binding protein that enhances fusion in a Ca^{2+} dependent manner (Becherer and Rettig, 2006). So far, its mechanistic role in the membrane fusion event is unknown.

5. PI(4,5)P_2 and lipid binding in exocytosis

Phosphatidylinositol phosphates (PIPs) are known to be key factors in membrane fusion regulation (Vicinanza *et al.*, 2008). PIPs have been shown to be important for membrane trafficking by activating, recruiting and assembling of the molecular membrane fusion machinery (Vicinanza *et al.*, 2008). It has been proposed that the local production of PIPs might act as a coordinator for the function of Rho GTPases, by activating them at the site of secretion. In this exocytic signalling model, activation of the Rho GTPases leads to actin cytoskeleton regulation and assembly of the exocytic machinery at the sites of secretion (Yakir-Tamang and Gerst, 2009b). In yeast there are four major PIPs, which localise to different compartments (Figure 6). PI(3)P is predominantly found on prevacuolar compartments and the endosomes, PI(3,5)P_2 on the vacuole and the endosome, PI(4)P on the Golgi apparatus and PI(4,5)P_2 on the plasma membrane (Yakir-Tamang and Gerst, 2009b). In yeast PI(4,5)P_2 is generated on the plasma membrane at sites of polarization by the Phosphatidylinositol-4-phosphate 5-kinase Mss4p (Audhya *et al.*, 2004, Figure 6). Defects in Mss4p function lead to actin depolarization and inhibition of secretion (Yakir-Tamang and Gerst, 2009a).

In the recent years, PIPs have been implicated in many steps of the vesicle targeting to the plasma membrane. In yeast, vesicles are transported along the actin cytoskeleton, whose dynamics are maintained by actin binding and remodelling proteins and their PIP mediated membrane binding (Saarikangas *et al.*, 2010). Once a vesicle buds from the Golgi apparatus high PI(4)P concentration in the vesicle membrane inhibit Sec2p binding to the Exocyst subunit Sec15p, but not to the GTPase Ypt32p.

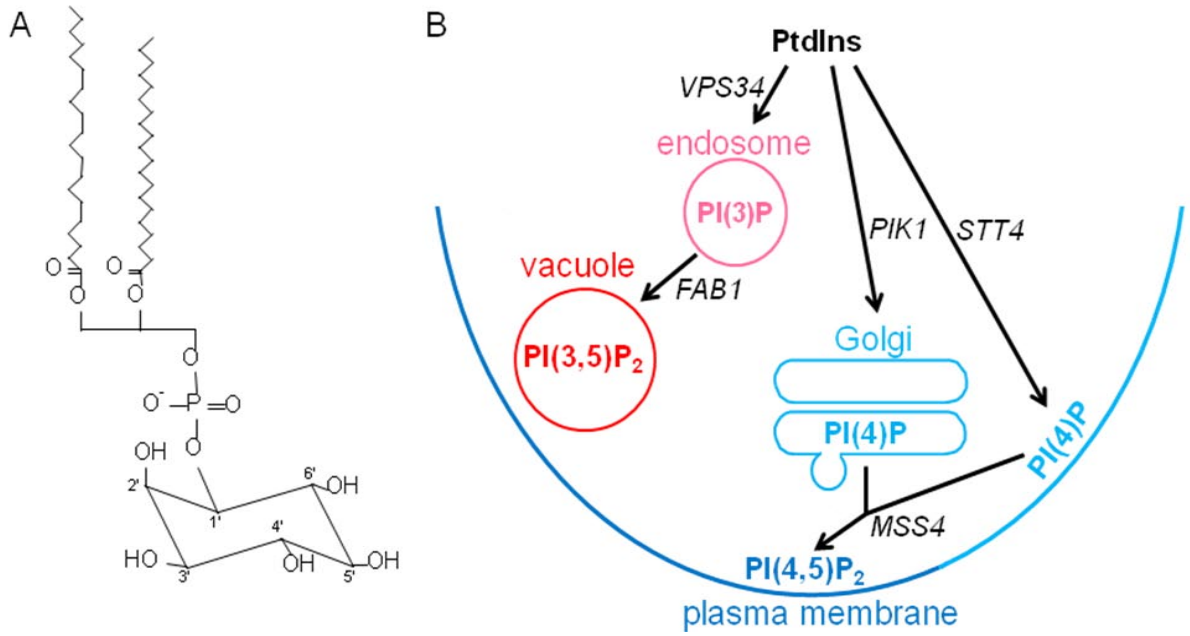

Figure 6. Schematic presentation of the major PIPs and their kinases. A. Chemical structure of 1,2 Diacylglycerol Phosphatidylinositol. B. Vsp34p synthesises PI(3), which is further phosphorylated to PI(3,4)P_2 by Fab1p at the vacuolar membrane. Pik1p creates PI(4)P at the Golgi, while Stt4p creates PI(4)P at the plasma membrane. PI(4,5)P_2 is synthesised at the plasma membrane by Mss4p.

It has been proposed that decreasing concentrations of PI(4)P during vesicle maturation mediate a switch of the binding partner of Sec2p from Ypt32p to Sec15p (Medkova *et al.*, 2006; Mizuno-Yamasaki *et al.*, 2010). Upon vesicle arrival at the plasma membrane, the targeting of the Exocyst complex has been shown to be mediated by the PI(4,5)P_2 binding properties of Exo70p and Sec3p (He *et al.*, 2007; Liu *et al.*, 2007). It has been shown that PI(4,5)P_2 generation at the sites of polarization triggers the recruitment of the Exocyst complex, suggesting a prominent role of PIPs in Exocyst function (Yakir-Tamang and Gerst, 2009b).

At the layer of SNARE complex formation, Sso1p and Sso2p have been shown to bind to lipids separately from their transmembrane helix. However, the Habc domain of Sso1p binds to PI(4,5)P_2 three times better than Sso2p. Taken into account that only Sso1p is required during prospore membrane formation in

sporulation (Jantti *et al.*, 2002), this result suggested a novel regulatory mechanism in prospore membrane formation mediated by PI(4,5)P_2 (Mendonsa and Engebrecht, 2009). Furthermore, it has been shown that the minimal lipid requirement of the SNARE complex for efficient vacuole fusion *in vitro* contains PI(3)P (Mima and Wickner, 2009). Collectively, these results show a prominent role of PIPs in all steps of exocytosis, starting at the vesicle targeting from the Golgi to the fusion of the vesicle with the plasma membrane.

AIMS OF THE STUDY

The aim of the study was to gain better understanding on the molecular mechanisms of the membrane fusion machinery in exocytosis by using yeast *Saccharomyces cerevisiae* as the model system. The study focused on the functional analysis of Sec1p and its interaction partner Mso1p previously shown to participate in membrane fusion (Aalto *et al.*, 1997; Brummer *et al.*, 2001; Jantti *et al.*, 2002).

The specific aims:

1. to investigate the interaction between Mso1p and Sec1p.
2. to explore the function of the C-terminal extension in Sec1p, which is common in fungal homologues, yet missing in the mammalian homologue Munc18.
3. to discover possible novel interaction partners for Mso1p.

MATERIALS AND METHODS

The methods used in this study are listed in the table below. Detailed description of the methods can be found in the publications (roman numbers). The methods personally performed are highlighted in bold.

Method	Publication
Electron microscopy	I
Fluorescence Anisotropy of DPH	IV
Fluorescence microscopy	I-IV
Genetic methods	I
Homology model of Sec1p	II
Immunoprecipitations	I, II, III
***In vitro* gel mobility shift assay**	III
In vitro binding assay	II
***In vitro* pull down assays**	I, III
***In vivo* pull down experiments**	I
Light scattering assay	IV
Membrane fractionation	III
Plasmid construction	I-IV
Production of recombinant proteins	I-IV
Ras rescue assay	IV
SDS PAGE	I, II, III
SEC1 insertion library	II
Vesicle co-sedimentation assay	IV
Western blotting	I, II, III
Yeast two hybrid assay	I, II, III
Yeast strain construction	I-IV

RESULTS AND DISCUSSION

The study used the yeast *Saccharomyces cerevisiae* as a model organism in order to investigate the mechanism of membrane fusion at the plasma membrane.

1. The Mso1p-Sec1p interaction

1.1. Mso1p-Sec1p interaction site is dependent on the Rab GTPase Sec4p and the SNARE complex (I and II)

Mso1p and Sec1p localise at the bud tip and the septum of vegetatively grown yeast cells (Scott *et al.*, 2004, I Figure 2A). In addition, Sec1p and the SNARE proteins Sso1p, Sso2p and Sec9p localise also along the plasma membrane of the growing bud and along the mother cell plasma membrane (Brennwald *et al.*, 1994; Scott *et al.*, 2004).

We made use of the Bimolecular Fluorescence Complementation (BiFC) technique to identify and characterise the Mso1p-Sec1p interaction site *in vivo* (Hu *et al.*, 2002; Kerppola, 2006; Skarp *et al.*, 2008). In vegetatively grown yeast cells, Mso1p and Sec1p were detected to interact at the plasma membrane of the emerging bud, growing daughter cell and at the septum of dividing cells (II Figure 1A and C). Interestingly, the Mso1p-Sec1p interaction signal also labelled the former bud site in haploid and diploid cells (II Figure 1A and C, stars), suggesting that at least some of the components of the secretion machinery remain at this site after the bud closure.

In order to evaluate the *in vivo* dependency of the Mso1p-Sec1p interaction on the secretion machinery, the Mso1p-Sec1p BiFC signal was analysed in different secretion mutants. A significant change in the Mso1p-Sec1p interaction site was observed in *sec4-8* and *sec2-41* cells. In these cells, the Mso1p-Sec1p interaction site no longer localised to the sites of cell growth at the restrictive temperature, instead an over 5-fold increase in fluorescence signal in the cytosol was detected (II Figure 1D, Table 2). In line with this finding, in co-immunoprecipitation experiments using *sec4-8* mutant cells the association between Mso1p and Sec1p was not affected (I Figure 7B). However, at the same time, no interaction with the SNARE complex components was detected using this technique. These results suggest that Mso1p and Sec1p interact independently of a functioning Sec4p GTPase, but are not

associated with the plasma membrane when Sec4p is defective or not GTP loaded. One possible explanation for this distribution of the Mso1p-Sec1p complex may be the disassembly of SNARE complexes in *sec2-41* and *sec4-8* cells (Grote and Novick, 1999; Grote *et al.*, 2000). This could suggest that Mso1p-Sec1p complexes preferably associate with assembled SNARE complexes and not with monomeric Sso proteins at the plasma membrane. Alternatively, a lack of upstream signalling might cause the phenotype.

Changes in the localisation of the Mso1p-Sec1p complex were also observed in strains defective in the SNARE complex function. In the *sec18-1* strain, defective for *cis*-SNARE complex disassembly at the restrictive temperature, the Mso1p-Sec1p interaction site accumulated as dotty structures at the plasma membrane (II Figure 1D, arrows). These dots could represent accumulated *cis*-SNARE complexes, to which the Mso1p-Sec1p complex stays bound. In the t-SNARE mutant strains *sso2-1 Δsso1* and *sec9-4* the Mso1p-Sec1p interaction site was partially mislocalised throughout the plasma membrane at the restrictive temperature (II Figure 1D, arrows and dotted line, Table 2), suggesting a defect in the polarization of the Mso1p-Sec1p complexes in these cells.

1.2. An N-terminal peptide of Mso1p binds to the putative N-peptide binding site in Sec1p domain 1 (I and II)

Mso1p and Sec1p appear to form a rather stable 1:1 complex with an approximate dissociation constant (K_D) of ~3 nM in *in vitro* binding studies (III Supplementary Figure S3). In order to better understand the structure and function of the Mso1p-Sec1p complex the interaction interfaces in the proteins were determined.

Initial mapping of the interaction domain of Mso1p with Sec1p was performed using yeast two hybrid analysis of Mso1p fragments, which revealed an amino-terminal peptide (amino acid 38-59) to be necessary for the interaction with Sec1p (I Figure 3A). The interaction was confirmed *in vitro* with bacterially expressed components (I Figure 3C), *in vivo* by pull down experiments (I Figure 3B) and by using the BiFC technique (II Figure 1B). Furthermore, this segment of Mso1p was necessary for the ability of Mso1p to multicopy suppress *sec1-1* and *sec1-11* mutations (I Figure 4). Within the interaction surface, Threonine 47 turned out to be critical for Mso1p *in vivo* function, as a T47A mutation in Mso1p resulted in specific genetic interactions with *sec1* mutations (I Figure 5).

Interestingly, in contrast to synthetic lethal combinations of *Δmso1* with *sec2* and *sec4* mutations, the T47A mutation was only synthetically lethal with *sec1* mutants. This suggests that molecular determinants that functionally link Mso1p with Sec2p and Sec4p are not located in the Sec1p binding site of Mso1p. So far, the contribution of the T47A mutation *in vivo* is unclear, yet in confirms the specific interaction for Mso1p(39-59) with Sec1p.

In order to map the Mso1p binding site in Sec1p, we performed yeast two hybrid screens with a *sec1* mutant library and selected Sec1p domains. These approaches identified Sec1p domain 1 as Mso1p binding site (II, Figure 3). In order to identify potential binding sites in Sec1p domain 1, a model of yeast Sec1p was created. Using this model, two potential binding surfaces within Sec1p domain 1 were identified: the putative N-peptide binding site and the Syntaxin binding site (II Figure 4). In order to address the Mso1p binding site in Sec1p in a more subtle way point mutations were generated, which according to homology should disrupt these binding surfaces. The combined use of yeast two hybrid, co-immunoprecipitation, BiFC, and genetic techniques revealed that mutations corrupting the putative N-peptide binding area (Q113L, F115A and L125D) in Sec1p domain 1 resulted in significantly reduced Mso1p binding to Sec1p (II Figure 5, Supplementary Figure S4). Furthermore, these mutations led to an inhibition of prospore membrane formation during sporulation (II Table 3), suggesting an important role for this interaction surface in SNARE complex mediated membrane fusion *in vivo*. Interestingly, the Mso1p peptide (amino acid 38-59) interacting with Sec1p does not display obvious sequence similarity to the Syntaxin N-peptides. This proposes a novel interaction mode within the putative N-peptide binding area in yeast Sec1p.

1.3. Mso1p mimics the Syntaxin N-peptide binding mode (II)

A stabilizing role of Mso1p in the Sec1p-SNARE complex binding has been suggested by genetic results (I, Figure 4). This possibility is further supported by the specific temperature sensitivity caused by deletion of *MSO1* in the SNARE binding deficient mutant *sec1*(V55D) (II Supplementary Figure S5). Similarly the BiFC signal between Sec1p(L25D), which is compromised in SNARE binding, and Sso1/2p was significantly reduced when Mso1p was deleted (II Figure 6E). This ability of Mso1p, being non essential, yet stabilising the Sec1p-SNARE complex

association, is similar to the role of the Syntaxin N-peptide (Burkhardt *et al.*, 2008). A stabilising role of Mso1p could be explained by an association between Mso1p and Sso1/2p.

Several experiments support such an interaction. Mso1p was shown by the yeast two hybrid and BiFC technique to interact with Sso1/2p (II Figure 7A and C). *In vitro* binding studies revealed a weak interaction between Sso1p and Mso1p. No interaction with Sso2p was detected *in vitro* (II Figure 7B). The difference in interaction strength is in line with the yeast two hybrid results, where repeatedly a stronger interaction between Mso1p and Sso1p was observed. Interestingly, in the BiFC analysis, a qualitatively different distribution for the Mso1p-Sso1p and Mso1p-Sso2p interaction sites was observed. While the Mso1p-Sso1p complexes occupied predominantly the daughter cell plasma membrane, the Mso1p-Sso2p complexes were enriched in the mother cell (II Figure 7C-E). This finding supports a distinct selectivity of Mso1p for interactions with Sso1p and Sso2p.

A differential interaction with the paralogous Sso1/2p proteins is supported by previous data, which suggested that Mso1p is important for Sso1p functionality when Sso2p is functionally compromised, and not vice versa (Jantti *et al.*, 2002). Another support comes from the observation that Sso1p, but not Sso2p, is needed for prospore membrane formation during sporulation (Jantti *et al.*, 2002), suggesting a special cooperation between Mso1p and Sso1p.

Using the BiFC and yeast two hybrid techniques, an area between amino acids 59 and 94 of Mso1p was identified to be contributing to the interaction with Sso1/2p. Interestingly, this area in Mso1p is adjacent to the Sec1p interaction site and could enable a bridging between Sec1p and the SNARE complex (Figure 7), thereby enhancing their association. In earlier overexpression studies, it was obvious that an area of Mso1p, corresponding to the Sec1p plus Sso1/2p binding site, is needed for suppression of the *sec1-1* and *sec1-11* temperature sensitivity (I Figure 4). This finding further suggests that the property of Mso1p binding to Sec1p and Sso1/2p facilitates Sec1p-SNARE complex association similarly to the Syntaxin N-peptide.

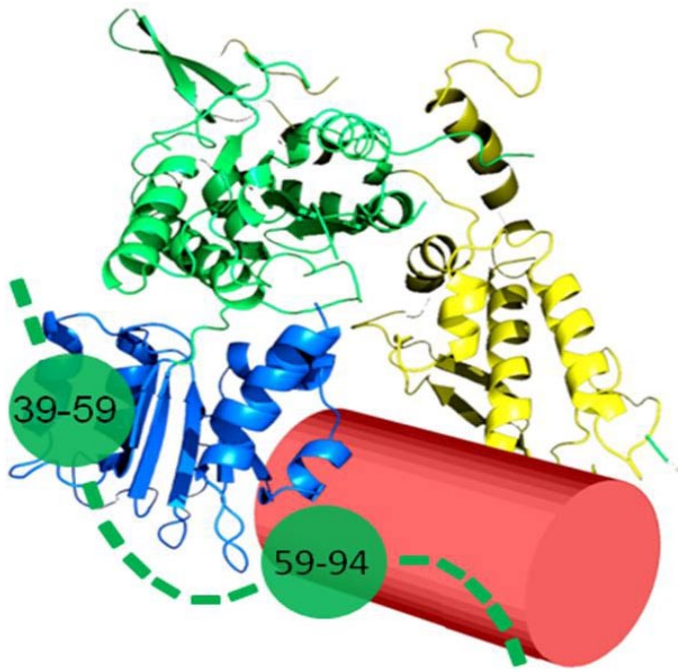

Figure 7. Model of the Mso1p-Sec1p-SNARE complex association. The yeast Sec1p structure is displayed as a ribbon presentation, with Sec1p domain 1 in blue, domain 2 in green and domain 3 in yellow. The SNARE complex is symbolised as a red cylinder. Mso1p is shown in green with the interaction patches to Sec1p(aa 39-59) and the SNARE complex (aa 59-94) shown as circles connected by a dotted line.

During exocytosis in *S. cerevisiae,* Sec1p interacts preferentially with the assembled SNARE complexes (Carr *et al.*, 1999; Scott *et al.*, 2004; Togneri *et al.*, 2006; Hashizume *et al.*, 2009). It is likely that the Mso1p-Sso1/2p interaction takes place within this larger protein complex, composed of Mso1p, Sec1p, Sso1/2p, Sec9p and Snc1/2p. Even though the interaction between Mso1p and the Sso1/2p proteins is weak *in vitro*, their affinity within the complex might create additional force for complex association. In agreement, point mutations in Sec1p affecting the SNARE binding did not abolish co-immunoprecipitation of Sso1/2p with Sec1p (II Figure 6B and C). It is possible that the affinity between Mso1p and Sso1/2p is at least partially responsible for the weak yet persisting co-immunoprecipitation.

Intriguingly, Mso1p is degraded upon disruption of the Sec1p function (II Figure 2A). The same was observed for the Syntaxin homologues Tlg2p and Ufe1p upon disruption of Vps45p and Sly1p, respectively (Bryant and James, 2001; Braun and Jentsch, 2007). Interestingly, in analogy to Mso1p, both Tlg2p and Ufe1p use the Syntaxin N-peptide binding mode for the interaction with their corresponding SM proteins. This specific dependence of proteins occupying the N-peptide binding site on SM protein function represents a regulatory mode whose function is so far unknown.

2. The importance of the Sec1p-tail for SNARE complex interaction (III)

Saccharomyces cerevisiae Sec1p possesses a 66 amino acid long C-terminal extension that does not exist in its higher eukaryote homologues. A C-terminal tail exists widely among fungal Sec1p homologues yet it does not possess any obvious sequence motifs that would reveal its functional role *in vivo* (III Figure 1A and B). Considering the conservation of the Sec1p-tail between different fungi, we studied its role in membrane fusion in yeast.

Deletion of the C-terminal tail in yeast Sec1p [Sec1p(1-657)] resulted in temperature sensitivity of haploid cells and a defect in sporulation and Bgl2p secretion (III Figure 1C-E). These results imply a significant function for the Sec1p-tail *in vivo*. When the SNARE binding of Sec1p(1-657) was addressed, a clearly reduced affinity to the SNARE components was observed in co-immunoprecipitation experiments (III Figure 2A). At the same time, overexpression of the Sec1p-tail enhanced Sec1p co-immunoprecipitation with the SNAREs (III Figure 4B and C). *In vitro* binding studies performed with purified components indicate that the Sec1p-tail interacts preferentially with binary Sec9p-Sso1p and ternary Snc2p-Sec9p-Sso1p complexes and enhances SNARE complex formation *in vitro* (III Figure 3A, Figure 4D and E).

Interestingly, when the Sec1p(1-657)-Sso1/2p binding was examined using the BiFC assay, it was obvious that the Sec1p-tail deletion affected more the interaction with Sso1p than with Sso2p (III Figure 3C). This selectivity is further supported by overexpression experiments, which show that *SSO2* is more efficient in suppression of the *sec1(1-657)* temperature sensitivity than *SSO1* (III Figure 3B). In line with these results, the Sec1p-tail alone binds more strongly to Sso1p in the yeast two hybrid and BiFC assays (III Figure 3C and D).

Considering these results, it is tempting to speculate that Sso1p and Sso2p occupy slightly different binding surfaces on Sec1p. It seems likely that Sso1p, but not Sso2p, uses a binding surface in Sec1p, which is at least partially created by the Sec1p-tail. Based on our model of the Sec1p structure, it appears feasible that the C-terminal peptide localises to the cleft surface of the Sec1p arch. Therefore, it is possible that the Sec1p-tail contributes to the SNARE binding. It has been shown that the surface of the SNARE complexes is typically negatively charged (Strop *et al.*, 2008). In neuronal SNARE complexes,

this surface is thought to be important for interaction with the SNARE complex regulator Synaptotagmin, which possesses a positively charged surface (Strop *et al.*, 2008). Interestingly, the Sec1p-tail, containing 16 Lysine and Arginine residues, has a net positive charge (pI 10.3) at the cytosolic pH. It is possible that, like in the case of Synaptotagmin, the Sec1p-tail uses these ionic interactions for mediating SNARE complex function. We suggest a model where the Sec1p C-terminal peptide positively regulates the assembly of SNARE complexes. In yeast, in the absence of other SNARE complex regulators such as Munc13, Complexin and Synaptotagmin, this additional regulatory mechanism together with the Sec1p interaction with Mso1p and Sso1/2p, could offer a framework of molecular interactions that enable the dynamic and directional assembly of SNARE complexes.

3. Identification of novel Mso1p interaction partners

3.1. The Rab GTPase Sec4p (III)

In order to identify potential regulators involved in the Mso1p-Sec1p complex function, we used the SNARE mutant strain *sso2-1Δsso1* in combination with the SNARE binding deficient Sec1p(1-657) and Mso1p in the BiFC technique. While wild type Sec1p-Mso1p complexes mislocalise along the plasma membrane in *sso2-1Δsso1* cells, a distinct polarised localisation to the bud and septum of the Mso1p-Sec1p(1-657) complexes was observed. This polarised targeting was dependent on the Mso1p C-terminus, as its deletion [Mso1p(1-188)] caused a shift of the Mso1p(1-188)-Sec1p(1-657) complexes to the cytosol (III Figure 5A and B). These results imply that the Mso1p C-terminus can mediate targeting of the SNARE binding deficient Sec1p(1-657) to sites of polarised membrane transport in *sso2-1Δsso1* mutant cells.

Previous results suggested a genetic link between Mso1p and the small Rab GTPase Sec4p. It was suggested that the Mso1p-Sec4p connection is independent of the Sec1p binding surface and might be mediated via the Mso1p C-terminus (Castillo-Flores *et al.*, 2005, I Figure 5B). Therefore we wanted to test whether Sec4p is involved in the targeting of Mso1p.

This possibility is supported by *in vitro* pull down assays, which showed a direct interaction between Mso1p and Sec4p (III Figure 6A). Furthermore, the BiFC technique revealed a signal between Mso1p and Sec4p in intracellular structures at the growing bud and septum

in vivo (III Figure 6B, C and D). Noteworthy, the Mso1p-Sec4p positive structures were found to be mobile. It appears possible that these structures are vesicles or accumulations of vesicles moving to the plasma membrane. The BiFC signal was especially prominent for the presumably GTP-locked form of Sec4p(Q79L), while for the GDP-locked form of Sec4p(S34N) only a weak signal was detected (III Figure 6B). Further evidence for the importance of the nucleotide binding state of Sec4p for the BiFC signal with Mso1p came from the finding that the Mso1p-Sec4p signal was clearly reduced in *sec2-41* cells defective of the Sec4p GEF Sec2p (Walch-Solimena *et al.*, 1997, III Figure 7C). A similar interaction profile with Sec4p was observed for Sec9p, a known effector of Sec4p (Brennwald *et al.*, 1994), suggesting that Mso1p might also be an effector of Sec4p.

In line with the finding that Mso1p can target the SNARE binding deficient Sec1p(1-657) in *sso2-1Δsso1* cells, the Mso1p-Sec4p BiFC signal was unaffected in the SNARE mutant *sso2-1Δsso1* (III Figure 7B). This suggests that the Mso1p-Sec4p cooperation occurs prior to SNARE complex function on intracellular vesicular structures before their arrival at the plasma membrane.

Interestingly, having an adaptor protein bridging SM proteins to a GTPase seems to be a common feature in eukaryotic cells. The other known yeast SM protein binding proteins Vac1p and Ivy1p have been shown to interact with the Rab GTPases Vps21p and Ypt7p, respectively (Tall *et al.*, 1999; Lazar *et al.*, 2002). The mammalian Sec1p homologue Munc18 has several interaction partners, which have been proposed to link Munc18 function to a GTPase. The Mint1/2 homologue Mint3 interacts with Rab6 via its PTB domain (Teber *et al.*, 2005). Additionally, the Munc18 binding protein Granuphilin has been shown to bind to GTP loaded Rab3 (Coppola *et al.*, 2002). Furthermore, Munc13, a priming factor in exocytosis, interacts with GTP loaded Rab27 (Shirakawa *et al.*, 2004). This redundancy in mammalian exocytosis might reflect a tighter regulation specialised for certain exocytosis modes in different tissues.

SM proteins themselves have not been reported to interact with GTPases; instead they seem to possess adaptor proteins, e.g. Mso1p, which interact with GTPases. These adaptor proteins could mediate the signal transmission from the GTPase to a SM protein, in order to regulate SNARE complex dynamics. In yeast, the Rab GTPase Sec4p has been additionally linked to the Exocyst and SNARE

complex via interactions with their subunits (Brennwald *et al.*, 1994; Guo *et al.*, 1999). The novel interaction partner of Sec4p, Mso1p, adds a new level of temporal and spatial modulation of exocytosis.

3.2. PIPs and other lipids (IV)

Mso1p shares homology with the PTB domain of the mammalian SM protein binding Mint proteins (I Figure 8). In Mint1, the PTB domain has been shown to mediate PIP_2 binding.

The homology between Mso1p and the Mint1 PTB domain prompted us to test the possibility that Mso1p interacts with lipids. The *in vivo* Ras rescue assay and *in vitro* lipid binding and insertion assays were employed to address the potential lipid binding of Mso1p. The results revealed that Mso1p can bind to PIP containing membranes (VI Figure 1A and C). This lipid binding appears to involve a general affinity of the Mso1p C-terminus (amino acid 40-210) to lipids and a specific insertion into lipid bilayers mediated via the Mso1p N-terminus (amino acid 1-39) (IV Figure 1B and D). It is possible that these two lipid binding areas in Mso1p mediate slightly different functions *in vivo*. The N-terminus of Mso1p appears to interact with the plasma membrane, while the C-terminus of Mso1p seems to localise to vesicular structures with Sec4p (IV Figure 5). *In vitro*, Mso1p can cluster vesicles by employing the N- and C-terminal lipid binding areas (IV Figure 6) making it tempting to speculate that *in vivo* Mso1p might participate in membrane fusion by bridging the vesicular and plasma membrane.

Within the Mso1p N-terminus, Leucine 26 and Leucine 30 are conserved between Mso1p and Mint1 (IV Figure 4A). Mutations changing the hydrophobicity of these amino acids result in a decrease in lipid bilayer insertion for both Mso1p and Mint1 (IV Figure 4B and C). In the *in vivo* Ras rescue assay, the mutations result in a reduced plasma membrane interaction of Mso1p (IV Figure 4D). These findings suggest a similar mode of lipid insertion for these two proteins.

Using the BiFC technique, we discovered that the lipid insertion of Mso1p is needed for Mso1p membrane localisation and consequently the Mso1p-Sec1p complex membrane localisation (IV Figure 5A and B). Furthermore, for the *in vivo* function of Mso1p, the lipid insertion is essential, as shown by the loss of sporulation of the *mso1(40-210)/mso1(40-210)* homozygous diploid strain (IV Table 3). It is tempting to speculate that the lipid insertion property of Mso1p can mediate anchoring

of Sec1p at the plasma membrane. Such a function could be beneficial, e.g. for placing the fusion machinery in position.

In yeast, the main phosphatidylinositol phosphate at the plasma membrane is PI(4,5)P_2. It has been proposed to be produced by Mss4p at the sites of secretion and thereby to label the point for the membrane fusion machinery assembly. We made use of the temperature sensitive *mss4ts* mutant strain to address the contribution of PI(4,5)P_2 on Mso1p-Sec1p localisation. In these cells the Mso1p-Sec1p signal was less concentrated at the bud and septum and instead it was enriched along the plasma membrane. This phenotype became even more obvious at the restrictive temperature (IV Figure 3). At the same time, the general membrane localisation was not disturbed, suggesting that Mso1p stays anchored in the lipid bilayer. One possible explanation for the mislocalisation is that reduced PI(4,5)P_2 levels at the bud tip result in lower affinity of Mso1p for this membrane location and thereby cause a loss of focus and diffusion of the Mso1p-Sec1p complex. This finding is supported by *in vitro* lipid insertion experiments, which showed that while Mso1p prefers membranes containing PIPs, PIPs are not essential for its membrane insertion (IV Figure 1C).

In addition to Mint1 and Mint2, the mammalian Munc18 binding protein Doc2 and the priming factor Munc13 have been shown to interact with lipids at the plasma membrane. The membrane binding of both proteins is dependent upon Ca^{2+} influx, which causes a structural reorganization resulting in higher membrane affinity (Friedrich *et al.*, 2010; Shin *et al.*, 2010). In yeast, the two other known SM protein binding proteins Vac1p and Ivy1p have been shown to interact with phospholipids (Tall *et al.*, 1999; Lazar *et al.*, 2002). For these proteins and now Mso1p, the lipid binding has been proposed to affect the functionality of the membrane fusion machinery at the particular stage of the secretory pathway. We speculate that the lipid binding of subcomponents of the secretion machinery is a common mechanism to ensure stabilised localisation of the necessary components and their assembly.

Our work focused on the membrane fusion machinery at the SM protein and SNARE complex level. Intriguingly, also at upstream events lipid binding has been shown to be important. Two members, Sec3p and Exo70p, of the vesicle tethering complex (the Exocyst) in yeast exocytosis have been shown to bind PI(4,5)P_2s. Similarly to Mso1p being needed for Mso1p-Sec1p localisation, the lipid interaction of Sec3p and Exo70p is important for the Exocyst localisation at the plasma membrane (He *et al.*, 2007; Liu

et al., 2007). These similar results from different steps of the membrane fusion machinery suggest a common requirement of anchoring of the machinery at the plasma membrane.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The present work sheds new light into the riddle of the different Sec1/Munc18 binding modes to the SNARE components. Previous results showed an interaction between Sec1p and the assembled SNARE complex (Carr *et al.*, 1999; Scott *et al.*, 2004). However, little information existed about the regulation of this interaction. Previous models suggested that Sec1p does not utilise its putative N-peptide binding area for SNARE complex interaction (Hu *et al.*, 2007). Now, we propose that Mso1p mimics the putative Syntaxin N-peptide binding for its interaction with Sec1p. In other SM proteins, this N-peptide binding site is occupied by the N-terminal peptide of the Syntaxin homologous SNARE components. Yeast Sso1p and Sso2p do not possess such an N-terminal extension and therefore can not bind to Sec1p via the N-peptide binding mode. Instead, the Sec1p binding protein Mso1p possesses affinity to the N-peptide binding site in Sec1p and for the Syntaxin homologues Sso1p and Sso2p. Our results suggest that in yeast, the N-peptide binding mode is provided by an additional protein, Mso1p. An interesting target for the future will be to reveal how well Mso1p mimics the N-peptide binding mode. In order to resolve this question, the three dimensional structure of the Mso1p-Sec1p-SNARE complex or subcomplexes would need to be resolved.

In the course of this study, the Sec1p C-terminal tail was identified as an essential mediator in SNARE complex formation regulation. While in mammalian cells there exists a large number of additional proteins regulating Sec1p and SNARE complex function, e.g. Complexin, Synaptotagmin and Munc13, in yeast so far Mso1p represents the only non-SNARE Sec1p interacting protein. It is possible that yeast circumvents the need for many regulators by assigning many functions to one protein. The Sec1p-tail, which does not exist in the mammalian homologues, might be one example. It creates an additional surface in Sec1p thereby allowing more and/or different interaction modes. It is intriguing that the Sec1p-tail appeared biased for Sso1p interaction. Further studies should reveal why there is a difference between Sso1p and Sso2p. Are they used in different exocytosis modes? One other potential target for future studies is to screen for other Sec1p and SNARE complex interaction partners. Considering the vast array of Munc18 and

SNARE regulators in mammalian cells, this approach could reveal novel regulators of SNARE function.

The other part of the work focused on the role of Mso1p in membrane fusion. We identified two novel interaction partners of Mso1p: the small Rab GTPase Sec4p and the plasma membrane lipids.

The Sec4p-Mso1p interplay appears to take place on the secretory vesicle prior to docking at the plasma membrane. Our results suggest that Mso1p and Sec4p cooperate in the establishment of polarised secretion. The dependence of this interplay on the nucleotide binding state of Sec4p suggests that Mso1p functions as an effector of Sec4p (Figure 8).

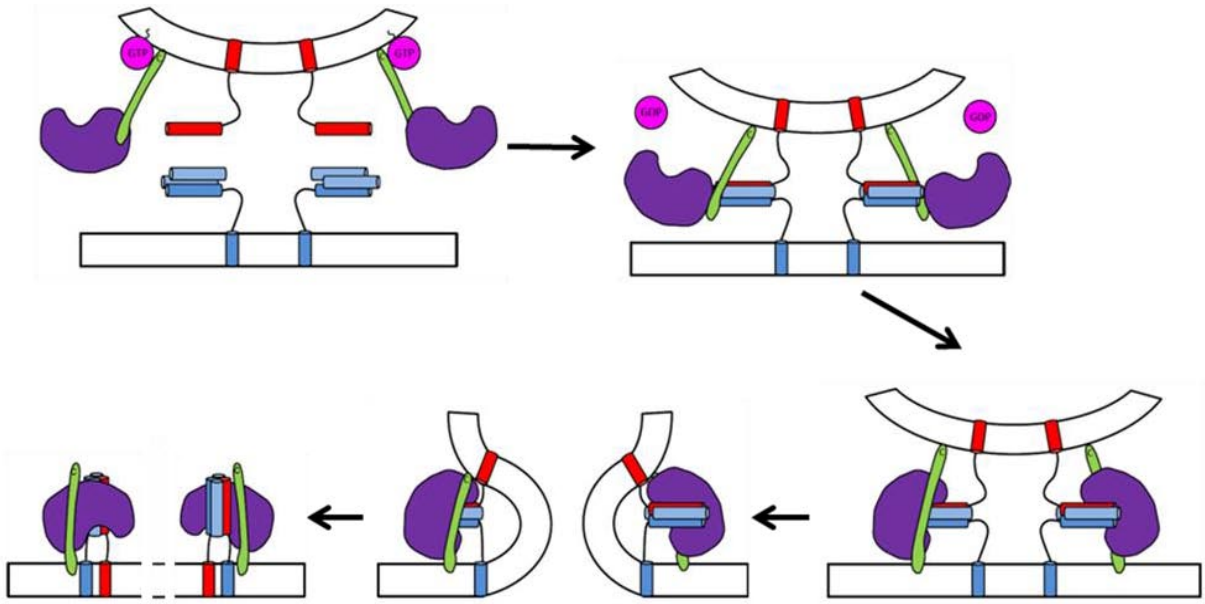

Figure 8. A Schematic model of the membrane fusion during yeast exocytosis. In the model, Mso1p (green, C marks the C-terminus) and GTP-Sec4p (pink) interact on the arriving vesicle (top left panel). GTP hydrolysis of Sec4p releases Mso1p from Sec4p and makes it available for interaction with the N-peptide binding area of Sec1p (purple) and with Sso1/2p (top right panel). Binding of Sec1p to the SNARE components (Snc1/2p in red, Sso1/2p in dark blue, Sec9p in light blue) triggers SNARE complex assembly (bottom right panel) leading to membrane fusion (bottom middle panel). During this process, we propose that Mso1p stays bound to the vesicle membrane via its C-terminus and inserts to the plasma membrane with its N-terminus. After fusion, Mso1p and Sec1p stay bound to the *cis*-SNARE complex until the SNARE complex is disassembled (bottom left panel).

Our results indicate that the cooperation between Mso1p and Sec4p occurs prior to Mso1p's function as an adaptor between Sec1p and the SNARE complex. The detailed sequence of events in the Mso1p-Sec4p interplay and the mechanistic switch to Mso1p-Sec1p-SNARE complex formation remain to be resolved in future work.

At a later step in membrane fusion, Mso1p appears to be interacting with plasma membrane lipids. The most prominent binding was observed for PIPs containing membranes. This interaction is necessary for the Mso1p *in vivo* function. We propose that the interlinking of Sec1p and the assembling SNARE complex via Mso1p to the plasma membrane is crucial for fixing the secretion machinery at the site of membrane fusion (Figure 8).

In vitro Mso1p has weak vesicle clustering capability. It is possible that Mso1p provides a bridge between the vesicular and plasma membrane. Mso1p interacts with Sec4p on the vesicle via its C-terminus. The SNARE and Sec1p interaction is mediated by central amino acids, with the Sec1p interaction site more N-terminal. At the very N-terminus of Mso1p there is the phospholipid insertion area. Taken these interactions into the context of the membrane fusion machinery, Mso1p can be positioned right between the vesicle and the plasma membrane. NMR and gel filtration studies indicated that Mso1p is an elongated unstructured protein (Konstantin Chernov, unpublished data). Mso1p could be able to work like a spring zipping up the membrane fusion machinery while interacting with its partners from the vesicle to the plasma membrane. We propose a model for Mso1p as an adaptor protein in membrane fusion. Given the different interaction partners, Mso1p could be part of a network to facilitate protein-protein interactions which control the different steps of the membrane fusion. A future challenge will be to reveal the place and time of these interactions in the order of events in the membrane fusion regulation.

In yeast, the proteins Vac1p and Ivy1p have similar interaction properties as Mso1p. Furthermore, in mammalian exocytosis, there is a vast array of regulatory proteins interacting with lipids and small GTPases of the secretion machinery. We propose that Vac1p and Ivy1p are functional homologues of Mso1p. Similar to Mso1p, they might function as adaptors in vacuolar and endosomal membrane fusion. In mammalian cells, the function of connecting the secretion machinery is fulfilled by many proteins, partially overlapping in their interaction capabilities. The presented work sheds

new light in understanding the function of SM proteins in the interplay with the SNARE complex and the adaptor proteins. The identified interactions of the SM binding protein Mso1p with membrane lipids and the small Rab GTPase Sec4p reveal that these types of interactions are also involved in yeast exocytosis. We propose that the present study reveals a new level of evolutionary conservation in the membrane fusion process from yeast to mammalian cells.

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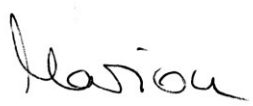

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