Ssp1p is a lipid binding protein involved in shaping of the prospore membrane during meiosis in *S. cerevisiae*

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Summary

Meiosis is a special form of cell division which results in the formation of haploid cells. At the end of meiosis in yeast four new cells, the so-called spores, are formed inside the boundaries of the mother cell. This morphogenetic process requires *de novo* formation of the cell membranes. Surprisingly it is the spindle pole bodies (SPBs) which organize the formation of these prospore membranes (PSMs). Membrane vesicles, probably redirected from the secretory pathway, accumulate at the cytoplasmic face of the SPB and fuse to form a continuous membrane system. In our lab we have identified a protein complex which localizes to the tip of this growing membrane, the leading edge protein coat (LEP coat).

In this thesis I demonstrate that the *S. cereviaiae* protein Ssp1p is a novel component of the LEP coat. It is even the most important constituent of the LEP coat as it recruits all other components to this structure. I can show here that Ssp1p is a lipid binding protein and this affinity is probably required for anchoring the protein complex in the membrane. Ssp1p would then act like a hinge, connecting the PSM to Ady3p, another protein of the LEP coat. Deletion of *SSP1* results in a block of sporulation. Here I show that this is most probably due to a defect in membrane shaping. In the deletion mutant the PSM sticks very tightly to the nuclear envelope and closes without incorporating cytoplasm. This later on leads to a defect in spore formation and to a loss of viability. One possible explanation for this phenotype would be that the LEP coat serves as a scaffold which provides some structural stability to the membrane while it grows. The protein ring at its tip might also keep the membrane open until all constituents required are enclosed. In addition to its lipid binding activity it is demonstrated that Ssp1p localizes to the plasma membrane of the bud when it is overexpressed in mitotic cells. This is a hint that Ssp1p might be involved in membrane vesicle targeting. Ssp1p could either be directed to target membranes by their lipid composition or by other interacting proteins. As overexpression of Ssp1p in mitotic cells is toxic some interference with the secretory pathway machinery is proposed.

Zusammenfassung

Meiose ist eine spezielle Form der Zellteilung, als Endprodukte entstehen haploide Zellen. In der Bäckerhefe *S. cerevisiae* werden am Ende der Meiose vier Tochterzellen, die sogenannten Sporen, innerhalb des alten Kompartiments der Mutterzelle gebildet. Dieser Differenzierungsprozess erfordert die komplette Neubildung der äußeren Membranen. Überraschenderweise wird diese Neubildung der sogenannten Prospormembranen von den Spindelpolkörpern organisiert. Membranvesikel, die vermutlich aus dem sekretorischen Pfad stammen, werden umgeleitet und akkumlieren am Spindelpolkörper, wo sie miteinander fusionieren und eine kontinuierliche Membran bilden. In unserem Labor haben wir haben einen Proteinkomplex identifiziert, der an der Spitze dieser wachsenden Membran lokalisiert und dort eine ringförmige Struktur ausbildet.

In dieser Arbeit konnte ich zeigen, daß Ssp1p eine weitere Komponente dieses Proteinkomplexes ist. Ssp1p ist sogar die wichtigste der bisher identifizierten Komponenten, da die Lokalisierung aller anderen Proteine an diese Membranstruktur von Ssp1p abhängt. Die Entdeckung, daß Ssp1p ein Lipid-bindendes Protein ist, erklärt den Aufbau dieser Struktur. Ssp1p interagiert auf der einen Seite mit Ady3p, einem Bestandteil dieses membranständigen Proteinkomplexes, auf der anderen Seite verankert es mittels seiner Fähigkeit zur Lipidbindung den ganzen Komplex in der Membran. Wird *SSP1* deletiert so verliert die Zelle die Fähigkeit zur Sporulation. Ich konnte zeigen, daß der Grund hierfür wahrscheinlich in einer Störung der Ausbildung der Prospormembran liegt. In der Deletionsmutante liegt die Prospormembran extrem eng auf der Kernmembran und der Abschluß erfolgt bereits, bevor Cytoplasma eingeschlossen werden konnte.

Dies führt dann später zu einem Defekt der Sporenbildung und einem Verlust der Lebensfähigkeit. Eine mögliche Erklärung für die Funktion dieses membranständigen Proteinkomplexes wäre, daß durch sein stabiles Gerüst der wachsenden Membran eine gewisse strukturelle Rigidität gegeben wird. Es ist auch denkbar, daß der Proteinring die Membran geöffnet hält bis alle notwendigen Bestandteile aufgenommen wurden. Zusätzlich zur Lipidbindungsaktivität von Ssp1p wurde entdeckt, daß Ssp1p, wenn es mitotisch überexprimiert wird, an der Plasmamembran der Tochterzelle (Knospe) lokalisiert. Dies könnte bedeuten, daß Ssp1p eine Rolle bei der Lenkung der sekretorischen Membranvesikel spielt. Durch die Bindung an spezielle Lipide könnte Ssp1p in Membranbereiche gelenkt werden, die Ziele für solche sekretorischen Vesikel darstellen. Diese spezielle Lokalisierung könnte aber auch durch Proteininteraktionen bewirkt werden. Da die Überexpression von Ssp1p für mitotische Zellen toxisch ist liegt es nahe anzunehmen, daß dieses Protein einen Einfluß auf die Funktion der Proteine des sekretorischen Pfades hat.

Abbreviations

aa’s	amino acids
AD	activating domain
APC	anaphase promoting complex
BD	binding domain
BSA	bovine serum albumine
cc	coiled coil
CIP	calf intestine phosphatase
DAPI	4’-6-diamino-2-phenylindoledihydrochloride
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DSB	double strand break
DTT	dithiothreitol
EDTA	ethylenediamintetra acidic acic
EGTA	ethylene-bis(oxyethylene-nitrilo)tetra acetic acic
EM	electron microscopy
EtOH	ethanol
5’FOA	5-fluoroorotic acid
g	gravitation constant
GFP	green fluorescent protein
h	hours
HA	hemagluttinine
HU	high urea
IPTG	b-D-thiogalactopyranoside
KD	Kilo Dalton
LEP coat	leading edge protein coat
M	molar
MD	Mega Dalton

mg	milligramme
min	minutes
ml	milliliter
mM	millimolar
NE	nuclear envelope
NSF	N-ethylmaleimide-sensitive-factor
mRNA	messenger ribonucleic acid
OD	optical density
P	pellet
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	negative decadic logarithm of $[H^+]$
PH	pleckstrin homology
PIP	phosphatidylinositolphosphate
ProA	protein A
PSM	prospore membrane
PX	phox homology
RE	raw extract
rRNA	ribosomal ribonucleic acid
S	supernatant
SC	synaptonemal complex
SC medium	synthetic complete medium
SDS	sodium dodecyl sulfate
SPB	spindle pole body
spo	sporulation
TBS	tris buffered saline
TCA	trichlor acetic acid
tSNARE	target soluble NSF attachment protein receptor
w/v	weight per volume

Chapter 1

Introduction

1.1 Sex and the single cell: Meiosis in Yeast

Meiosis is a specialized cell cycle which results in the formation of four haploid progeny from one diploid zygote. The outcome of this process, haploid gametes, are required for sexual reproduction. Meiosis ensures genetic diversity in the population and is therefore central to eukaryotic life.

In meiosis, one round of DNA replication is followed by two consecutive rounds of DNA segregation (Figure 1). This results in the reduction of the chromosome complement by half. In the first division, the replicated, homologous chromosomes are segregated to opposite poles. Meiosis I is also called the reductional division. In meiosis II, the sister chromatids are separated, this resembles more the mitotic division.

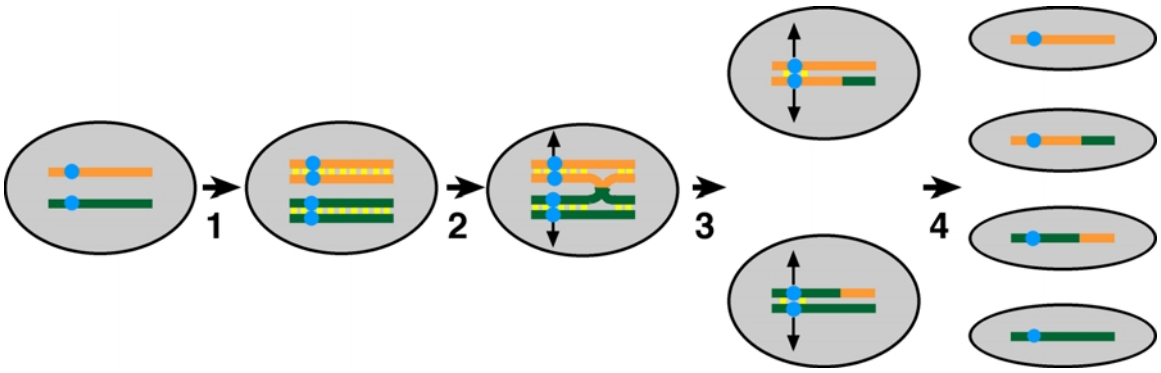

Figure 1:
Chromosome segregation during meiosis. Depicted are two homologous chromosomes in green and orange. (1) DNA replication during meiotic S phase, cohesion is established between the sister chromatids (symbolized as yellow squares). (2) Recombination events ultimately lead to strand exchange between the two homolgous chromosomes (3) Meiosis I, reductional division, homologs are separated; cohesins are cleaved along the arms but persist in the centromeric region. (4) Meiosis II, equational division, sister chromatids are separated; the residual cohesins are cleaved.

Special features of the meiotic cell cycle

Compared to the standard mitotic division, several modifications to the cell cycle machinery have to be made in meiosis (Lee and Amon, 2001).
In mitosis, sister chromatids are segregated to each daughter cell. The mitotic spindle attaches to the two sister kinetochores of one chromosome and pulls the sister chromatids apart. This pulling force has to be resisted by an association between the chromatids until the onset of anaphase. This association is mediated by a multiprotein complex called cohesin. In *S. cerevisiae*, loss of sister chromatid cohesion depends on Esp1p (Separin) induced proteolytical cleavage of the cohesin subunit Scc1p (Uhlmann et al., 1999). Interestingly, in meiosis the cohesin Scc1p is replaced by its meiosis specific homolog Rec8p. At anaphase of meiosis I, Rec8p is only cleaved along the chromosome arms but not in the centromeric region (Klein et al., 1999). This allows recombination along the chromosome arms but prevents sister chromatid separation in meiosis I. By an unknown mechanism Rec8p in the centromeric region is protected from cleavage until anaphase II, when the sister chromatids then are segregated.
Additonally, before meiosis I, homologous chromosomes have to pair and establish physical interactions. This is achieved by recombination events, which are discussed in more detail in the next chapter.
Another major difference in meiosis I, compared to the mitotic division, is that the sister kinetochores have to attach to microtubules emanating from the same spindle pole body (SPB). Although the mechanism by which this is achieved remains elusive, a mutation that prevents this co-orientation has recently been identified (Toth et al., 2000).

Meiosis in *Saccharomyces cerevisiae*

Although many of the recent findings have been made in yeast, either in *S. cerevisiae* or in *S. pombe*., for cohesins and for other meiotic proteins homologs exist in higher eukaryotes. This means that many landmarks of meiosis are conserved from yeast to mammals. Nevertheless, there are of course also major differences between the species concerning meiosis. In higher eukaryotes, only a specific subset of cells, the germ cells, are able to undergo meiosis. The outcome, namely sperm and oocytes, are the only haploid cells in higher eukaryotes. In contrast, yeast can reproduce rather indistinguishably in either a haploid or a diploid life cycle. If haploid yeast from the two opposite mating types, the sex equivalents in yeast, happen to meet under rich nutritional conditions, they mate to form a diploid yeast cell. The two haploid nuclei fuse to give rise to one diploid nucleus, a process called karyogamy.

The transition from diploid to haploid stage is induced by poor nutritional conditions, namely starvation for glucose and nitrogen. It results in the formation of four haploid progeny, the spores, inside the boundaries of the mother cell. Meiosis in yeast is therefore also called sporulation. The spores can then germinate to enter again the haploid life cycle (Figure 2).

This mechanism is thought to have evolved because the spores are more resistant to harsh environments and starvation might signal the onset of such conditions. Another major difference to meiosis in higher eukaryotes is that in yeast meiosis the nuclear envelope does not break down during the divisions and that there is no cytokinesis after the first meiotic division. Instead, the cellularization of the four doughter cells occurs coordinated at the end of meiosis II.

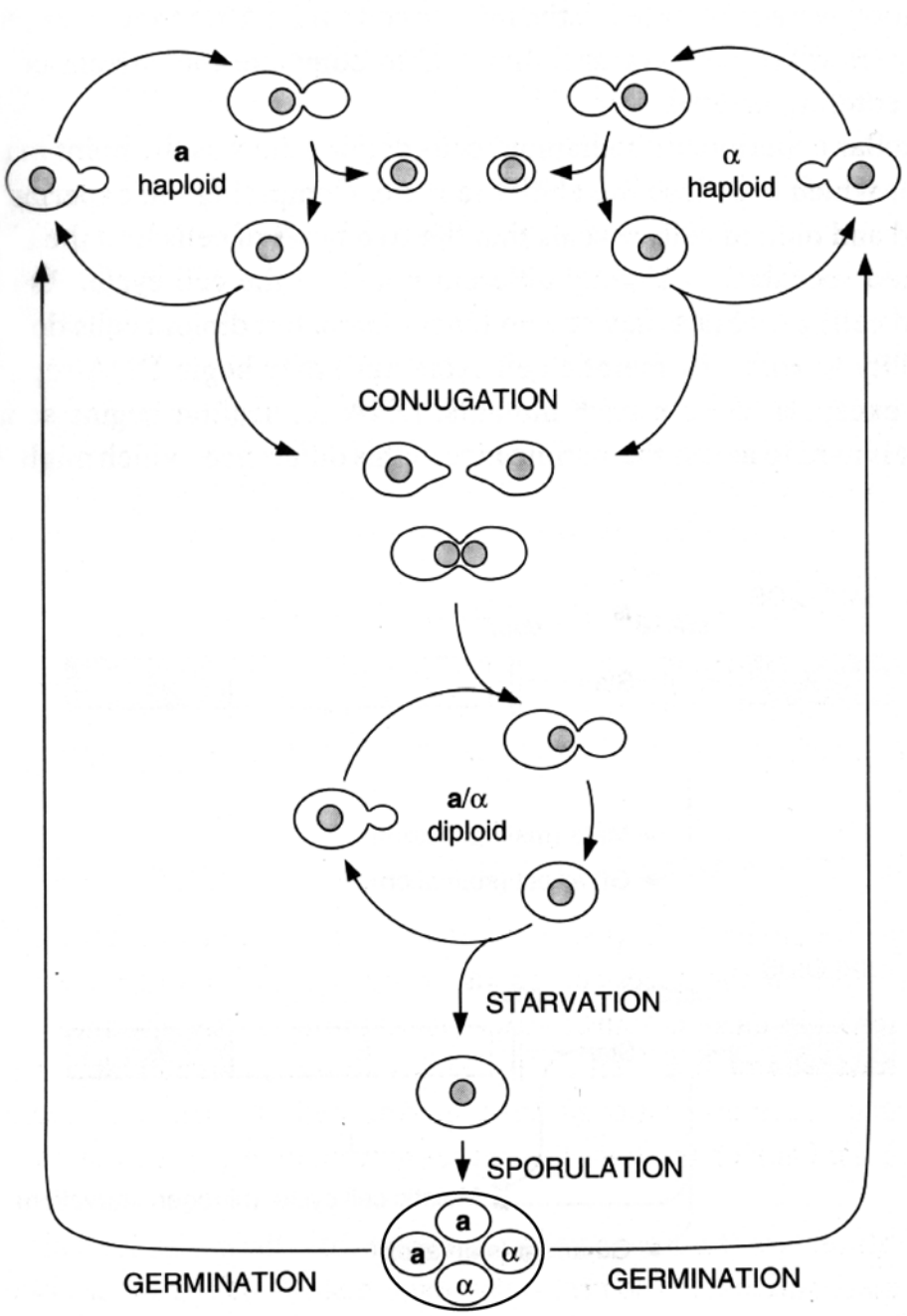

Figure 2:
Life cycle of the budding yeast *S. cerevisiae*. Transition from the haploid to the diploid state is called mating or conjugation. Upon starvation a diploid cell forms four haploid spores. This process is termed sporulation and requires the meiotic cell cycle. This model is adapted from Murray and Hunt, 1993.

1.2 Prophase of meiosis I: Recombination events during assembly and disassembly of the Synaptonemal Complex

Preceding the first meiotic division, recombination allows the exchange of genetic material between the two parental homologous chromosomes. This process generates offspring with a reassortment of parental genes. The wide distribution of sexual reproduction among eukaryotes demonstrates the great advantages of the meiotic cell cycle in achieving genetic diversity.
In the lengthy prophase of meiosis I a sequence of highly coordinated events culminates in the formation of chiasmata between homologous chromosomes (Roeder, 1997; Zickler and Kleckner, 1998). These chiasmata are required for proper chromosome segregation at the anaphase of meiosis I.
Prophase of meiosis I can be divided into several substages according to recombination events and chromosome morphology (Figure 3).

The recombination pathway in meiosis

In Leptotene, meiotically induced Double Strand Breaks (DSBs) at certain hot spots of recombination appear. The formation of both possible end products of recombination, crossover and gene conversion, initially depends on DSBs. A number of yeast mutants are deficient in the formation of DSBs. One of them, Spo11p, a protein with homology to type II topoisomerases, was identified as the strand cleaving enzyme (Keeney et al., 1997). A *spo11* mutant produces inviable spores, this is due to random chromosome segregation at meiosis I as a result of lacking of crossovers (Borts et al., 1986).
Double strand cleavage is followed by exonucleolytic digestion to produce single stranded tails with 3' termini.

The 5’ to 3’ exonucleolytic activity required for the resection of the ends of DSBs is dependent on Rad50p and Mre11p (Alani et al., 1990). The next step of DSB repair is the invasion of a single stranded tail into the neighboring homologous DNA duplex. This reaction is catalyzed by homologs of the bacterial strand exchange protein recA, including Rad51p and Dmc1p (Bishop et al., 1992; Shinohara et al., 1992). The following repair synthesis and branch migration results in the formation of Double Holliday junctions. DSB repair processes take place in the Zygotene stage.

In a Double Holliday junction the two initial helices are held together by reciprocal exchange of two of their four strands. Double Holliday junction resolution during Pachytene requires the cleavage of the two strands connecting the two helices. In a regulated set of rotational movements Double Holliday junctions can isomerize. This leads to a conversion of crossing strands into non-crossing and vice versa. Depending on which strands are cleaved, different recombination products are made. If the two originally crossing strands are cut, only the short segment corresponding to the length of the heteroduplex DNA is exchanged, this is termed gene conversion. However, if after isomerization the pair of originally non-crossing strands is cut, then a major segment of double stranded DNA is reciprocally exchanged (see also Figure 1). This event is called crossover. Crossover are not only more important for establishing genetic diversity, they are also required for faithful chromosome segregation in meiosis I. Crossovers lead to the formation of chiasmata, stable connections between homologs which persist after the Synaptonemal Complex (SC) has disassembled. Chiasmata are cytological markers which correspond to the reciprocal breakage and rejoining of two nonsister chromatids. These chiasmata resist the pulling force of the meiosis I spindle in anaphase to signal the correct attachment of the two homologs to microtubles emanating from opposite spindle poles.

Gene conversion has no effect on the fidelity of chromosome segregation.

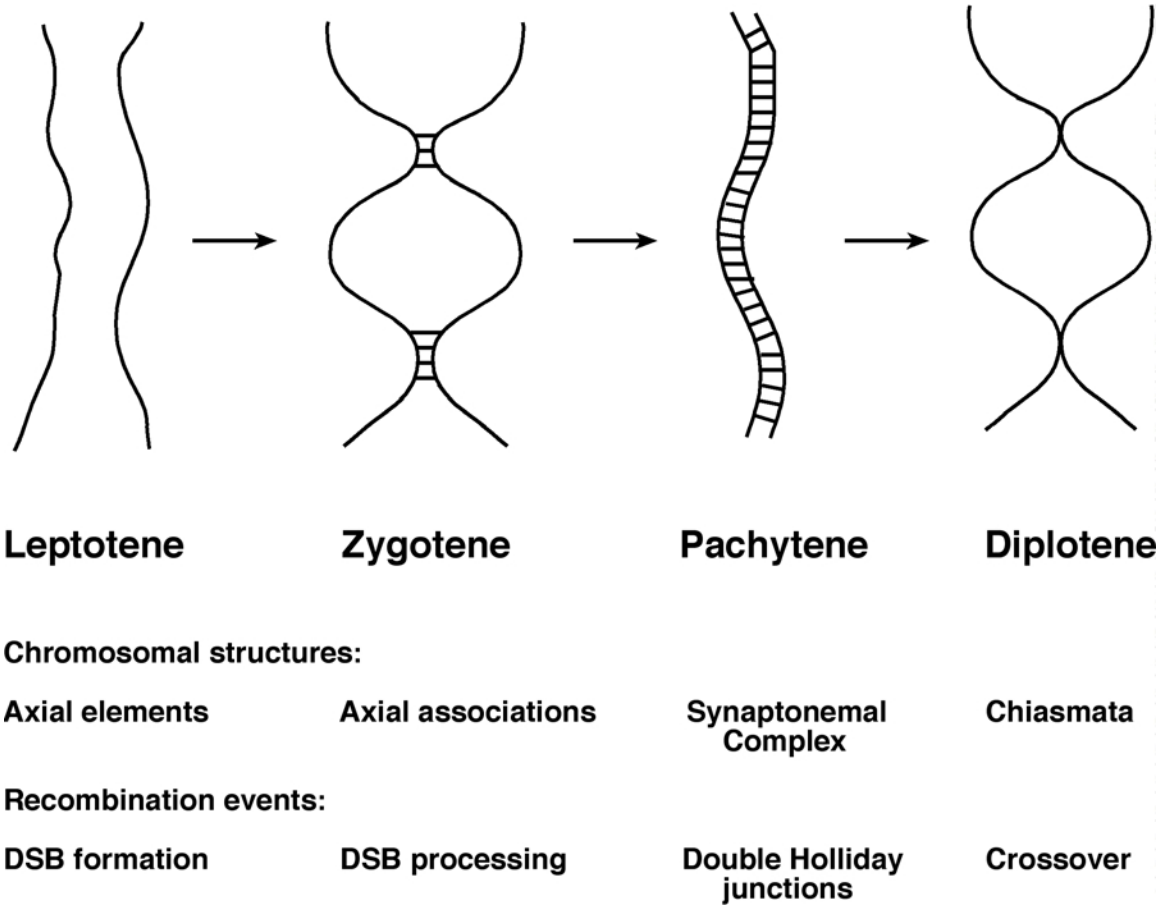

Figure 3:
Cell cycle stages in the Prophase of the first meiotic division. The two curved lines correspond to Axial elements, the proteinaceous cores of the two homologous chromosomes. Connections between the homologs are mediated by either DSB repair proteins (Zygotene) or Synaptonemal Complex proteins (Pachytene)

Chromosme morphology in meiotic prophase

During prophase of meiosis I the chromosome morphology changes dramatically (Figure 3). First, as a prerequisite for later steps, homologous chromosomes have to find and recognize each other in early prophase in a process called homologous pairing. Pairing refers to the side by side alignment of homologs at a variable distance. Although it is generally believed that homologous pairing involves a genome wide search for sequence homology, no clear picture of this process has yet emerged. Homologous pairing precedes synapsis and is separable from it, as several mutants pair but fail to synapse (Loidl et al., 1994). In some organisms, e.g. budding yeast and fission yeast, homologs are also associated to a certain extent in nonmeiotic diploid cells (Scherthan et al., 1994; Weiner and Kleckner, 1994). In contrast, homologs are not paired premeiotically in mouse and men (Scherthan et al., 1996).
At Leptotene, each pair of sister chromatids develops a common proteinaceous core. From this linear axis the chromatin loops out and later the loops of both sisters come to lie on the same side of the axis (von Wettstein, 1984). This structure is called an axial element and is unique to the meiotic division. The proteins which localize to axial elements and promote their formation in budding yeast are Red1p and Hop1p (Hollingsworth et al., 1990; Rockmill and Roeder, 1990; Smith and Roeder, 1997). Axial elements of homologous chromosomes then periodically come into close vicinity to form axial associations. These association sites are also thought to be the sites of recombination (Albini and Jones, 1984). In axial association, homologs approach to a distance of around 0.4 µm.

The Synaptonemal Complex

Starting at the association sites the SC polymerizes along the whole length of two axial elements to hold the homologs in intimate association at a distance of around 0.1 µm. Pachytene is the cell cycle stage with fully assembled SC.

The SC is a zipper-like structure with many transverse filaments lying perpendicular to the length axis (Figure 4). These transverse filaments comprise the central region of the SC which contains very little DNA. In mature SC, the axial elements are now referred to as lateral elements.

In *S. cerevisiae*, the best characterized protein of the SC is Zip1p. Zip1p localizes along the entire length of fully synapsed chromosomes, but not to unsynapsed axial elements (Sym et al., 1993). The idea that Zip1p might be the transverse filament of the central region was confirmed in a convincing set of experiments. Zip1p consists of two globular domains at either N- and C-terminus which are linked by an extended coiled coil domain (Figure 4). Coiled coil domains are known to promote dimerization and complexes containing coiled coil proteins often serve as structural scaffolds (Zimmerman et al., 1999). If the coiled coil domain of Zip1p is artificially elongated, the width of the central region of the SC is increased (Sym and Roeder, 1995). Experiments designed to elucidate the organization of Zip1p in the central region of the SC propose an arrangement of parallel homodimers (or higher multimers) which are linked to the lateral elements with their C-termini and having their N-termini overlapping in the middle of the central region (Dong and Roeder, 2000).

In Δ*zip1* strains SC structures are never observed. However, homologs are paired and axial elements are formed. This results in the activation of a checkpoint at the exit of Pachytene triggering a cell cycle arrest (Roeder and Bailis, 2000). The Pachytene checkpoint is most likely activated by recombination intermediates. Mutants, which are unable to engage in recombination, e.g. Δ*spo11*, are not affected by this checkpoint (Klapholz et al., 1985).

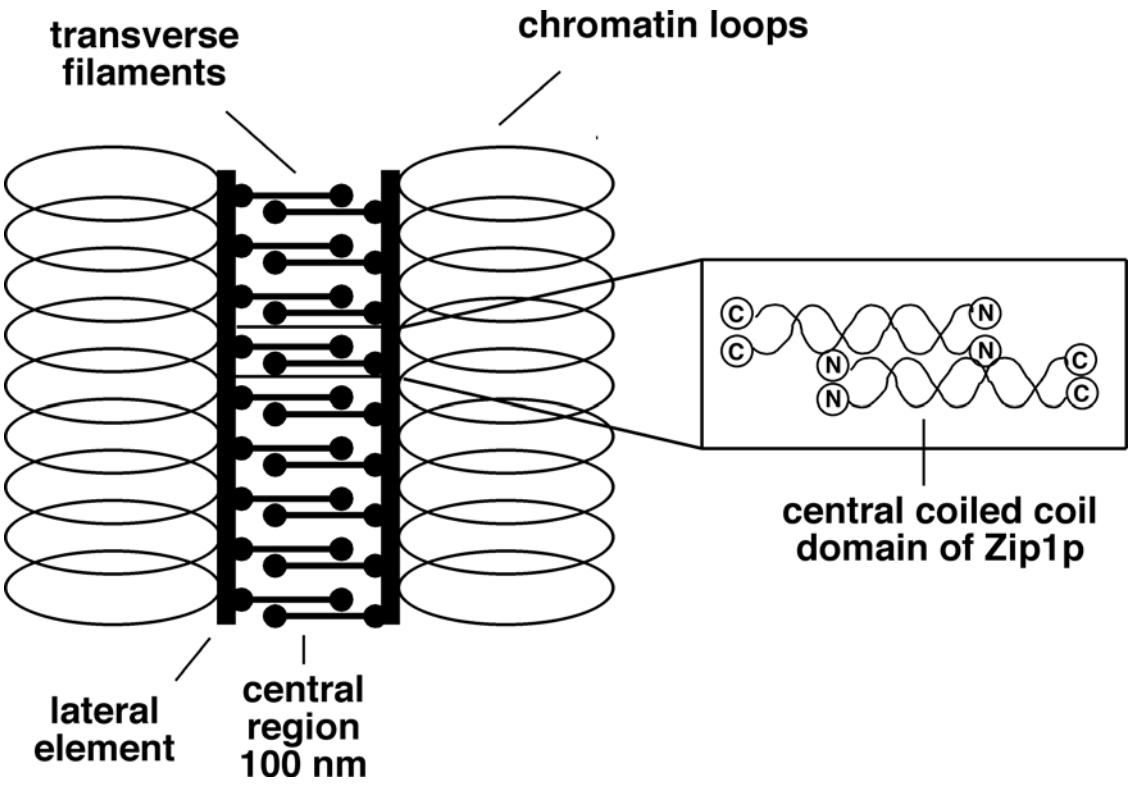

Figure 4:
Model for the assembly of Zip1p in the Synaptonemal Complex. The two homologous chromosomes are held together by this protein complex. Chromatin loops out to one direction from the proteinaceous lateral elements. The two lateral elements are linked by numerous transverse filaments, which consist presumably of parallel Zip1p homodimers arranged in the indicated way. Model adapted from Sym, Engebrecht and Roeder, 1993.

Even though in the Δ*zip1* mutants no SC is formed, commitment to recombination is nearly at wild type level (Sym et al., 1993).

After Pachytene, desynapsis begins as SC proteins dissociate and the two homologs lose their contacts along the whole length. In Diplotene, the homologs are now held together by chiasmata, which are formed at crossover sites. It is important to note that before the first meiotic chromosome segregation the SC has completely disassembled (Roeder, 1997).

Function of the Synaptonemal Complex

For a long time it was believed that the SC promotes recombination by bringing homologs into spatial proximity. Now this view is challenged by the finding that there is recombination without SC. On the other hand, analysis of mutants which are unable to induce DSBs shows that all of them are deficient in formation of the SC (Roeder, 1995). This means that SC formation is dependent on recombination, and not vice versa. However, then the function of the SC becomes enigmatic.
Other experiments propose a role for the SC in crossover interference (Sym and Roeder, 1994). Distribution of crossovers is nonrandom in the sense that two crossovers on the same chromosome arm rarely occur close together. This phenomenon could involve the transmission of an inhibitory signal from one crossover site to a nearby potential crossover site (Maguire, 1988). In the Δ*zip1* mutant crossover interference is completely abolished and crossovers are distributed randomly among all chromosomes (Sym and Roeder, 1994). Small chromosomes then often fail to crossover resulting in nondisjunction of those chromosomes. How crossover interference works remains unclear but the SC might provide the conduit for it.

1.3 Building new cells within old ones: The morphogenetic program at the end of meiosis involves *de novo* membrane formation

Looking at the formation of daughter cells at the end of the second meiotic division, there is one striking difference to most other reproduction events: The new membranes and cell walls form *de novo* instead of arising from a preexisting system, as for example during budding (Kupiec, 1997). At the end of meiosis, four daughter cells are formed inside the boundaries of the mother cell. The plasma membrane of the mother cell is not affected during this differentiation process.
The nuclear envelope remains intact during the meiotic divisions and the nucleus is not divided at the end of meiosis I. In anphase II, the nucleus is still undivided and consists of four nuclear lobes with one spindle pole body (SPB) situated at the top of each lobe (Figure 6).

Spindle pole bodies meet an additional task in meiosis

It has been noticed for a long time that the SPB plays a crucial role in meiotic differentiation which is completely different from its regular determination (Moens and Rapport, 1971). SPBs, the centrosome equivalents in yeast, usually serve as microtubule organizing centers. In late meiosis II, however, they are modified to become the site for organization of the newly forming prospore membrane. The outer plaque of the SPB is enlarging and does no longer nucleate cytoplasmic microtubules (Figure 5). The function of cytoplasmic microtubules in mitotic cells is to align the spindle along the mother-bud axis and to position the nucleus at the bud neck. It is obvious that this function is no longer required in meiotic cells. Nuclear microtubules, however, are still anchored at the inner plaque of the SPB during meiosis.

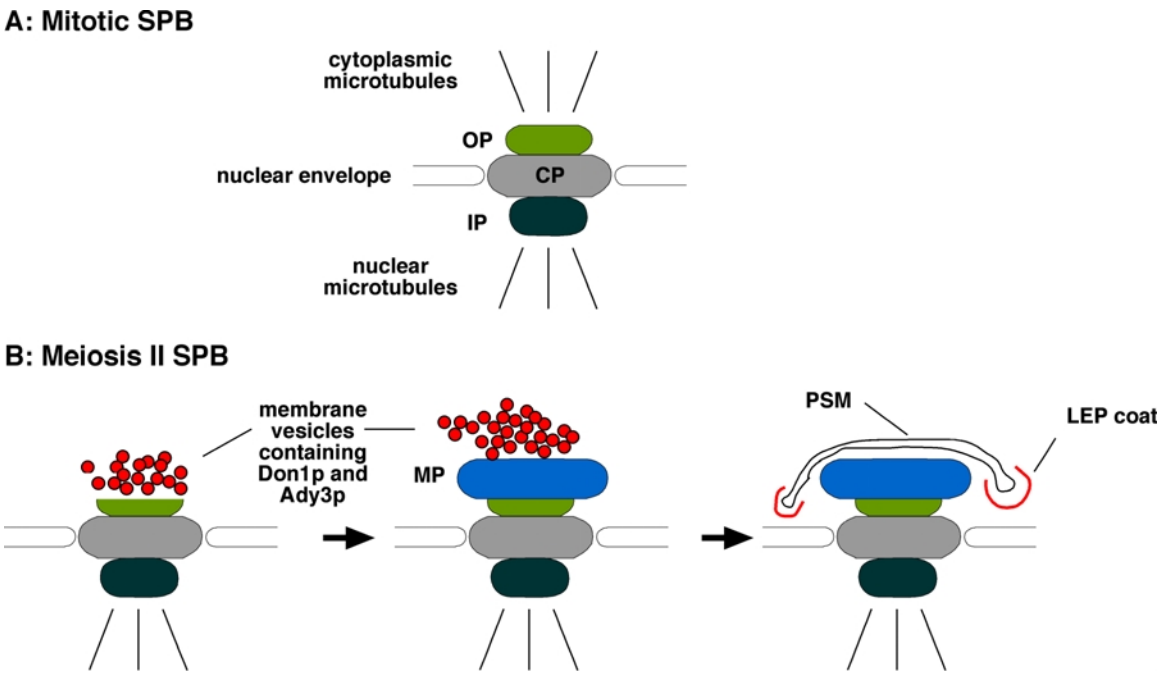

Figure 5:
Model for the modification of the spindle pole body in meiosis. OP = outer plaque, CP = central plaque, IP = inner plaque, MP = meiotic plaque. In meiosis, the microtubule binding capability of the cytoplasmic side (part of the OP) is lost and replaced by the meiotic plaque. This modification is required for the formation of the prospore membrane.

At anaphase II, Mpc54p and Mpc70p, meiosis specific components of the outer plaque, replace the γtubulin binding protein Spc72p at the cytoplasmic face of the SPB (Knop and Strasser, 2000). The so-called meiotic plaques are then required as attachment sites for the prospore membranes (PSMs). These membranes engulf the dividing nucleus and later mature to form the spore walls. In deletion strains for either Δ*mpc54* or Δ*mpc70* the assembly of vesicles into a continuous membrane is inhibited and no PSMs are formed (Knop and Strasser, 2000).

Discovery of proteins localizing specifically to a substructure of the prospore membrane

The morphological processes during meiotic differentiation have been described already a long time ago, mainly in electron microscopy (EM) studies (Moens, 1971). Membrane vesicles fuse at the SPB to build a continuous membrane system which engulfs the postmeiotic nucleus (Figure 6). After incorporation of cytoplasm the PSMs close and spore wall material is deposited between the two double membranes to form the inner layer of the prospore walls. Prospore walls aquire additional outer layers and mature to spore walls.

Although the cytological pathway was known, the molecular components required for this process remained unidentified for a long time. This changed recently when the meiotic plaque components Mpc54p and Mpc70p were identified (Knop and Strasser, 2000). The localization pattern of their GFP fusion proteins allowed to identify them as SPB components. This was done in a large scale genomic GFP-tagging study for meiosis specific coiled coil proteins. In the same screen, another protein, Don1p, showed a very distinct localization pattern during meiosis (Knop and Strasser, 2000). Don1p-GFP can be detected first in late meiosis I as a punctate staining in the cytosol. As shown by membrane specific staining with antibodies against the tSNAREs Sso1/2p, these dots correspond to membrane vesicles (Moreno-Borchart et al., 2001). In early meiosis II these Don1p-GFP containing vesicles cluster at the SPBs of the two short spindles. In anaphase II, as the spindles elongate, Don1p-GFP moves inward to build four ring like structures, two of them surrounding each bundle of microtubules (Knop and Strasser, 2000). This unique, donut-like staining also initiated the name for the new protein. Each ring corresponds to the growing tip of one PSM which is still open and surrounds one spindle axis (Figure 6).

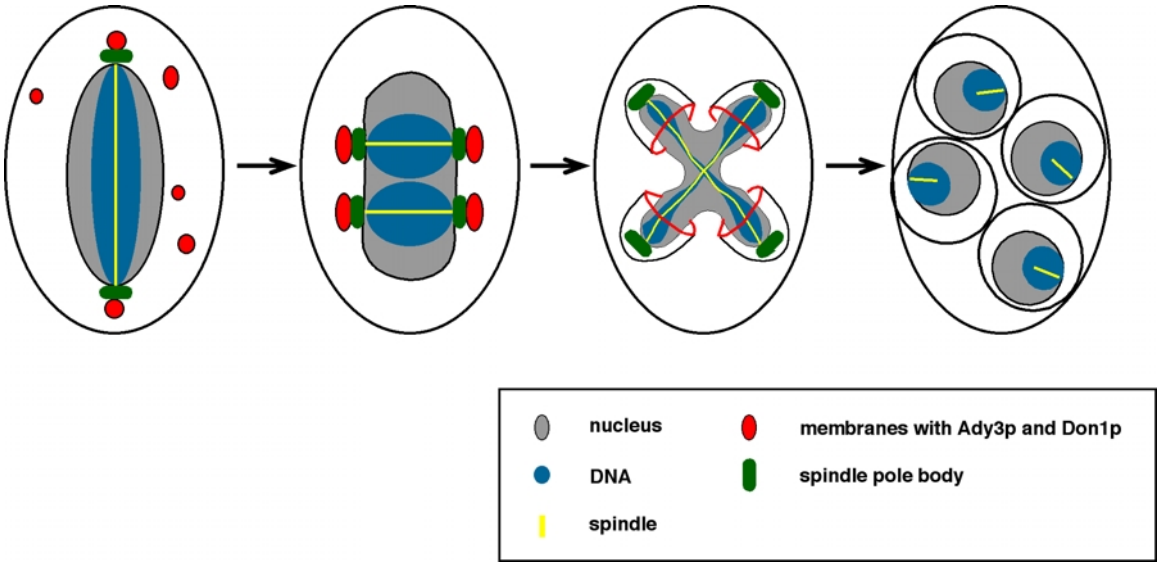

Figure 6:
Model for the morphogenetic processes at the end of meiosis. Precursor membrane vesicles accumulate in the cytoplasm in late meiosis I, they are redirected to the spindle pole body in early meiosis II. As the spindle elongates in later stages of meiosis II the proteins of the LEP coat, Ady3p and Don1p, relocalize from the SPB to this ring structure. As the PSM closes the LEP coat is disassembled.

In the following, this structure is termed the leading edge protein (LEP) coat. The LEP coat represents a new protein complex which localizes transiently, only during the period of PSM enlargement, to the tip of this membrane.

Membrane growth is mediated by ongoing fusion of membrane vesicles to the PSM. The exact order of events at the end of this differenciation process remains unclear. As the prospore membranes close the LEP coat rings disassemble and Don1p-GFP is found more diffuse in the prospore wall. In Δ*don1* strains no detectable phenotype is observed, PSM assembly and spore formation occur at wild type level.

Another meiosis specific protein, Ady3p, was identified as a Mpc54p-ProA binding partner as it copurified with preparations of Mpc54p-ProA (Moreno-Borchart et al., 2001). The same protein was shown to be required for efficient sporulation in an independent genomic approach (Rabitsch et al., 2001). The name of this protein stems from the deletion phenotype to produce many dyads with two spores in one ascus instead of four (accumulation of dyads). Viability for the spores formed in the Δ*ady3* background is normal and this excludes a defect in chromosome segregation. In immunofluorescence experiments, Ady3p revealed a perfect colocalization with Don1p-GFP at all stages, therefore representing the second component of the LEP coat. The interaction of Ady3p with the SPB component Mpc54p must be of transient nature as in anaphase II Ady3p is relocalized from the SPB to the LEP coat. Immuno-EM pictures detecting Ady3p at the tip of the PSM provided final proof that Ady3p is associated with the prospore membrane (Moreno-Borchart et al., 2001). The phenotype of inefficient spore formation in the Δ*ady3* strain is not due to a defect in PSM assembly, as the PSMs are formed at wild type level. This points to a role of Ady3p in either PSM closure or spore maturation, but the exact mechanism is still unclear. Moreover, the binding of cytosolic precursors of the PSM to the SPB seems to depend on Ady3p. However, this effect is only dominant in mutants missing the meiotic plaque (Moreno-Borchart et al., 2001). The colocalization of Ady3p and Don1p suggests an interaction of the two proteins and in fact this has been observed in a two hybrid screen (Ito et al., 2000). Furthermore, in the Δ*ady3* strain Don1p localizes dispersed in the cytosol and does not form the characteristic LEP coat rings (Moreno-Borchart et al., 2001). Conversely, Ady3p localization is not altered in a Δ*don1* deletion strain.

Ssp1p was shown by two groups to be a two hybrid interactor of Ady3p (Ito et al., 2000; Uetz and Hughes, 2000). The phenotype of the Δ*ssp1* deletion strain in meiosis is severe in the sense that spore formation is completely abolished (Nag et al., 1997).

The predicted interaction between Ssp1p and Ady3p prompted us to investigate the role of Ssp1p in the process of spore formation. It was tempting to speculate that Ssp1p might localize to the same structures as Ady3p (Figure 6).
The protein sequence of Ssp1p neither has detectable similarity to any known proteins in other organisms nor does it contain any predicted domains. Here we show that Ssp1p is another component of the LEP coat. Recruitment of the other two proteins, Don1p and Ady3p, to the LEP coat depends on Ssp1p function (Moreno-Borchart et al., 2001). We further demonstrate that Ssp1p binds to phospholipids and that this interaction is most likely required for the assembly of these proteins on the membrane. In the Δ*ssp1* deletion strain, which is devoid of a LEP coat, PSMs are misshaped and fail to incorporate cytoplasm before closure. This observation explains the failure to form mature spores and the loss of viability later in the course of sporulation.

During meiosis membrane vesicles of the secretory pathway are redirected to the spindle pole body

Among the most important open questions concerning this differentiation process is the targeting of membrane vesicles to the spindle pole body. Where do those vesicles suddenly come from and what signal directs them to the SPB?
When yeast cells grow under rich nutritional conditions they divide every hour. This requires a massive membrane build-up at the bud site, so the main route of vesicle trafficking under these conditions is directed to the plasma membrane of the bud. The secretory pathway controls this vesicle transport and many of the proteins required for this process are known (Guo et al., 1999).
In meiosis vesicle transport to the plasma membrane is not needed anymore. Instead, membrane vesicles should now cluster at the SPB and fuse there to form the prospore membrane. New insights how this could work come from a study which finds that spore formation is blocked in certain mutants of the late secretory pathway (Neiman, 1998).

Genes implicated in this process are *SEC1*, *SEC4* and *SEC8*. They are responsible for transport of late secretory vesicles derived from the Golgi to the plasma membrane. These findings support a model in which the vesicles at the SPB in meiosis are of post-Golgi origin and become redirected to this new target. Formation of prospore membranes would then be a developmentally regulated side branch of the secretory pathway (Neiman, 1998).
However, the molecular components which allow to redirect secretory vesicles to the SPB have not yet been identified.

Aims of this study

The overall interest of our group is to understand gene function in the process of meiosis. The starting point for my work was to search for new Synaptonemal Complex components. As this approach did not lead to satisfactory results I shifted my focus of interest on the process of membrane biogenesis in meiosis. Meanwhile the LEP coat has been identified as a new protein complex by my colleagues in the lab. The two hybrid interaction between Ssp1p and Ady3p, a component of the LEP coat, directed me to study the role of *SSP1* in prospore membrane formation. As Ssp1p was found to be the protein responsible for the organization of the protein complex on the membrane the Δ*ssp1* deletion mutant reflects the situation of PSM formation without LEP coat. The main part of this thesis deals with the problem to affiliate a function with this protein complex.

Chapter 2

Results

2.1 Studies on the Synaptonemal Complex protein Zip1p

Zip1p is the only known structural component of the SC in *S. cerevisiae*. Sometimes Red1p and Hop1p are also referred to as SC components, but they localize more clearly to unsynapsed axial elements and their localization to the lateral elements in the SC is somewhat discontinuous (Smith and Roeder, 1997). In fact, the most continuous staining of Red1p along axial elements is observed in Δ*zip1* strains, in the absence of the SC. It seems like mature SC would partially displace the Red1p protein from the chromosomes. Therefore Red1p and Hop1p are considered to be associated to the lateral elements (Heyting, 1996).
In rats, four structural components of the SC are known. Two of them, *SCP2* and *SCP3*, are parts of the lateral elements. The other two, *SCP1* and an unnamed 48 kD protein, localize to the central region of the SC (Dobson et al., 1994). *SCP1* and *ZIP1* show a similar secondary structure concerning the central coiled coil domain. However, they do not show sequence similarity beyond the level expected to be found between any two coiled coil proteins, while *SCP2* and *SCP3* do not resemble any of the candidate components of the yeast SC.
For this reason it was presumed that other structural components of the SC in yeast are still undiscovered. One way to identify additional SC proteins was to search for Zip1p binding partners by biochemical and genetic means.
To launch this project we first created tools to investigate Zip1p properties.

Expression of Zip1p in a meiotic time course

Constructing a strain which expresses a Zip1p-ProA fusion protein allowed us to visualize and affinity purify the protein. Via homologous recombination of a tagging cassette amplified by PCR (Knop et al., 1999) a C-terminal ProA-tag was introduced at the genomic locus of *ZIP1*.

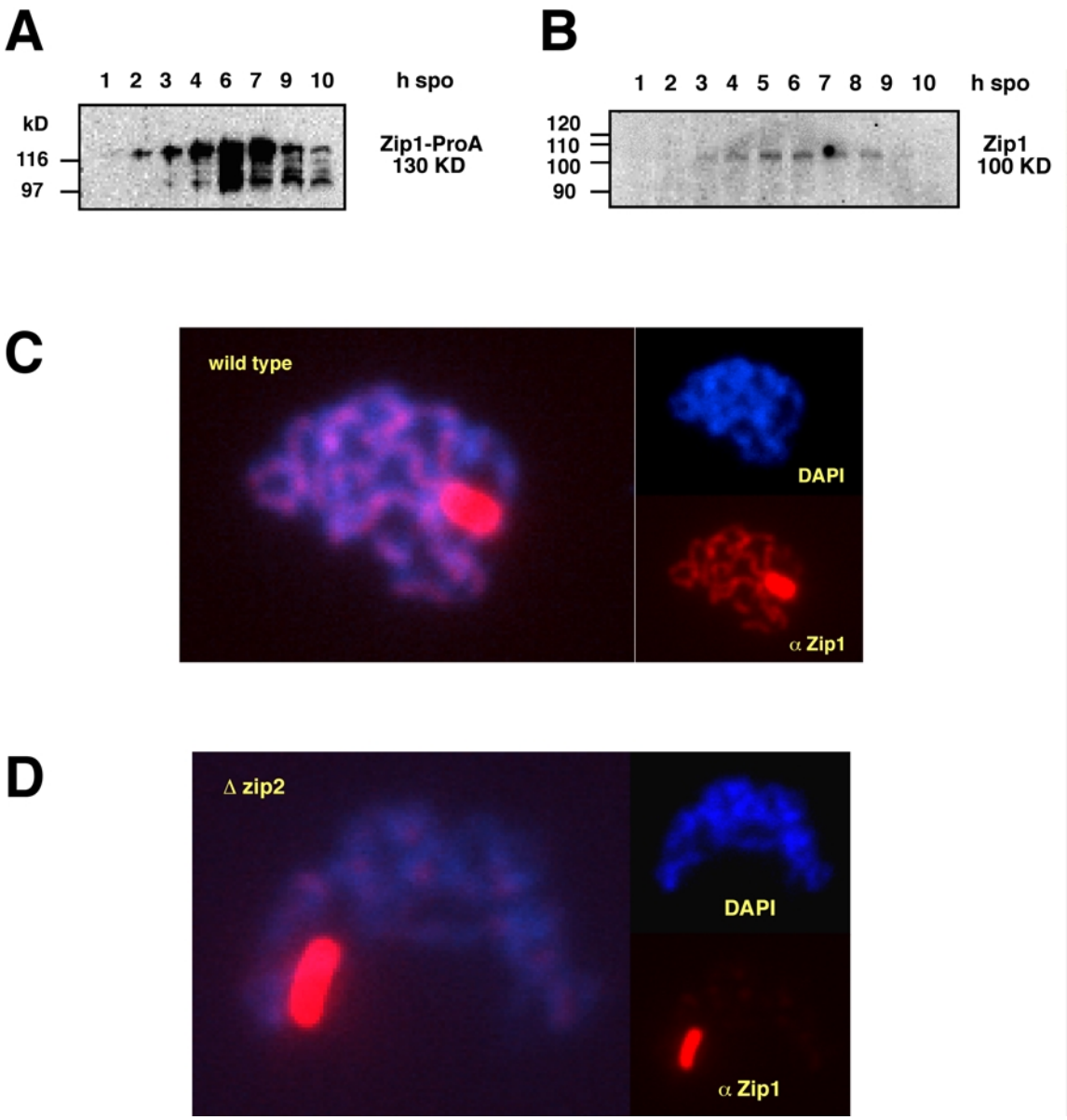

Figure 7:
Meiotic expression and chromosomal localization of Zip1p. (A) Time course of Zip1p-ProA meiotically expressed from the endogenous promoter (strain YMK314-1, h spo means hours sporulation). The fusion protein was detected in a western blot using IgG. (B) Detection of endogenous expressed Zip1p in a sporulation time course from wild type strain (YMK367-1) with the polyclonal antibody against Zip1p. (C) Localization of Zip1p along synapsed meiotic chromosomes in a meiotic spread experiment with wild type (YMF13-1). Pachytene nuclei after 6 hours sporulation were surface spread, Zip1p was detected with the antibody, DNA was stained with DAPI. (D) Meiotic spread experiment localizing Zip1p in the Δ*zip2* strain (YMF45-2-1), same conditions as in (C)

The ProA-tag from *Staphylococcus aureus* recognizes most IgGs and fusion protein expression can be monitored in western blot experiments. Yeast were harvested at several time points after induction of sporulation by transfer to sporulation media. Extracts were prepared with the TCA method and western blots probed against ProA (Figure 7A). The protein is not present in mitotic cells (data not shown), but is detectable from two hours after induction of sporulation on. The Zip1p-ProA expression peak is around 6 hours, from 6 to 10 hours protein expression decreases. *ZIP1* therefore belongs to the early meiotic genes. Under the sporulation conditions used the time point 6 hours corresponds to the prophase of meiosis I preceding the two rounds of chromosome segregation. The expression pattern observed for Zip1p is in line with the data from a screen using DNA microarrays to identify meiotically upregulated genes (Chu et al., 1998). Meiosis specific expression is a common feature for many genes with a role in sporulation. Actually, sporulation is induced by a transcriptional cascade (Rubin-Bejerano et al., 1996).

In order to detect unmodified, endogenous Zip1p, a polyclonal antibody from rabbit against the N-terminus of Zip1p was raised and purified. With this antibody, Zip1p could be detected in a meiotic time course (Figure 7B). The distribution over time is very similar to the Zip1p-ProA experiment with an expression peak around 6 hours. With these experiments we have made sure that we can detect the Zip1p protein.

Zip1p localization along synapsed chromosomes and to polycomplexes in meiotic spread experiments

The ability to visualize Zip1p on synapsed chromosomes would allow us to colocalize a putative binding partner and to look whether Zip1p localization would be disturbed in the corresponding deletion mutant. Therefore, making use of the polyclonal antibody against Zip1p, we tried to localize Zip1p in meiotic spread experiments. When cells are lysed and their nuclei are surface spread on a glass slide, the DNA adheres to the glass surface.

Proteins which bind to DNA can then be detected by immunofluorescence. When pachytene nuclei of wild type yeast were subjected to this method, Zip1p could be detected all along the synapsed chromosomes (Figure 7C). It seems even possible to distinguish single chromosomes in these experiments, what is generally regarded difficult in yeast. The Zip1p staining pattern is confined to a more narrow region of each bivalent compared to the DNA staining pattern. This observation is in line with the model in which the DNA loops out from the SC central region containing Zip1p (Figure 4). The other structure which the antibody detects looks like a big blob. These aggregates are referred to as polycomplexes and consist of multiple stacks of SC components which are not associated with DNA (Sym and Roeder, 1995). The function of these polycomplexes is however unclear.

Zip2p is a meiosis specific protein which is required for the initiation of chromosome synapsis. In the *zip2* mutant no SC is formed (Chua and Roeder, 1998). Zip2p appears to be no structural component of the SC, but localizes to discrete foci on meiotic chromosomes which are probably the sites of initiation of synapsis. In the Δ*zip2* deletion mutant Zip1p no longer localizes along the chromosomes. Instead all the protein is concentrated in those polycomplexes (Figure 7D). These localization experiments confirmed the published subcellular localization of Zip1p.

Two Hybrid interactions among Zip1p domains

Based on domain structure predictions of Zip1p with the extended central coiled coil domain and its structural role in the formation of the SC, it was long expected that Zip1p interacts with itself (Sym et al., 1993). This hypothesis was tested in a two hybrid assay with the regions of Zip1p cloned into the corresponding two hybrid vectors to produce fusion proteins with either the lexA DNA binding domain or the Gal4 transcriptional activation domain. Zip1p was divided into three parts as suggested by its secondary structure: N-terminus, coiled coil (cc) domain and C-terminus. These constructs and combinations of them were tested in a two hybrid assay for protein-protein interaction.

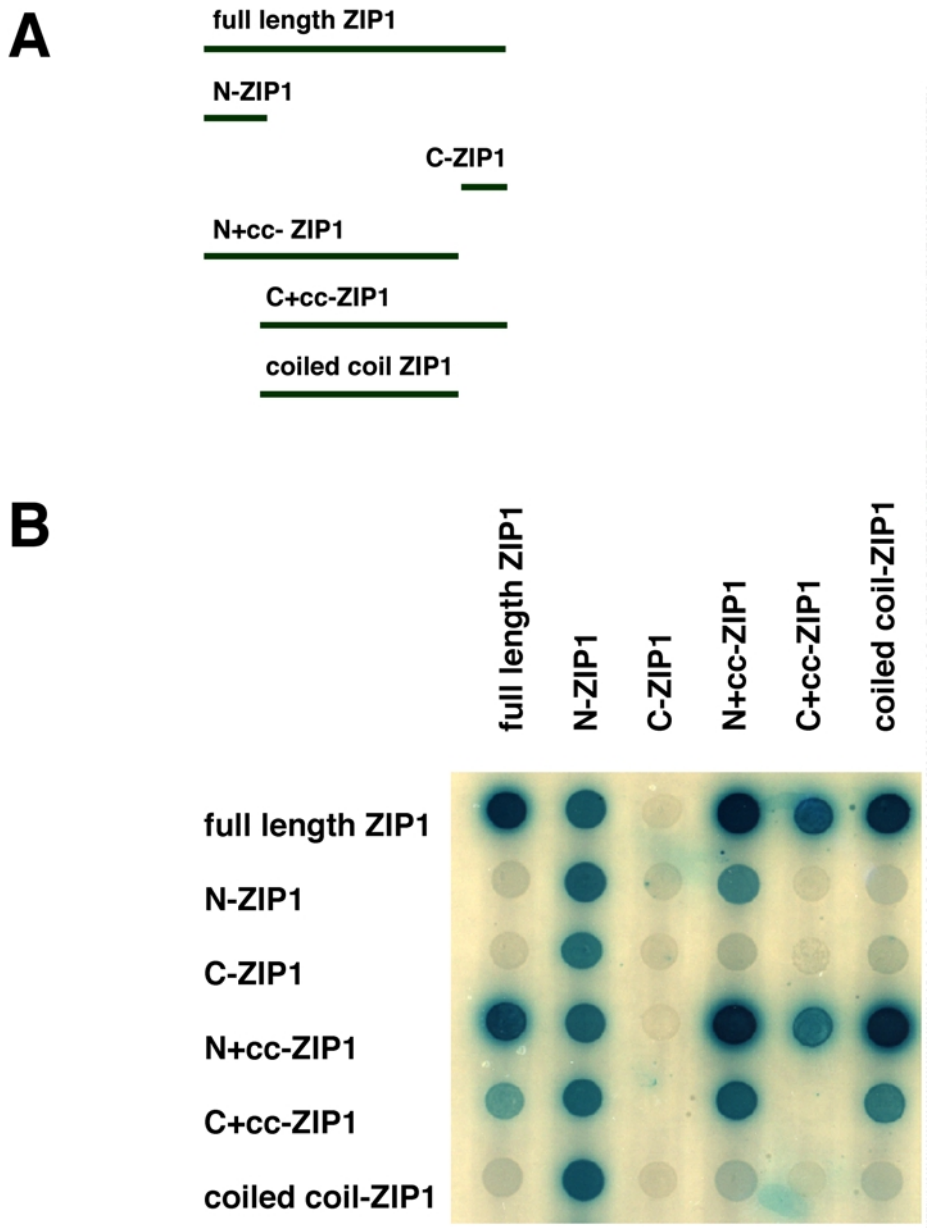

Figure 8:
Yeast two hybrid interactions among the Zip1p subdomains. (A) Schematic drawing of the Zip1p constructs cloned into two hybrid vectors. (B) Two hybrid assay with the corresponding domains of *ZIP1* cloned into pMM5 (fusion with lexA-BD, vertical row) and pMM6 (fusion with Gal4-AD, horizontal row). *ZIP1* domains are full length (aa's 1-876), N (aa's 1-184), C (aa's 746-876), N+cc (aa's 1-746), C+cc (aa's 166-876) and cc (aa's 166-746). Two hybrid reporter yeast strains (SGY37 and YPH500) were transformed with one plasmid (derived from pMM5 or pMM6) and then mated with the strain containing the other plasmid and plated on double selection media. βGalactosidase activity was measured in an overlay assay.

Interaction of the fusion proteins brings DNA binding activity and transcriptional activation activity together to result in a transcriptional activation of the βGalactosidase gene. βGalactosidase in turn cleaves X-Gal to produce a reaction product which is visible as a blue dye. As predicted, there were many interactions found among the Zip1p constructs (Figure 8). The strongest interactions were observed between the full length proteins themselves, between N+coiled coil and itself as well as full length and between coiled coil and full length plus N+coiled coil. One of the fusion proteins, lexA-BD-N-Zip1p, showed autoactivation, it also activated transcription when cotransfected with empty vector (data not shown).

Taken together, the homo-multimerization activity of Zip1p resides in the part comprising the N-terminus and the coiled coil domain, whereas the C-terminus is not involved in this process. Both domains alone, the N-terminus and the coiled coil domain, showed interaction (N $\leftrightarrow$ N+cc; cc $\leftrightarrow$ C+cc), albeit this activity seems much weaker than if the two domains act together.

If the present model of Zip1p organization in the SC is correct (Dong and Roeder, 2000), then the regions responsible for homo-multimerization are located opposite of the C-termini which are associated with the lateral elements (Figure 4). Concerning the search for a possible binding partner for Zip1p, these findings attract the attention more to the C-terminal part of the protein.

Search for a Zip1p interacting partner with biochemical and genetic methods

The major goal of the work done on Zip1p was to find interacting proteins. The first approach was to find Zip1p binding partners by biochemical means. With the strain expressing Zip1p-ProA I hoped to copurify interacting proteins when the fusion protein was purifies with magnetic IgG beads. Larger quantities of cells were lysed by glass bead lysis in the presence of various protease inhibitors. These extracts were incubated with IgG beads and the eluates of the beads put on an SDS gel (data not shown).

In any case the full length Zip1p-ProA fusion protein could be detected after purification. Additional bands which were not present in the negative control lanes were cut out and analysed by mass spectrometry (data not shown). I obtained the sequence of several bands, including various Zip1p degradation products, the yeast *HSP70* homolog Ssa1p, ribosomal proteins Rpl2p and Rpl8Ap, eukaryotic initiation factors Tif1p and Tif2p and the yeast LA virus major coat protein, which seems to be a common contamination in protein purifications from sporulating cells (Ciosk et al., 2000).

The experiments failed in discovering proteins which might have a role in SC assembly. Neither could I identify proteins with a predicted function in meiosis nor proteins with unknown function which could be potential new SC candidates.

The other method of choice for finding new protein interactors is a two hybrid screen. The C-terminus of Zip1p (aa's 746-876) was cloned into a two hybrid vector as a bait and a yeast genomic library was screened for interactors. Interaction was monitored by induction of three different reporter genes, all induced by *GAL4*: GAL1-*HIS3*; GAL2-*ADE2* and GAL7-lacZ (James et al., 1996). While selection on medium without Histidine still gave rise to tenthousands of clones, selection for the more restrictive Adenine marker resulted in 162 clones, all of them growing very slowly. Plasmid loss tests for the bait plasmid with 5'FOA demonstrated that the growth on medium without Adenine was indistinguishable for yeast containing only the library plasmid compared to those containing both plasmids, library and bait. Some of the library plasmids were sequenced and shown to contain random yeast genomic sequences (most sequences either non coding, in the wrong orientation or not in frame to the activation domain sequence). Thus the two hybrid screen only found background activation and did not discover any specific Zip1p interactors.

2.2 Biochemical analysis of the meiosis specific protein Ssp1p

After the attempt to identify novel components of the Synaptonemal Complex has failed I concentrated my research on the process of prospore membrane formation in meiosis. Starting point for the interest in Ssp1p was the finding that this protein is a two hybrid interactor of Ady3p (Ito et al., 2000; Uetz et al., 2000). The first goal was to reproduce this interaction by other means, either biochemically with a coimmunoprecipitation or by colocalization with Ady3p to the LEP coat structure in immunofluorescence experiments. It has to be mentioned that Ssp1p has proven to be extremely sensitive to fusion with any other protein sequence in terms of functionality (K. Strasser and M. Knop, unpublished results). Insertion of any protein tag at either the C- or the N-terminus resulted in a phenotype identical to the deletion of the whole gene, these strains were unable to form spores in meiosis.
A polyclonal antibody against the full length Ssp1p protein was generated (work performed by K. Strasser). With this tool some of the features of the protein concerning meiotic expression and biochemistry could then be investigated.

Expression of Ssp1p in a meiotic time course

Yeast cells were harvested at several time points after induction of sporulation and extracts were prepared with the TCA method. In western blot experiments with the purified antibody against Ssp1p a band around 70 KD could be detected, corresponding well to the calculated molecular weight of 65.8 KD for Ssp1p (Figure 9A). The appearing band is specific for Ssp1p as no protein could be detected in 6 hours sporulating samples from the Δ*ssp1* strain (Moreno-Borchart et al., 2001). Expression of the protein is induced by sporulation as it could not be detected in vegetative cells (0 hours). The kinetics of protein expression identify Ssp1p as a middle meiotic gene with a peak of expression around 6 hours, i.e. approximately at the time of the second meiotic division (data not shown).

These results confirm the observations for the meiotic expression of the *SSP1* mRNA (Chu et al., 1998; Nag et al., 1997).
Please note that the time point 6 hours here corresponds to a later meiotic stage than the same time point in Figure 7A as different sporulation conditions were used.

Fractionation of Ssp1p reveals modification of the protein

In a fractionation experiment the solubility of Ssp1p was investigated. Only cleared supernatant could be used for the following gel filtration, this experiment reveiled if Ssp1p was still present in this fraction. Extracts prepared by glass bead lysis (raw extract RE) were subjected to two subsequent centrifugation steps at increasing velocity. Samples from the supernatant (S) and the pellet (P) were taken and loaded on a gel for western blot analysis with the antibody against Ssp1p. The solubility of Ssp1p is relatively high. After low speed centrifugation, an equal amount of the protein remained in the supernatant and even after high speed centrifugation, a smaller part is still soluble (Figure 9B). Another striking observation in this experiment is that Ssp1p exists in different forms as indicated by the different migration properties of several distinct Ssp1p bands. This is a strong hint for posttranslational modification of the protein. From the fractionation experiment it becomes apparent that this modification also affects the solubility of the protein. Whereas the fastest migrating, probably unmodified form of Ssp1p, which appears as weak band at the position of the 62 KD marker, is present in all fractions, this is not true for the two main forms: the main band consists of two forms and the slowest migrating form, which runs at around 85 KD, is no longer present in the high speed supernatant. This could indicate that modification renders the protein insoluble. The other main form, which runs at around 75 KD, remains soluble under these conditions.

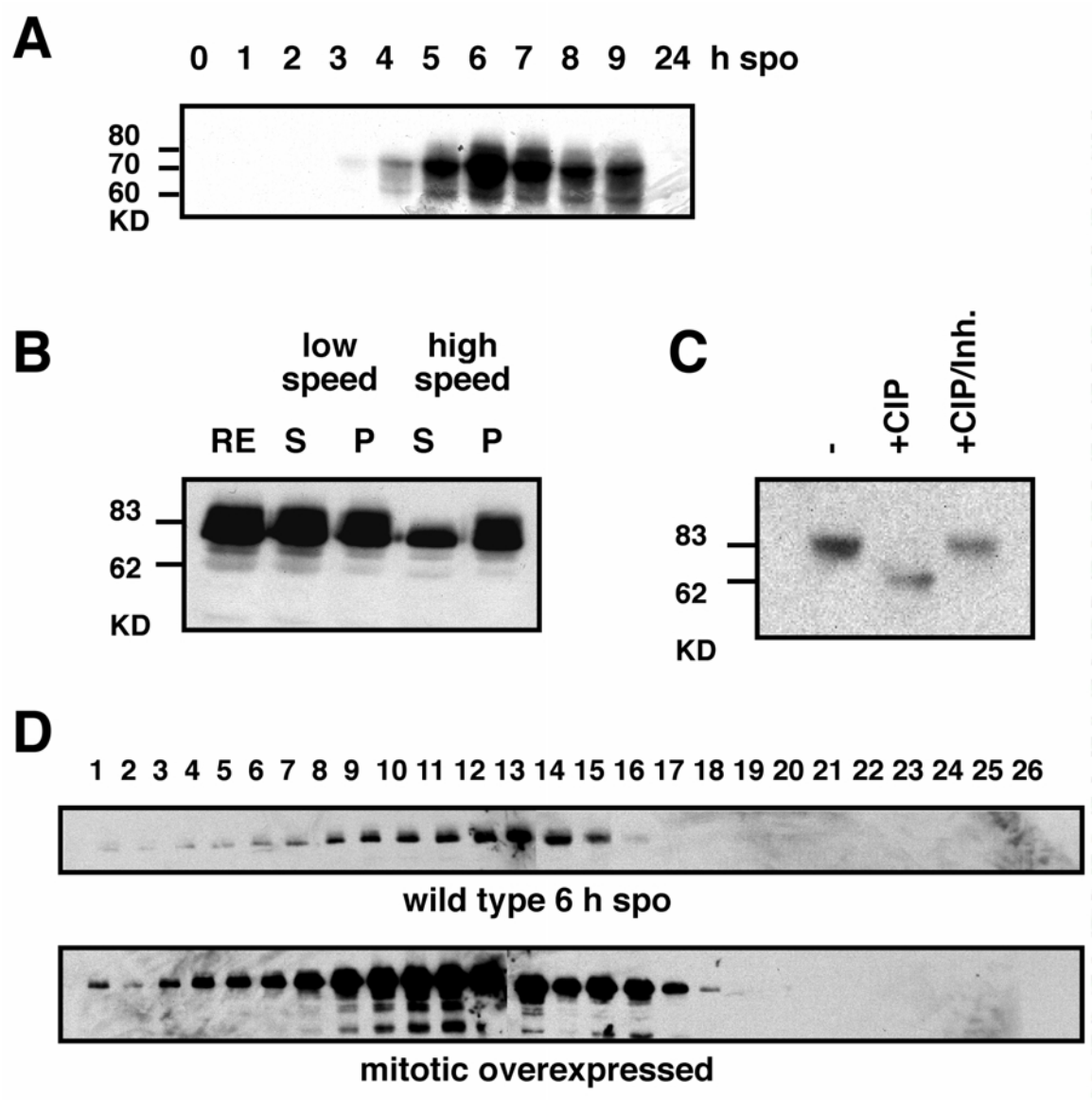

Figure 9:
Biochemical analysis of the Ssp1p protein. (A) Time course of meiotically expressed Ssp1p in wild type strain (YKS32). (B) Fractionation of Ssp1p in two centrifugation steps. Glass bead extracts of sporulating yeast (6 hours) were centrifuged in two steps with increasing speed and samples from supernatant S and pellet P were taken (C) Dephosphorylation assay: the slower migrating form of Ssp1p was extracted from the gel and treated with either nothing (-), with calf intestine phosphatase (CIP) or with the enzyme in the presence of phosphatase inhibitors (CIP/Inh.). (D) Gel filtration experiments with extracts from sporulating wild type (YKS32, 6 hours) or with a strain mitotically overexpressing Ssp1p (YKS178-1). Marker proteins were detected in the following fractions: 2 MD in fraction 1, 670 KD in fraction 7 and 440 KD in fraction 11. All western blots in this Figure were probed with the antibody against Ssp1p.

Dephosphorylation experiments prove that Ssp1p is a phosphoprotein

The observations described above prompted me to analyse the nature of the Ssp1p modification. As protein phosphorylation is the most widespread form of modification, this possibility was tested first in a dephosphorylation assay. The slower migrating form of Ssp1p was isolated from a gel and the protein was split into three parts: one remained untreated, one was incubated in the presence of phosphatase and the third was incubated with phosphatase together with phosphatase inhibitor. The western blot with these three samples provides clear evidence that Ssp1p is modified by phosphorylation at multiple sites. The slower migrating, phosphorylated form at around 85 KD in the fractions untreated and treated with phosphatase plus inhibitors shifts to about 62 KD, which corresponds to the unphosphorylated form in the fraction treated with phosphatase (Figure 9C). It is possible that different grades of Ssp1p phosphorylation exist but it cannot be ruled out that the lowest bands in Figure 9B are degradation products.

It was interesting to find out which kinase is responsible for the Ssp1p phosphorylation. As an educated guess it was tested whether Ssp1p is still phosphorylated in a deletion mutant Δ*sps1*. *SPS1* codes for a meiosis specific kinase and deletion of *SPS1* leads to a defect in late steps of spore formation (Friesen et al., 1994). Since this phenotype resembles the effect of a *SSP1* deletion in sporulation (Nag et al., 1997), a functional link between the two proteins was suspected. However, Ssp1p phosphorylation was not affected in the Δ*sps1* mutant strain (data not shown). So up to now it is unknown which kinase phosphorylates Ssp1p and at what sites.

Gel filtration experiments of Ssp1p

These experiments were designed to elucidate whether Ssp1p is associated in a specific protein complex and what stochiometry such a complex could have. From the results of previous studies it was suspected that Ssp1p is associated with its two hybrid interactor, Ady3p, and maybe further proteins. Extracts from sporulating yeast were loaded onto a Superose 6 column and fractions from the eluate were collected for western blot analysis. Large protein complexes should elute in early fractions (fraction 1: exclusion volume, 2 MD), whereas smaller complexes take more time to pass the gel matrix (marker 440 KD: fraction 11). Meiotically expressed Ssp1p was eluted from fraction 1 to fraction 16 with a peak around fraction 13 (Figure 9D, upper panel). We then repeated this experiment with Ssp1p mitotically overexpressed under the Gal1 promoter. This resulted in a very similar pattern of bands with a broad distribution from fraction 1 to 18 (Figure 9D, lower panel). As meiosis specific binding partners like Ady3p are not present in the mitotic sample, the distribution of Ssp1p from a range of 2 MD to approximately 200 KD is most likely due to selfinteraction of the protein. A specific protein complex with a distinct size could not be observed in this experiment.

One possible explanation for this behaviour arises from the different fractionation of the phosphorylated forms of Ssp1p. In order not to congest the column with debris only cleared high speed supernatant can be loaded on the column. However, this fraction contains only the hypophosphorylated form of Ssp1p. One hypothesis is now that only the hyperphosphorylated form exists in the complex. Unfortunately, this form was never tested in gel filtration because it is not soluble. To overcome this problem we should have used another method, e.g. separation on a glucose gradient, where we also could have taken the low speed supernatant as input.

To our disappointment we were never able to confirm the interaction between Ssp1p and Ady3p by coimmunoprecipitation. All experiments performed to show this interaction biochemically failed, which might be due to the insolubility of the protein complex bound to the membrane.

2.3 Identification of Ssp1p as another component of the leading edge protein (LEP) coat

The characterization of the two meiosis specific proteins Don1p and Ady3p led to the discovery of a new protein structure: the leading edge protein (LEP) coat on the tip of the growing prospore membrane forms a ring which surrounds the meiotic spindle (Figure 6). Both proteins exhibit a stage dependent localization: to dots in the cytoplasm and to the SPBs in late meiosis I, more prominently staining to the SPBs in early meiosis II and finally to the LEP coat from anaphase II on until the closure of the PSMs when the staining becomes more diffuse. Don1p and Ady3p show perfect colocalization at all stages (Moreno-Borchart et al., 2001). The following experiments were designed to localize the putative Ady3p interactor, Ssp1p.

Ssp1p localizes all along the prospore membrane

Making use of the polyclonal antibody against Ssp1p we detected the protein in immunofluorescence experiments. Cells from a sporulating culture around meiosis II (6 hours after transfer to sporulation medium) were fixed and permeabilized. These samples were treated with antibodies against Ssp1p and βtubulin and additionally with the DNA-binding dye DAPI. In a cell around anaphase II containing an elongated spindle, Ssp1p localized to four globes which emanate from the spindle poles and engulf the dividing nucleus as can bee seen by DNA staining (Figure 10A). This staining corresponds to a localization all along the prospore membrane, but not to the plasma membrane of the mother cell. At this meiotic stage with a continuous, elongated spindle the PSMs are still open and the protein could diffuse. Therefore, this staining pattern is also different from a cytoplasmic localization which would be visible all over the cell.

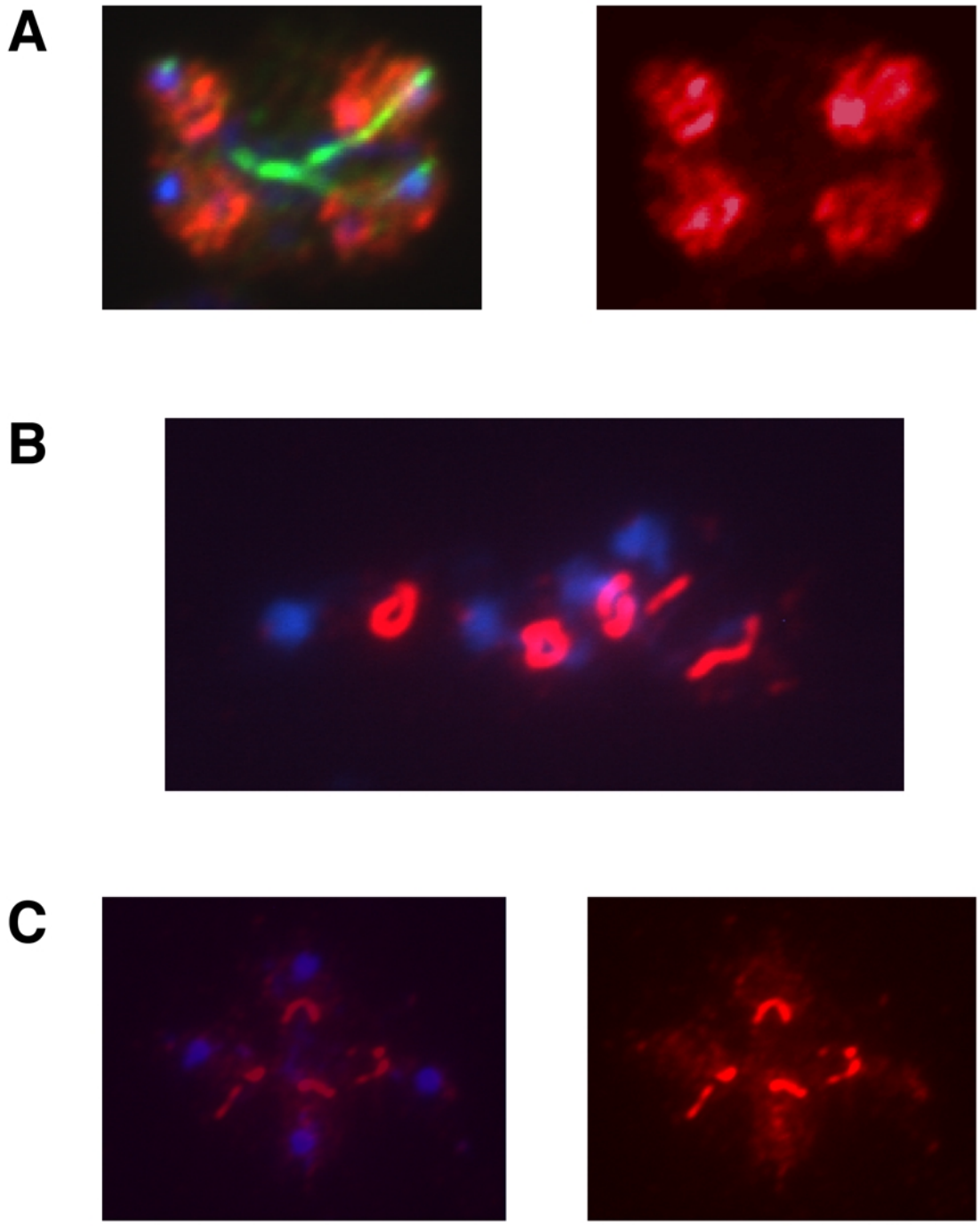

Figure 10:
Localization of Ssp1p in meiotic yeast cells. All these pictures were taken from wild type yeast (YKS32) in anaphase of meiosis II, 6 hours after induction of sporulation.
(A) Immunofluorescence experiment using antibodies against Ssp1p (red) and βtubulin (green) and DAPI to detect DNA (blue) (B) and (C) Meiotic spread experiment using the antibody against Ssp1p (red) and DAPI to detect DNA (blue).

Clearly, the staining pattern observed for Ady3p shows four rings surrounding the spindle at that stage and thus looks very different compared to the one observed for Ssp1p. However, a localization all along the PSM does not eliminate the possibility that Ssp1p is also present at the LEP coat. Therefore these results cannot solve the problem whether Ssp1p and Ady3p colocalize or not. To adress this question we had to think of a new set of experiments.

Ssp1p forms ring-like structures visible in meiotic spread experiments

Meiotic spread experiments are usually applied to detect DNA binding proteins, such as transcription factors, proteins involved in DNA repair or synaptonemal complex proteins (see Figure 7C). In contrast to immunofluorescence, where the whole cell is subjected to antibody incubation, only the surface spread DNA of a lysed cell is tested in meiotic spreads. Cytoplasm and membranes are eliminated by this method, but it was shown that DNA associated structures like microtubules are retained on the slide (Loidl et al., 1998). This prompted us to investigate whether Ssp1p is still detectable in meiotic spreads. We were still able to see a strong, specific signal from the antibody against Ssp1p. In many cases we were even able to observe Ssp1p-donut structures with a thin thread of DNA running through the central aperture (Figure 10B). Spreading DNA on a glass slide is a harsh treatment and therefore nuclei which have already started to divide during meiosis are often disrupted during this procedure. Scanning through the samples DNA blobs and Ssp1p-donuts appeared scattered all over the place. It is only in rare cases where we could still assign the observed signals to a certain cell (Figure 10C). Here we can see a meiosis II cell with four areas containing the DNA and a Ssp1p staining visible as half-rings which seem to seperate the developing spores. This localization of Ssp1p fits into the idea that the protein is associated with the entire prospore membrane and with the LEP coat.

Colocalization of Ssp1p with LEP coat components

The finding that Ssp1p forms ring-like structures was encouraging in terms of a possible colocalization of Ssp1p with other proteins of the LEP coat. For this experiment the strain with chromosomal tagged *DON1*-GFP was used because the antibodies against Ady3p and Ssp1p were both raised in rabbits and could not be used simultaneously. Since Don1p and Ady3p have been demonstrated to show a completely overlapping localization it is sufficient to detect either Don1p or Ady3p (Moreno-Borchart et al., 2001). In a late meiosis II nucleus with all four LEP coats preserved both proteins, Don1p-GFP and Ssp1p, could be clearly detected in these structures. The perfect match of the two signals shown in green and red is indicated by the bright yellow staining of the merged layers (Figure 11A). This experiment demonstrates that Ssp1p is another component of the LEP coat. This colocalization also supports the observed two hybrid interaction between *ADY3* and *SSP1* and gives a strong hint that this protein interaction is relevant *in vivo*.

Localization of Ssp1p to the spindle pole body and to the LEP coat does not depend on Ady3p

In order to find out how the proteins of the LEP coat assemble in the complex it was interesting to look if the localization of Ssp1p depends on Ady3p. One of the characteristics of the LEP coat components Don1p and Ady3p is their association with the SPB in early meiosis II, whereas at later stages with elongated spindles the proteins relocalize to the LEP coat and cannot be detected at the SPB any more. The same behaviour is also true for Ssp1p (Figure 11B, upper panel).
Among the three known components of the LEP coat there is a certain hierarchy in the interdependence of localization: Don1p localization to the LEP coat is abolished in an Δ*ady3* strain, whereas Ady3p is not affected by a deletion of *DON1*.

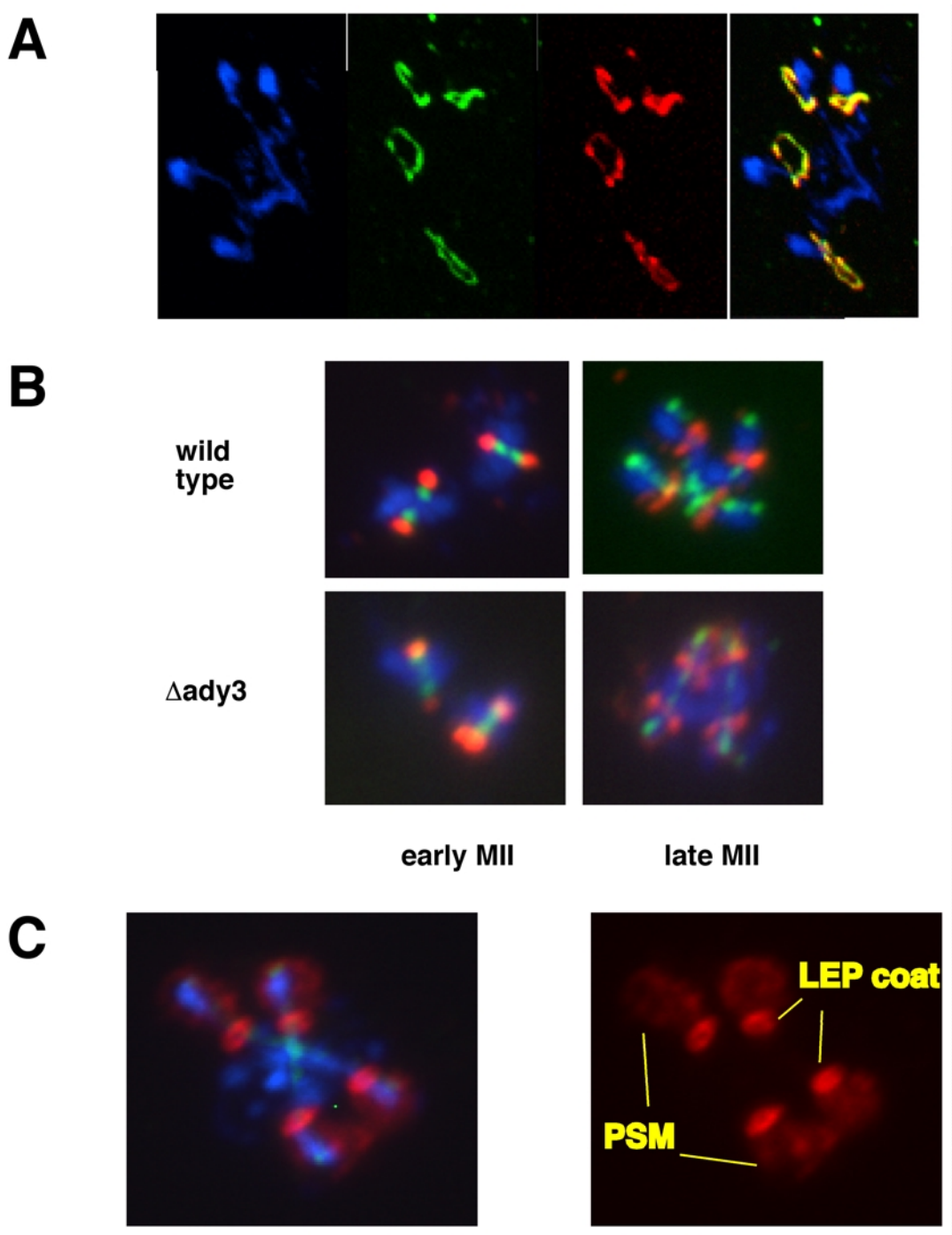

Figure 11:
Colocalization of Ssp1p with LEP coat components at several stages of meiosis. (A) Meiotic spread experiment with wild type strain (YKS32) 6 hours after induction of sporulation. Ssp1p (red), Don1p-GFP (green) and DNA (blue) were simultaniously detected. Overlap of the merged signals from Ssp1p and Don1p-GFP appears yellow. (B) Meiotic spread experiments with wild type (YKS32) or Δ*ady3* strain (YAM37-12) 5 hours (early meiosis II) or 6 hours (late meiosis II) after induction of sporulation. Signals refer to Ssp1p (red), βtubulin (green) and DNA (blue).
(C) Meiotic spread experiment with wild type strain (YKS32) at 6 hours sporulation detecting the same signals as in (B)

In a Δ*ssp1* strain both proteins, Don1p and Ady3p, cannot be detected at the LEP coat any more (Moreno-Borchart et al., 2001). On the other hand, Ssp1p localization to the SPB and to the LEP coat is not affected by a deletion of *ADY3* (Figure 11B, lower panel).

This puts Ssp1p on top of the hierarchy of LEP coat proteins as localization of all known components depends on *SSP1* function. In electron microscopy, an electron dense enlargement of the tip of the PSM can be detected only in the presence of Ssp1p (Moreno-Borchart et al., 2001). These findings altogether propose a function for Ssp1p in recruiting Ady3p to the LEP coat via protein-protein interaction. Ady3p then in turn mediates the association of Don1p to the LEP coat.

Ssp1p localizes to the LEP coat and additionally all along the prospore membrane

It has to be kept in mind that Ssp1p localizes to the entire prospore membrane in late meiosis II and not only to the LEP coat (Figure 10A). As the LEP coat comprises a substructure of the PSM a specific ring staining is not visible in immunofluorescence experiments. On the contrary, in meiotic spreads usally only the LEP coats are detected. This is due to the fact that prospore membranes are washed away by this procedure. However, under milder conditions with less detergent, sometimes parts of these membranes are preserved. In these cases a dual staining pattern is visible: a strong signal at the LEP coat but also localization along the prospore membrane (Figure 11C). This is a remarkable difference to the other LEP coat components. It also raises the question that if Ady3p would be recruited to the LEP coat via binding to Ssp1p, why would Ady3p then not also be bound to the whole prospore membrane. This indicates that our understanding of the process is not yet complete.

2.4 Phenotypic analysis of the Δ*ssp1* deletion mutant

The phenotype of the *ssp1* mutant in meiosis is its failure to form spores. Which step exactly in the process of spore formation is defective remains elusive. It was described that the *ssp1* mutant undergoes both meiotic divisions indistinguishable from those in wild type. Nevertheless, soon after meiosis II, the nucleus of the mutant becomes fragmented (Nag et al., 1997). The authors of this study speculated that this might be due to a defect of packaging the meiotic daughter nuclei into spores. They proposed a role for *SSP1* in the coordination of the meiotic cell cycle to spore formation and closure. As it is clear now that Ssp1p localizes to the prospore membrane, I wanted to study if the *ssp1* mutant shows a defect in the formation of this structure. Deletion of the other two components of the LEP coat, Ady3p and Don1p, has no effect on the formation of the prospore membrane. A first hint that this might be different for the *ssp1* mutant came from immunofluorescence microscopy experiments which detected the tSNAREs Sso1p/Sso2p in wild type and Δ*ssp1* strains. In meiosis these tSNAREs localize to the PSM and can therefore be used as a marker for the membrane (Neiman et al., 2000). In the *ssp1* deletion mutant the prospore membranes could still be detected but looked misshaped compared to the globular PSMs in the wild type (Moreno-Borchart et al., 2001). These results promted me to look at the PSMs in more detail in electron microscopy studies detecting intracellular membranes.

Prospore membrane formation and spore wall maturation in the wild type strain

To find differences for the mutant strain we first had to take a look at the wild type situation. Sections through sporulating yeast cells were contrasted and subjected to transmission electron microscopy. Prospore membranes visualized by the permanganate staining method appear like caps sitting on nuclear bulges. Sometimes the spindle pole body is in plane with the section and this structure appears as a characteristic imprinting in the nuclear envelope (Figure 12A and C). The meiotic plaque of the SPB lies further outside of the nuclear envelope and probably can be seen as an additional layer under the PSM in the region of the SPB (Figure 12A). This picture also shows the accumulation of membrane vesicles fusing with each other at the SPB to give rise to a continuous membrane system. This membrane then grows to engulf bigger nuclear lobes and to incorporate cytoplasm before it closes (Figure 12B-D). It is important to keep in mind that in the wild type the PSM never lies directly on the nuclear envelope and that the opening of the membrane at the tip is wide (Figure 12A-C). Before the PSM closes the developing spore has to incorporate cytoplasm and organelles. Upon germination of the spore the PSM becomes the plasma membrane of the cell and therefore it has to contain all vital functions. Incorporation of cytoplasm probably takes place after the nucleus has already pinched off, often the nucleus is located on one side while the cytoplasm is concentrated on the other side (Figure 12D). After the PSM has closed, spore wall material is deposited between the two double membranes of the PSM (Figure 12E-F). In these pictures it also becomes clear that only part of the space of an immature spore is occupied by the nucleus and much of the rest is filled with cytoplasm and organelles like mitochondria. The spore wall material, which contains mostly glucans, accumulates in the cytoplasm already at earlier stages (Figure 12B-D). In this method it appears as transparent aggregates which seem to have an affinity for the prospore membrane. As the spores mature they develop a characteristic thick spore wall (Figure 12F).

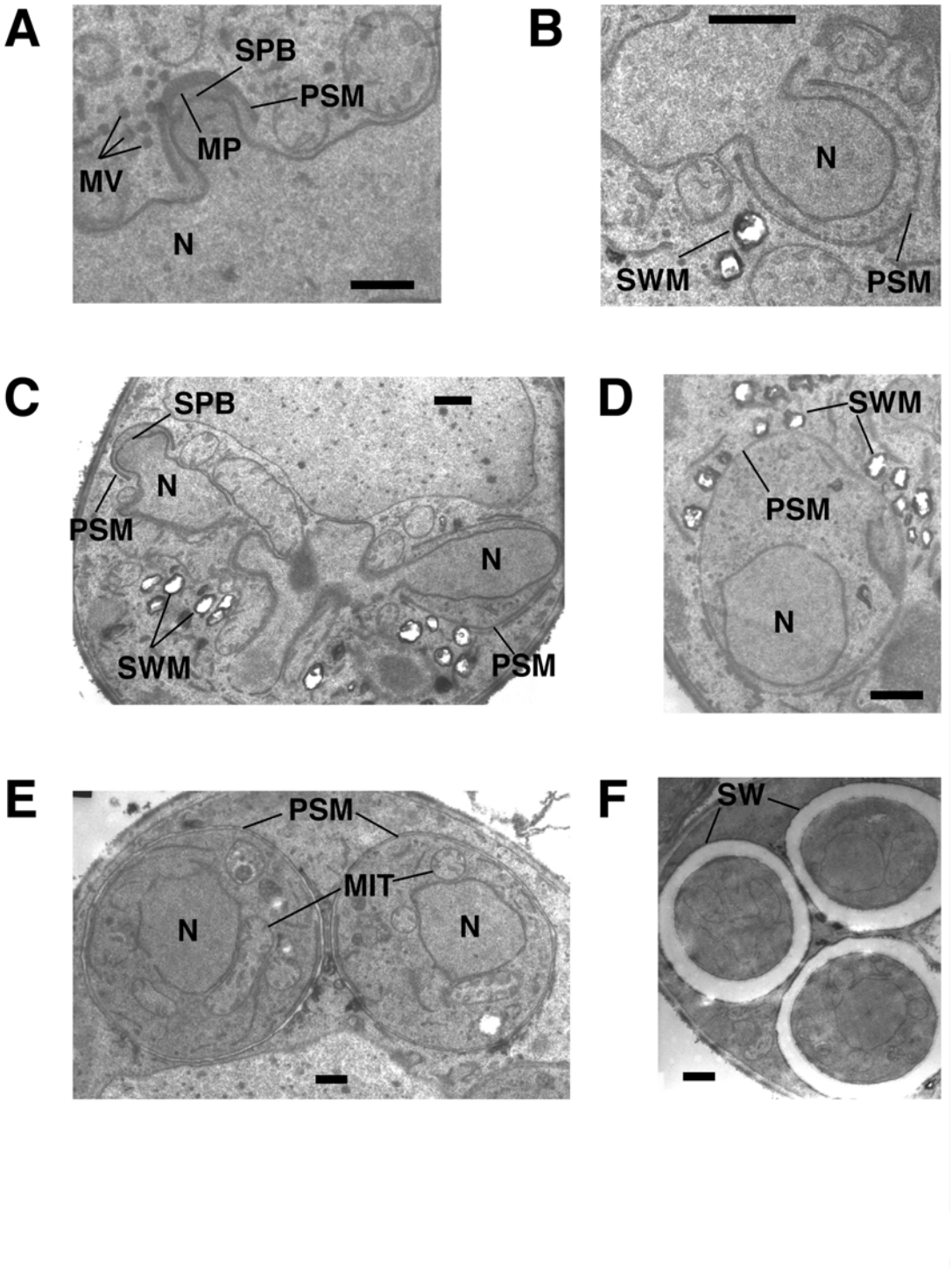

Figure 12:
Prospore membrane formation in the wild type strain. Transmission electronmicroscopy pictures of sections from sporulating yeast of wild type strain (YKS32) after 5.5 hours sporulation (A and B) or after 7.5 hours sporulation (C-F). Bar is corresponding to 500 nm. N=nucleus, PSM=prospore membrane, SPB= spindle pole body, MV=membrane vesicles, MP=meiotic plaque, MIT=mitochondria, SWM=spore wall material, SW=spore wall

In the *ssp1* mutant prospore membrane shaping is abnormal

Looking at the *ssp1* deletion strain with the same technique described in the previoius paragraph results in a completely different picture. A prospore membrane is still formed and the membrane is still organized by the SPB. The meiotic plaque underlying the membrane is visible (Figure 13A-C). However, in comparison to the wild type the localization of the PSM with respect to the nuclear envelope is clearly altered. In the *ssp1* mutant the PSM follows the surface of the nuclear envelope very tightly. It seems like the two membrane systems stick to each other and there is little space left between them (Figure 13A-C). Another major difference concerns the tips of the prospore membrane where the LEP coat usually resides. While in the wild type this opening is wide and keeps some distance to the cleavage furrow of the nuclear envelope, the tip of the PSM points directly to the site of constriction in the mutant (compare Figure 12A-C with Figure 13A-C). It is also apparent that in the mutant the diameter of the nuclear cleavage furrow is reduced and it seems like the daughter nuclei would pinch off prematurely. Looking at a whole cell with three PSMs visible they all appear close to pinch off while large parts of the nucleus, which spans the entire cell length, are not included (Figure 13C). Finally, it has to be mentioned that mature spores with their prominent, hard to overlook spore walls are never present in the mutant.

Atypical bodies surrounded by two membrane systems accumulate in the *ssp1* mutant

In contrast to the wild type, immature and mature spores containing cytoplasm were not observed in the Δ*ssp1* mutant. Instead another structure accumulates: bodies of variable size and shape which are surrounded by two systems of two double membranes (Figure 13B, D-F). Although there is no direct evidence this suggests that the outer membrane system is derived from the PSM while the inner system is the nuclear envelope.

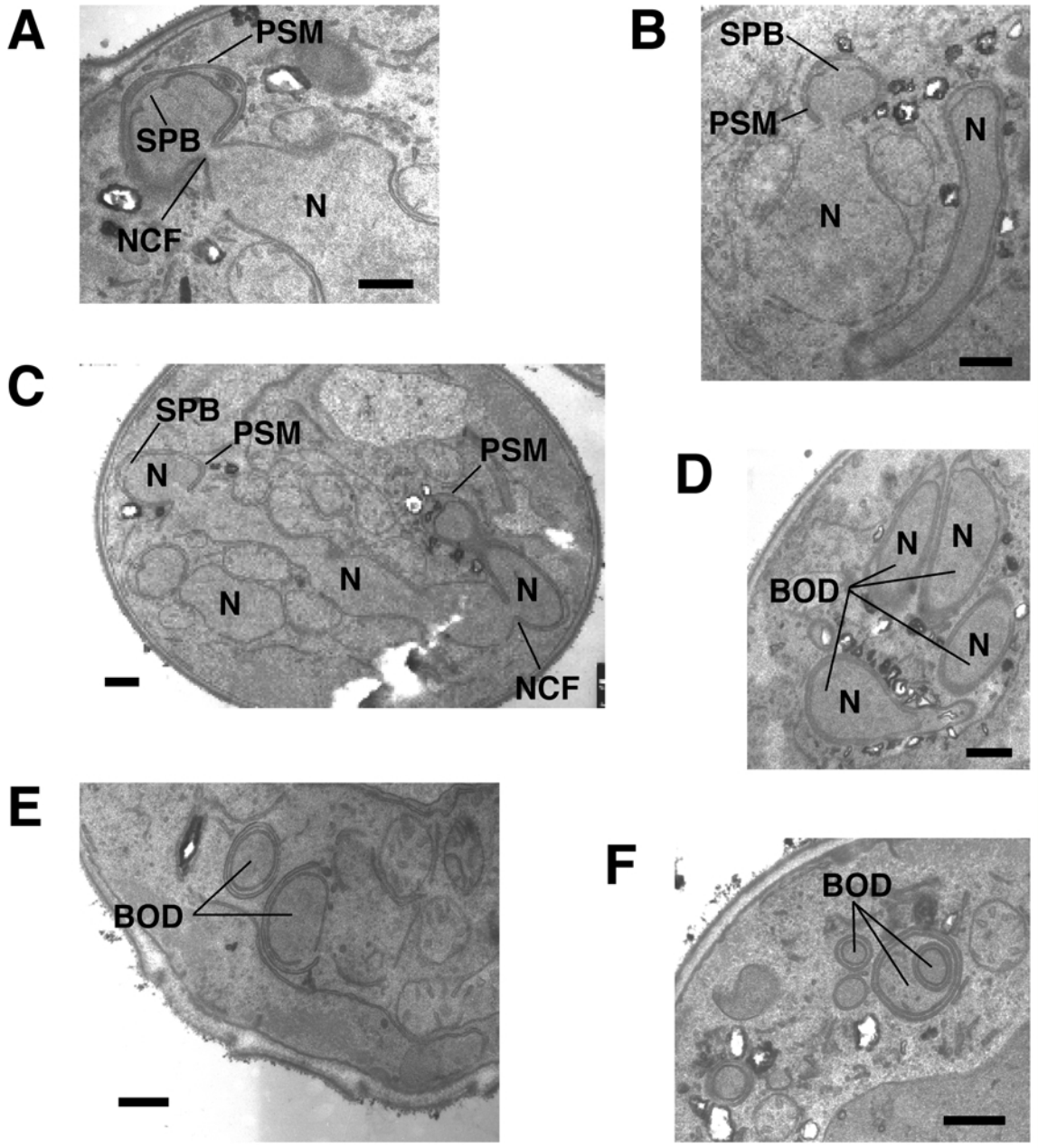

Figure 13:
Prospore membrane formation in the Δ*ssp1* stain. Transmission electronmicroscopy pictures of secions from sporulating yeast of strain Δ*ssp1* (YKS127) after 7.5 hours of sporulation (A-F). Bar is corresponding to 500 nm, N=nucleus, PSM=prospore membrane, SPB=spindle pole body, BOD=bodies, NCF=nuclear cleavage furrow

Formation of these bodies can be explained imagining that the daughter nuclei would pinch off and that the PSMs close before any cytoplasm is incorporated into these bodies (Figure 13C). Whether these bodies contain DNA or not is another open question, the electron microscopy technique applied does not allow DNA staining. However, if the bodies would contain DNA without cytoplasm, protein synthesis would not work which explains the observed loss of viability (Figure 14A).

What happens to the rest of the nucleus after parts have pinched off in these bodies remains also unclear. On one side, more then four bodies have never been detected in one cell. This could mean that the quantity of these bodies is limited by the number of SPBs. On the other side the bodies appear to be much more abundant then prospore membranes in the wild type. Sometimes membranes seem to float around randomly in the cytoplasm without being attached to the nuclear envelope (Figure 13E). In one case a body inside another one was detected, this can be explained only if membrane growth and engulfment continues after one body has pinched off (Figure 13F).

The phenotype we observe corresponds nicely to the proposed nuclear fragmentation (Nag et al., 1997). With our new data we are now able to give an explanation for this: *SSP1* is involved in shaping and closure of the PSM. In the absence of the protein parts of the nucleus pinch off but fail to develop into spores. The leading edge protein coat on the membrane is missing in the *ssp1* mutant, and this structure is probably required as a scaffold for shaping of the growing PSM. A model which could explain this phenotype is shown in the discussion. Although we cannot rule out this possibility we do not suggest a role of *SSP1* in the coordination of meiotic divisions and spore formation.

The *ssp1* mutant loses viability in the course of sporulation

In order to further analyse the *ssp1* mutant phenotype it was tested whether the viability of this strain is changed in a time course of sporulation. This experiment was also desinged to compare the viability in sporulation to other mutants (Figure 21).Yeast cells of wild type and Δ*ssp1* strains were induced to sporulate and at certain time points dilutions were plated on rich medium. The relative viability was determined by dividing the total number of colonies at the time indicated by the number of colonies at time zero for a given strain. It can be seen that the viability of the *ssp1* mutant declines over time, after 24 hours the viability is below 10 %, after 48 hours even below 1 % (Figure 14A). The viability of the wild type strain does not change significantly over time. This means that after some time the mutant yeast cannot recover from their defective sporulation even under good nutritional conditions. These results are in line with the published phenotype for *ssp1* mutants (Nag et al., 1997).

Deletion of *SSP1* does not block transcription of late meiotic genes

To further characterize the phenotype of the *ssp1* mutant the expression of meiosis specific genes which act later in meiosis than *SSP1* was investigated. The meiotic cell cycle depends on the timed expression of meiosis specific genes. Some genes with known function act after *SSP1*, they are expressed after the spore has closed (spore autonomously). *DIT1* for example is an enzyme required for the formation of the outermost Dityrosine layer of the spore wall (Briza et al., 1994). Another late gene which acts in spore wall maturation is *SPS100* (Law and Segall, 1988).
It was shown for the deletion mutant of *spo70/ama1*, which also fails to form spores, that the expression of these late genes is blocked (Cooper et al., 2000). It is likely that a checkpoint mechanism controls the packaging of daughter nuclei into spores. Triggering this checkpoint could result in a cell cycle arrest and inhibit transcription of late meiotic genes.

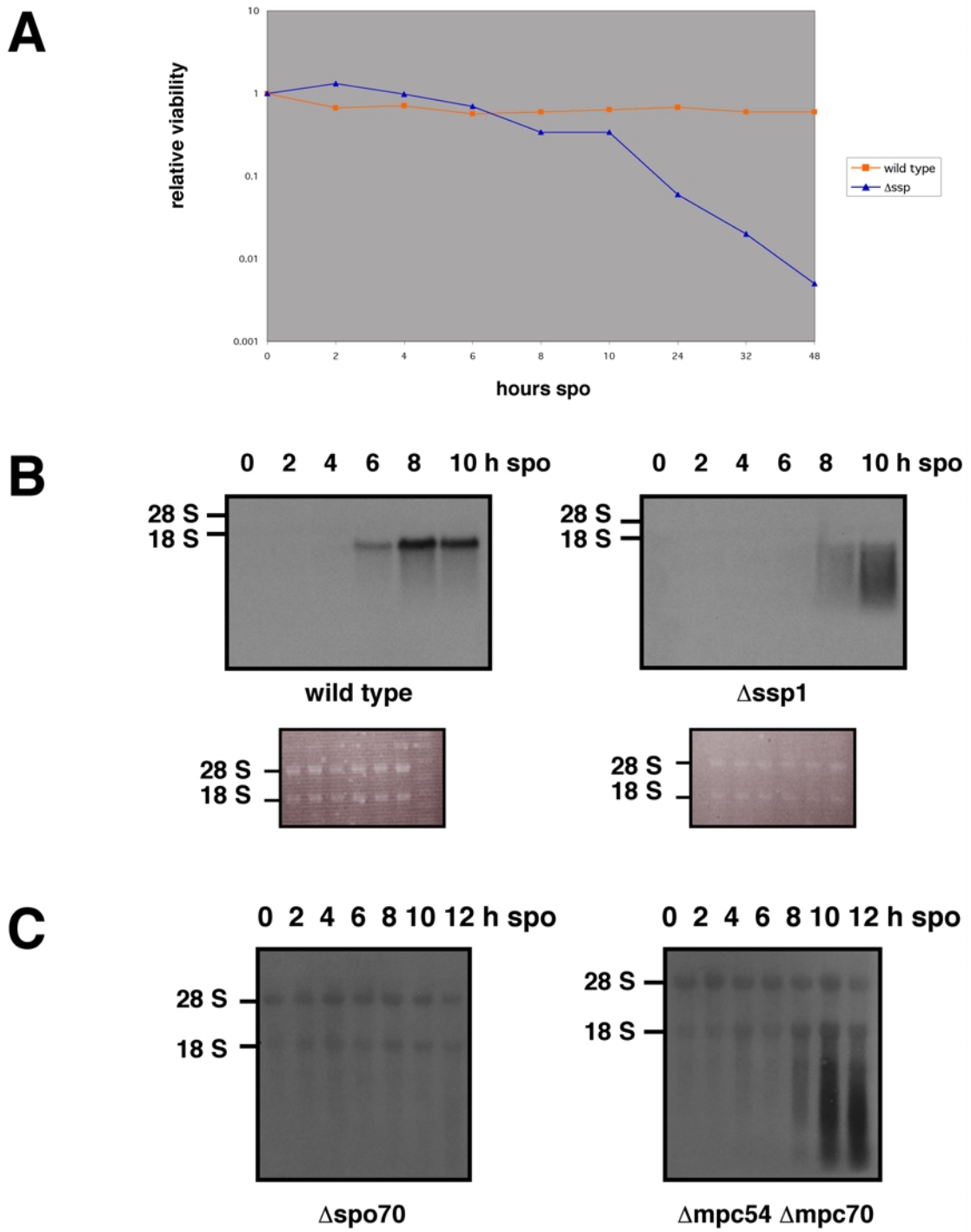

Figure 14:
Loss of viability and degradation of late meiotic mRNAs in the Δ*ssp1* stain. (A) Relative viability of strains wild type (YKS32) and Δ*ssp1* (YKS127) logarithmically plotted. (B) and (C) Northern blot experiments detecting *DIT1* mRNA in RNA preparations of a meiotic time course from strains wild type (YKS32), Δ*ssp1* (YKS127), Δ*spo70* (KN3279) and Δ*mpc54* Δ*mpc70* (YKS65). rRNA serves as marker and loading control either separate on agarose gels (B) or directly on the northern blot (C) In this experiment the *DIT1* probe apparantly also bound unspecifically to the rRNA, this serves as a n internal loading control.

Therefore experiments were made to investigate the expression of late meiotic genes in the *ssp1* mutant by northern blots. With hybridization to a radiolabeled probe the *DIT1* mRNA could be detected in RNA preparations from six hours sporulation on in the wild type strain. The *DIT1* mRNA (1608 bases) appears as a discrete band slightly below the 18 S rRNA band (1874 bases) (Figure 14B).

In the *ssp1* deletion strain the *DIT1* mRNA still accumulates but is apparently rapidly degraded, since only a smear below the full length product can be detected. In a loading control staining the rRNA in an agarose gel it could be shown that the RNA it the preparation was not degraded. This experiment was repeated several times with the same result. Furthermore we were not able to obtain reasonable RNA preparations from the *ssp1* mutant at late time points (24 hours sporulation). In these samples also the rRNA has been completely degraded (data not shown). mRNA instability was observed also for the *SPS100* mRNA (data not shown). From these results it can be concluded that late meiotic genes are still expressed in the *ssp1* mutant. However, the mRNA is unstable in the *ssp1* mutant strain. RNA degradation is generally associated with cell death, which explains the observation.

We also probed for the *DIT1* mRNA in strains with a deletion of *SPO70* and the meiotic plaque components *MPC54* and *MPC70*. If these meiotic plaque components are deleted, formation of a continuous PSM is completely abolished (Knop and Strasser, 2000). As expected, *DIT1* is no longer expressed in the Δ*spo70* strain. In the Δ*mpc54* Δ*mpc70* double mutant again a smear of degradation products but not the full length mRNA is detected (Figure 14C).

Taken together these data suggest that in deletion mutants of *SSP1* and *SPO70* a checkpoint, which prevents expression of late meiotic genes, is not activated. This is different from the phenotype observed when *SPO70* is deleted. In deletion mutants of *SSP1* and *MPC54/MPC70* the produced mRNA is rapidly degraded and the production of functional gene products is probably also inhibited.

2.5 Lipid binding properties and membrane localization of Ssp1p

It was demonstrated that Ssp1p localizes to the prospore membrane and that it recruits two other proteins, Don1p and Ady3p, to the LEP coat. This complex must somehow be anchored in the membrane. This could be achieved in several ways: one of the proteins could be a membrane protein or fatty acylated protein or on the other hand it binds to different membrane proteins or to membrane lipids.

Ssp1p does not contain known domain structures and has no putative transmembrane domain. Whether Ady3p contains a transmembrane region or not is doubtable, experimental data about this is ambiguous (A. Moreno-Borchart and M. Knop, unpublished data). However, this putative transmembrane domain of Ady3p cannot be responsible for anchoring the LEP coat in the membrane alone, as in the absence of Ssp1p the other proteins do not localize there anymore. As in the case of Ssp1p also no transmembrane domain is predicted for Don1p. We could not find any signal sequence for covalent modification with fatty acids (e. g. myristoylation or farnesylation) in the Ssp1p sequence.

Although it cannot be excluded that Ssp1p is associated with the membrane by binding to another, yet unidentified, membrane protein, data suggests that this is not the case: in electron microscopy pictures of sporulating yeast the LEP coat can still be seen as an electron dense structure on the tip of the membrane in a Δ*ady3* strain, but this structure is gone in the Δ*ssp1* mutant (Moreno-Borchart et al., 2001). In the Δ*ady3* strain Ssp1p is the only protein among the so far identified LEP coat components that still binds to this structure.

Therefore it seemed worthwile to investigate whether Ssp1p itself has lipid binding capabilities.

Full length Ssp1p binds to Phosphatidylserine, Cardiolipin and to all Phosphatidylinositolphosphates (PIPs)

Lipid binding of proteins can be monitored in a so-called lipid-protein overlay assay. Lipids in solvent are spotted on a nitrocellulose membrane and the membrane is incubated with a protein extract. Bound protein is then detected with a specific antibody like in western blot experiments.

For this experiment the full length *SSP1* gene was cloned into a bacterial expression vector (pET-3a). Since *SSP1* has proven to be extremely sensitive to tagging *in vivo*, the *SSP1* gene was cloned into the expression vector without modifications. The lack of a tag complicates purification of Ssp1p, therefore supernatant (S) from crude, unpurified bacterial extracts was used for the lipid binding assay. As the antibody against Ssp1p recognizes only this protein and does not crossreact with any bacterial proteins in the extract (Figure 15C), the signal in the assay must derive from Ssp1p.

We first tested standard lipids which are found in the yeast plasma membrane, such as Phosphatidylserine, Phosphatidylethanolamine, Phosphatidylinositol, Phosphatidylcholine, Ergosterol and Cardiolipin. Serial dilutions of these lipids were spotted on two membranes, one of them was incubated with protein extracts containing Ssp1p, the other with extract from E. coli transformed with an expression vector without the *SSP1* gene (empty vector). Signals could be detected for an interaction with Phosphatidylserine and Cardiolipin, for which the intensity decreases as the lipid concentration decreases and the negative control with empty vector does not give a signal (Figure 15A). This means that Ssp1p binds to these two phospholipids from the plasma membrane, what could explain the localization of the protein. It has to be mentioned that while Phosphatidylserine is a major constituent of the plasma membrane, Cardiolipin is found there only in traces. In fact Cardiolipin is much more abundant in the mitochondrial membrane, what appears a little strange for an Ssp1p lipid interactor.

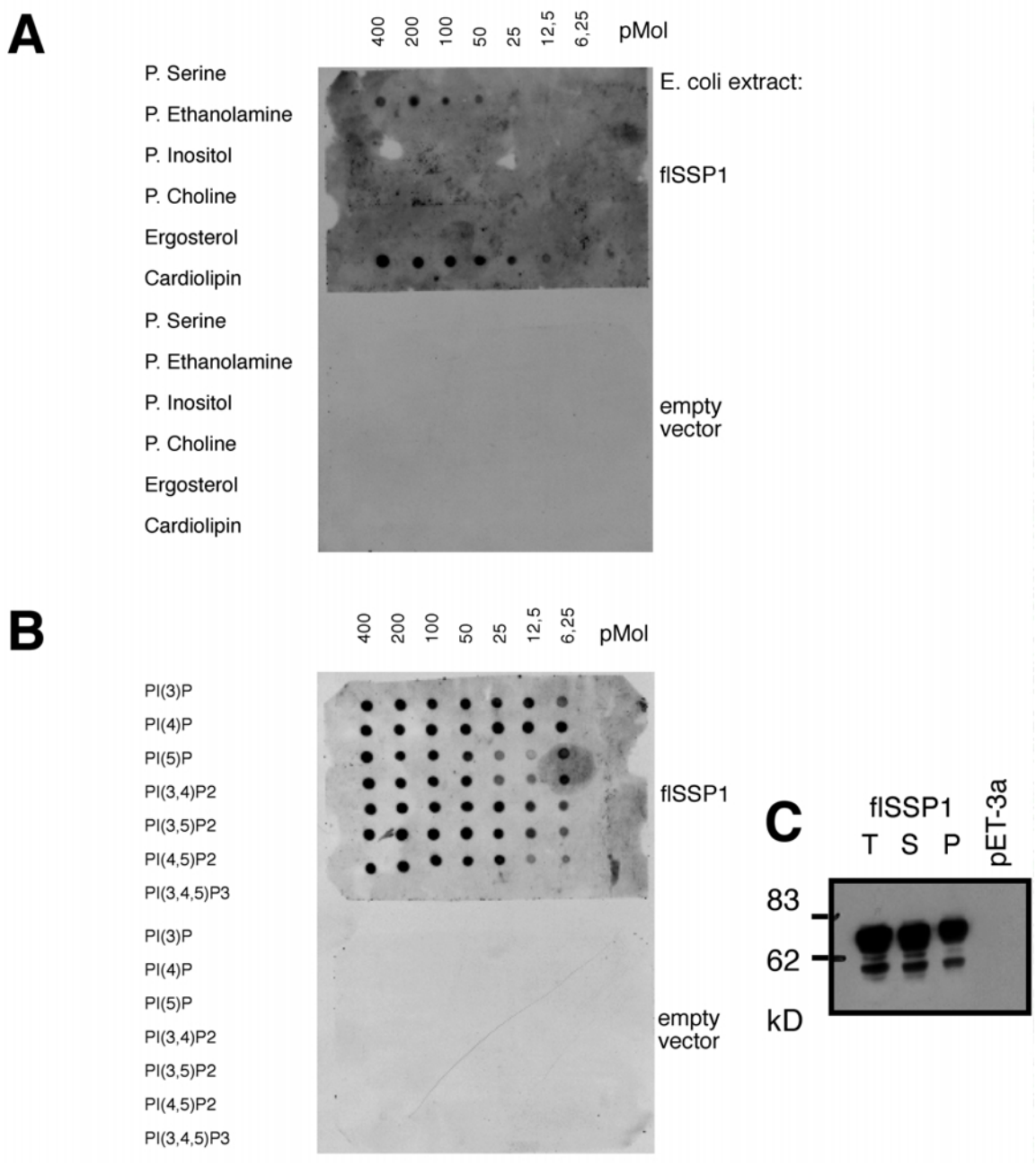

Figure 15:
Lipid-protein overlay assay with unpurified Ssp1p. (A) Lipid-protein overlay assay with standard plasma membrane lipids and bacterial extract overexpressing full length Ssp1p (pMF43). The protein was not purified. P. = Phosphatidyl- (B) Lipid-protein overlay assay with PIPs. (C) Western blot detecting the input of Ssp1p in bacterial extracts after centrifugation (T=total, S=supernatant, P=pellet, fl=full length). Right lane shows an extract from bacteria transformed with empty vector.

We applied the same assay also to a special class of lipids, the Phosphatidylinositolphosphates (PIPs). PIPs are not only structural membrane components but are also signaling molecules. PIPs regulate the function of proteins by directing them to the membrane to interact with membrane specific targets (Misra et al., 2001; Sato et al., 2001). Many of the proteins regulated in this way are involved in vesicle trafficking and therefore the distribution of PIPs is an important marker for the membrane (Simonsen et al., 2001). Ssp1p strongly binds to all different PIPs tested (Figure 15C). Even the lowest amount of lipid tested in the series (6.25 pMol) still binds Ssp1p strong enough to be detected. Besides the negative control with empty vector we also tested if Ssp1p would bind to solvent without lipids, what is not the case (data not shown). So it is concluded that Ssp1p really is a PIP binding protein, although it is a little surprising that Ssp1p seems to bind to all PIPs without making a difference. Usually proteins tested in this assay only bind to a subset of PIPs and the binding also diminishes when the concentration is lowered (Kanai et al., 2001). As this binding appears to be very strong and PIPs are known to be involved in vesicle trafficking, in the following experiments we concentrated on this interaction.

Purification of Ssp1p subdomains

After it was shown that Ssp1p in crude extracts binds to PIPs I wanted to demonstrate that this is also true for the purified protein. To this end I have cloned full length Ssp1p into the *E. coli* expression vector pET-28c, thereby introducing two short tags at the N-terminus of the protein: a 6 His tag for purification and a T7 tag for detection. After it was found that the tagged full length version of Ssp1p still binds to PIPs (Figure 17AI) and the tag does not interfere with this *in vivo* assay subdomains of the protein were also cloned into the same vector. The goal in this experiment was to find out which part of the protein is responsible for the PIP binding properties of Ssp1p.

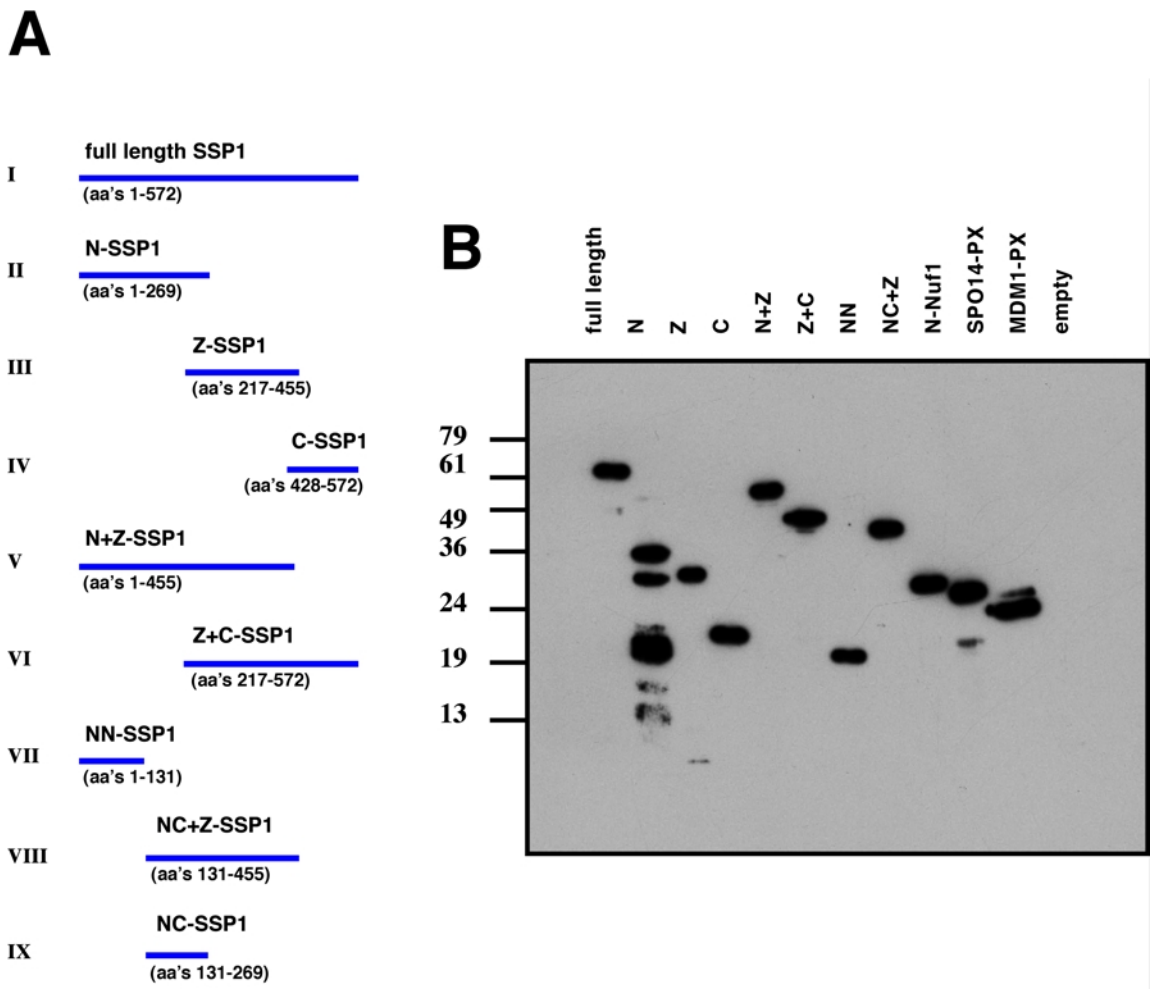

Figure 16:
Purified Ssp1p subdomains. (A) Schematic drawing representing the Ssp1p constructs. The length of the blue line corresponds to the area of the protein which the construct covers. The numbers indicated (I-IX) are also used in Figures 17-19, aa's = amino acids. (B) Western blot detecting the Ssp1p domains after purification with the antibody against the T7 tag. The molecular weights of the Ssp1p constructs plus tags are 70 KD (I), 37 KD (II), 32 KD (III), 22 KD (IV), 58 KD (V), 45 KD (VI), 20 KD (VII) and 43 KD (VIII)

Three slightly overlapping regions of Ssp1p were cloned into pET-28c: the N-terminus, the central region which we termed Z-domain and the C-terminus (Figure 16A). Expression of these constructs and constructs expressing two regions was induced and the fusion proteins were purified on a nickel column under native conditions. In some cases proteins around 70 kD (probably chaperones of the Hsp70 family) were copurifying. Therefore the protein amounts were normalized on a western blot probed with the T7 antibody (Figure 16B). The T7 antibody was used because it recognizes specifically the N-terminal T7 tag while the polyclonal antibody against Ssp1p could preferentially bind to certain parts of the protein although it was raised against full length Ssp1p. The N-terminus of Ssp1p, which is only expressed at very low level, could be purified only together with some degradation products. The expression of the NC-*SSP1* construct, the C-terminal half of the N-terminus, is not shown here but the protein was also normalized against other domains (data not shown).
In the same way the positive controls for PIP binding (*SPO14*-PX and *MDM1*-PX) and a negative control (N-*NUF1*) were purified. *SPO14* codes for Phospholipase D, while *MDM1* is an intermediate filament of the cytoskeleton. Both proteins contain a PX (phox homology) domain which has previously been shown to bind PIPs (Yu and Lemmon, 2001). As a negative control we used the N-terminus of *NUF1*, a component of the central plaque of the SPB, we assumed that this protein would probably not bind to PIPs.

The N-terminus of Ssp1p comprises the PIP binding activity

With all purified proteins including the controls again lipid-protein overlay assays were performed. The N-terminus could clearly be identified as the PIP binding domain (Figure 17A II), neither the Z-domain nor the C-terminus were active in PIP binding (Figure 17A III and IV, for numbers of the constructs see Figure 16A). The other two constructs which bind to PIPs contain the regions N+Z and NC+Z (Figure 17A V and VIII), in which probably the N-terminal part is responsible for the PIP binding activity, as the Z-domain alone was clearly not active.

However, being non-active in this PIP binding assay could also be due to a defect in protein folding, what is more likely for short domains. However, the construct Z+C, which comprises more than half of the protein, is also inactive (Figure 17A VI). It also seems that the N-terminus alone accounts for all binding activity of the full length protein.
In this experiment two negative controls were included: bacterial extracts from *E. coli* transformed with empty vector and purified N-Nuf1p. N-Nuf1p is a SPB component which was purified in the same way as the Ssp1p constructs. Both negative controls show no activity in the assay. The positive control Mdm1p-PX was active in the assay with a binding activity similar to Ssp1p full length and N-terminus, the same is true for Spo14p-PX (data not shown).
From these experiments we conclude that Ssp1p really is a PIP binding protein and that it is the N-terminus which is responsible for the binding activity. The affinity for certain PIPs in this assay diminishes with decreasing concentration (e.g. PI(5)P in Figure 17A II), although full length and N-terminus bind to all PIPs in higher concentration. As this assay is not quantitative, we cannot deduce any proportions between the binding activities for certain PIPs. To elucidate the binding specificity quantitatively, more sophisticated measurements, e.g. surface plasmon resonance studies, would be required (Yu and Lemmon, 2001).
Several conserved protein motifs which mediate PIP binding are known, including PX domains, PH (pleckstrin homology) domains and FYFE domains (Dowler et al., 2000; Gillooly et al., 2001; Song et al., 2001). Sequence databases cannot find any of those domains in the Ssp1p sequence. When we looked at the Ssp1p sequence in more detail we could find a region in the central Z-domain which resembled a PX domain. But the experiments we did show that this region is not required for PIP binding activity. We repeated the experiment several times and simply mixing the samples can now be excluded. When we looked at the N-terminus we could not find any similarity to the conserved motifs. However, it is remarkable that the N-terminus is especially rich in positively charged Lysines. An attractive model would be that these amino acids mediate the interaction with the negatively charged head groups of the PIPs. Experiments where we have mutagenized those residues are currently under way.

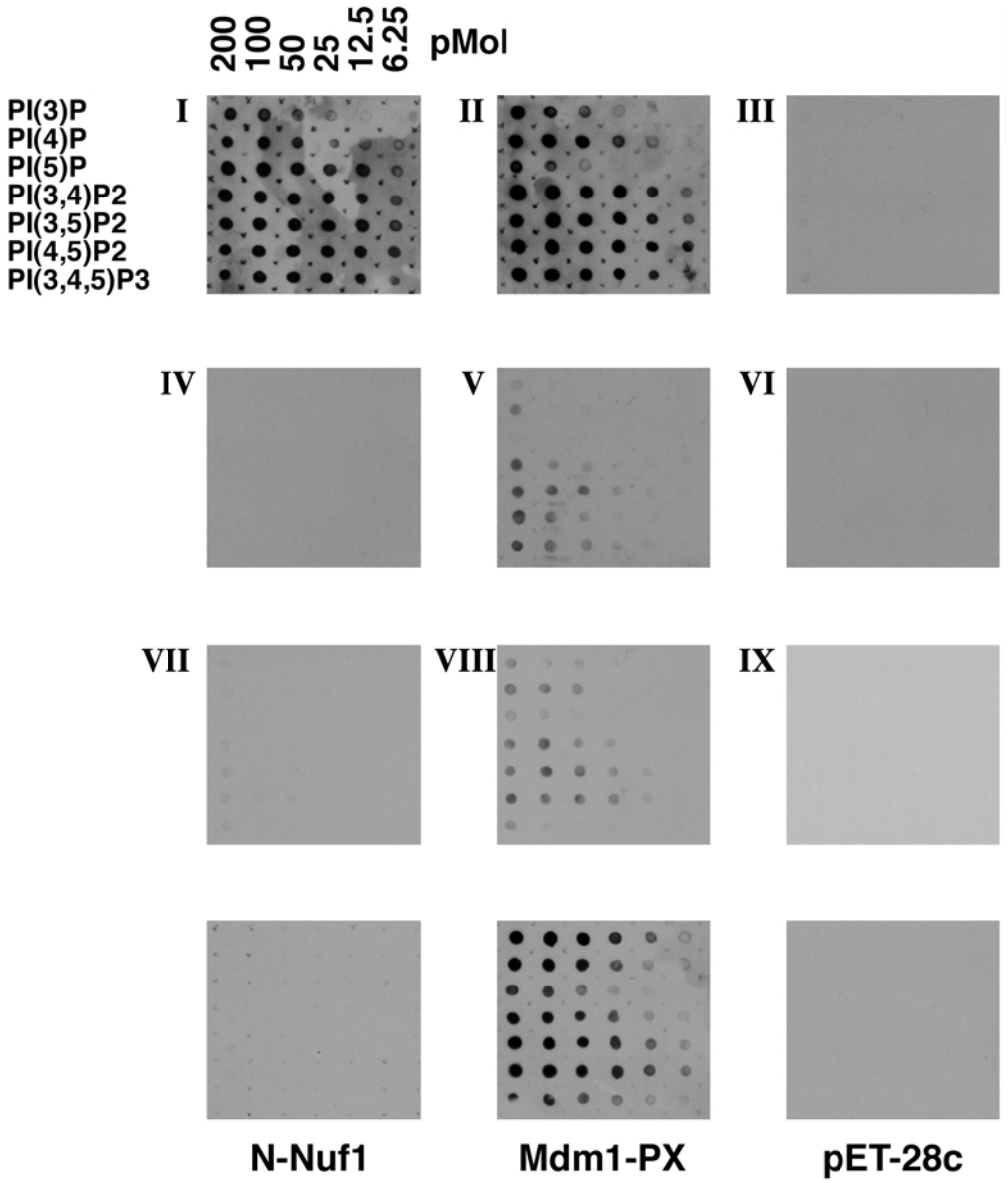

Figure 17:
Lipid-protein overlay assay of the purified Ssp1p constructs. Numbers indicated are I = full length, II = N, III = Z, IV = C, V = N+Z, VI = Z+C, VII = NN, VIII = NC+Z, IX = NC. Two negative controls N-Nuf1p and pET-28c plus one positive control Mdm1p-PX. In those domains which we could purify without contaminating bands, we could also determine the protein concentration used, which was 0.25 μg/ml for the C-terminus (pMF53). This is below the protein concentration of 1.0 μg/ml used for this assay in the literature (Kanai et al., 2001).

Mitotically overexpressed Ssp1p-GFP localizes to sites of secretory vesicle fusion at the bud plasma membrane

Given the PIP binding capabilities of Ssp1p I wondered where Ssp1p would localize when it is overexpressed in vegetatively growing yeast. Ssp1p subdomains were cloned into a yeast expression vector which is inducible and introduces a N-terminal GFP tag. Expression of all fusion proteins was confirmed by western blot experiments (data not shown). After two hours of induction for all constructs we could see a strong GFP signal. The localization pattern for the fusion proteins is very specific for the respective constructs. Full length Ssp1 localizes in speckles along the bud membrane and staining in the bud is much more prominent than in the mother cell (Figure 18A I, for numbers of the constructs see Figure 16A). Also the N-terminus localizes very specifically along the bud membrane. This staining is more continuous and additionally aggregates in the mother cell are visible (Figure 18A II). The Z-domain again forms speckle-like aggregates which are distributed randomly over the budding yeast (Figure 18A III). The C-terminus, Z+C and NC are dispersed in the cell. This staining probably corresponds to cytoplasmic localization and is undistinguishable from expression of GFP alone (Figure 18A IV, VI, IX). The construct N+Z again localizes to the bud membrane and forms aggregates. In this picture it becomes very clear that the staining is restricted to the plasma membrane of the bud as the fusion protein is not detected at the plasma membrane of the mother cell (Figure 18A V). A model is depicted to illustrate this specific localization (Figure 18B). For the staining of the construct NN we observed a high background but still a specific stronger staining along the bud membrane which again appears continuous (Figure 18A VII). The situation for the construct NC+Z is ambiguous. There are very prominent aggregates close to the bud neck, but some smaller aggregates also seem to localize to the plasma membrane (Figure 18A VIII). However, a specific staining to the bud membrane was not observed for this clone. In all strains which display a localization to the bud membrane in the case of unbudded yeast the aggregates are still visible but membrane staining was clearly diminished.

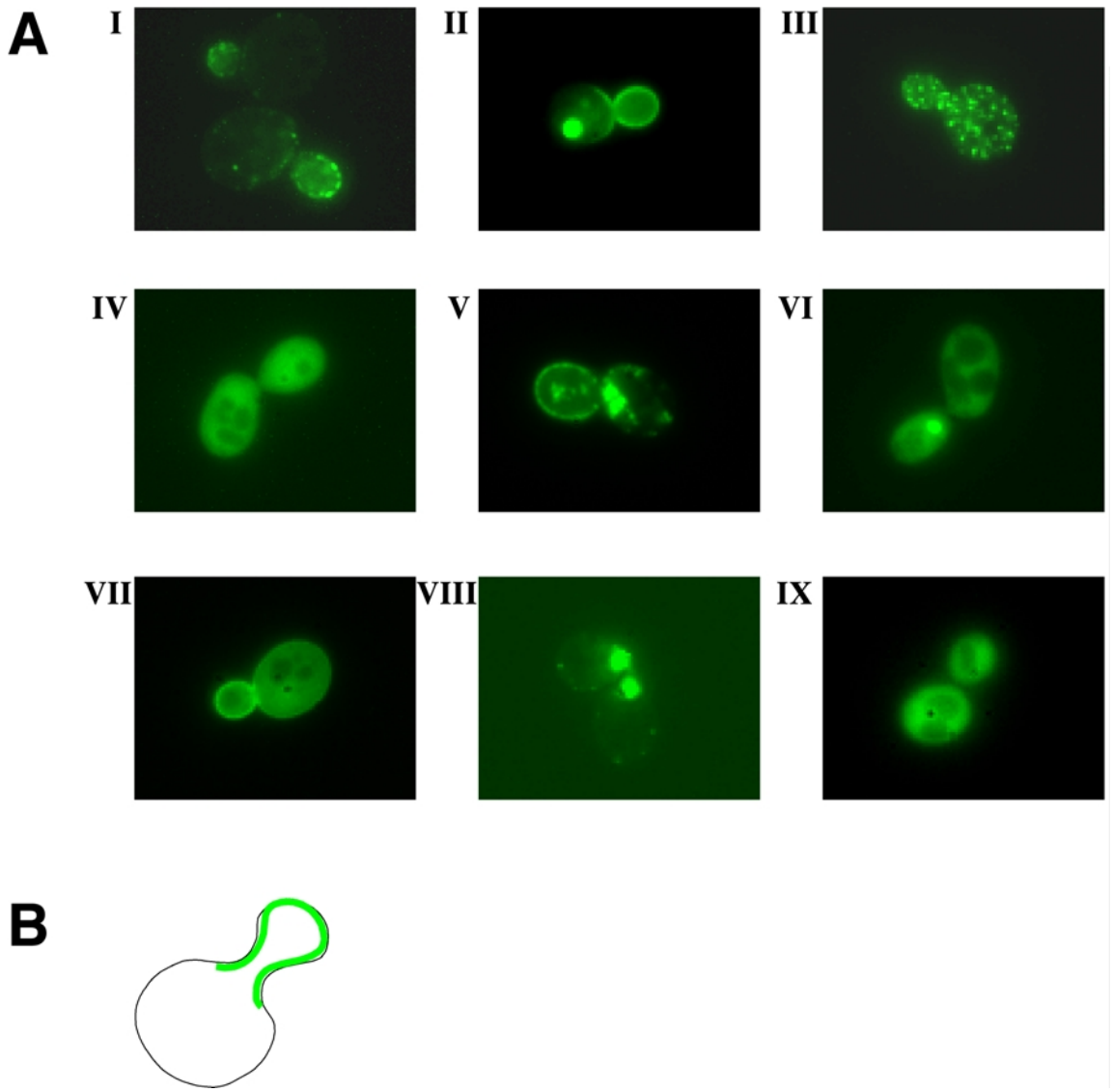

Figure 18:
Localization of GFP-Ssp1p subdomains overexpressed in mitotic yeast cells. (A) Living cell fluorescence microscopy of mitotically overexpressed Ssp1p-GFP fusion proteins. Domains are I = full length, II = N, III = Z, IV = C, V = N+Z, VI = Z+C, VII = NN, VIII = NC+Z, IX = NC. (B) Model for the localization of certain fusion proteins to the plasma membrane of the bud only, not to the plasma membrane of the mother cell.

The observation that full length Ssp1p and certain subdomains localize specifically along the bud membrane in mitotic cells demonstrates another link of Ssp1p to secretory vesicle trafficking. In mitotic cells the bud membrane is the site where vesicles from the late secretory pathway fuse to enlarge the membrane system. This is a parallel to the situation in meiosis were Ssp1p localizes to the prospore membrane (and not to the plasma membrane, see Figure 10A) while this is the site of vesicle fusion. Given the overlap of PIP binding activity and bud membrane localization for the Ssp1p domains (compare Figure 17A with Figure 18A, see also Table 1) it is tempting to speculate that sites of secretory vesicle fusion contain a specific lipid composition. These sites would then probably have high concentrations of PIPs what marks them as targets for membrane vesicles. If Ssp1p is overexpressed in mitotic cells, part of the protein is associated with these sites via the PIP binding activity. However, the experiments which could prove this model are very challenging: analysing the lipid composition of substructes from membranes would require their isolation without other, contaminating membranes. An *in vitro* assay which simulates prospore membrane formation is currently not available. The lipid binding properties of Ssp1p would also have to be analysed in more detail to find out which of the lipid really is required for membrane localization.

Ssp1p has cytotoxic effects when it is mitotically overexpressed in yeast

When we controlled the expression of the GFP fusion proteins we noticed that expression of certain clones apparently had a negative effect on the growth rate. After four hours the liquid cultures which induced Ssp1p had a much lower optical density then those not expressing the protein. In order to further elucidate this effect we cloned all Ssp1p subdomains into an inducible yeast expression vector (p426-Gal1). This vector system allows higher levels of expression and does not introduce a tag which itself could interfere with growth. Expression of all constructs was tested in western blots (data not shown). Dilution series of transformed yeast were spotted on selective plates containing as sugars either Galactose and Raffinose (induction of the Gal1 promoter) or Raffinose alone (no induction).

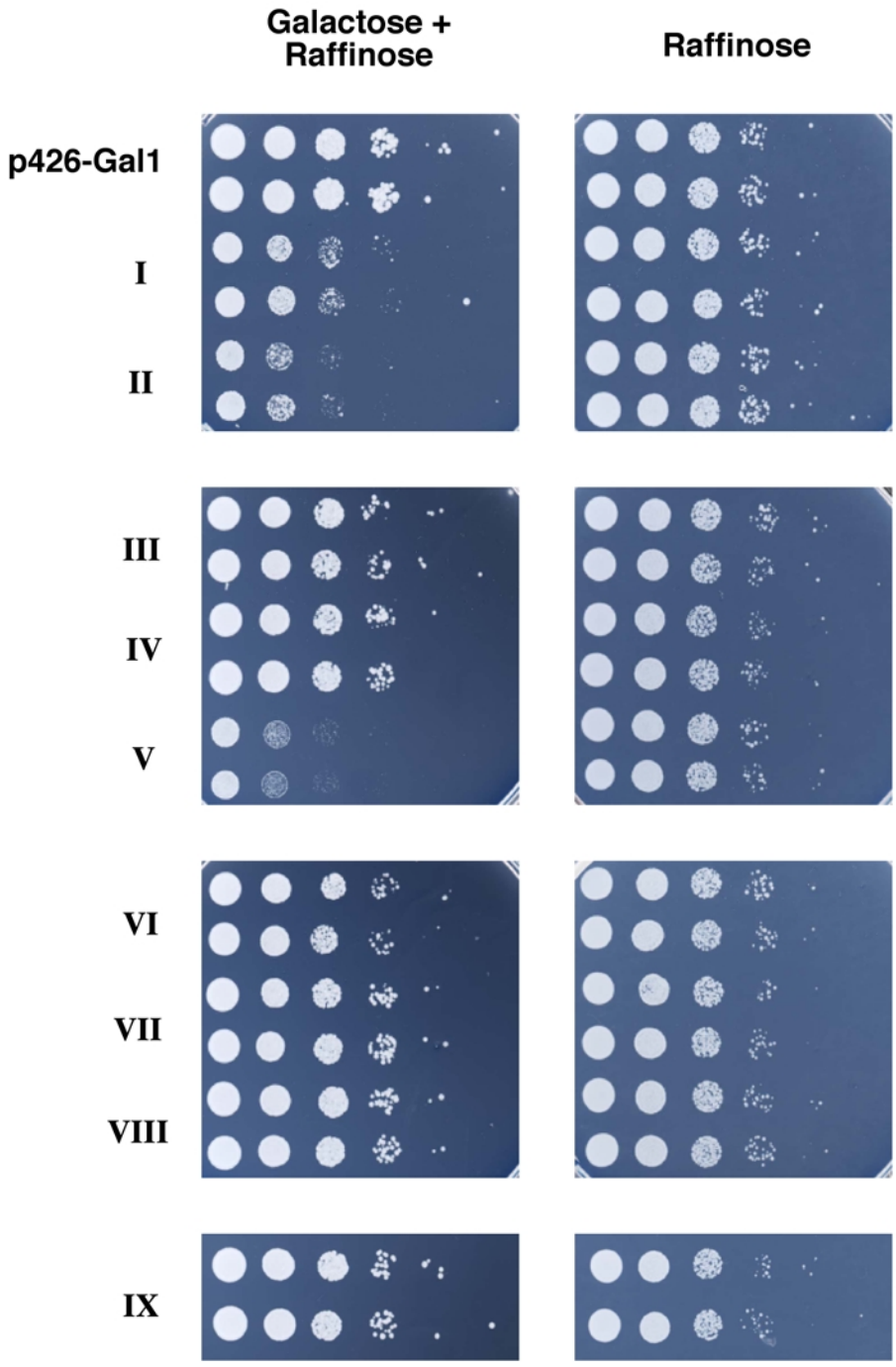

Figure 19:
Overexpression toxicity of the Ssp1p constructs in yeast. Dilutions series were spotted on plates which induce expression (left column) or not (right column). Two transformants from each clone were spotted. Ssp1p domains are I = full length, II = N, III = Z, IV = C, V = N+Z, VI = Z+C, VII = NN, VIII = NC+Z, IX = NC.

For each construct and the empty vector two dilution series of independent transformants were made. After two days incubation at 30 °C the plates were scanned. It can be seen that under non-inducing conditions all yeast strains grow at the same growth rate (Figure 19, right column). When Ssp1p is overexpressed, three clones manifest overexpression toxicity by showing a growth defect. These are full length Ssp1p, the N-terminus and the domain N+Z (Figure 19 I, II and V). Expression of all the other constructs had no effect since these strains grow at the same rate under induced or non-induced conditions. The growth defect for mitotic overexpression is not very strong, but it is highly reproducible. The fact that again the N-terminus is required for this effect indicates that Ssp1p localization to the membrane might be responsible for this growth defect.

Mapping of the region in Ssp1p responsible for Ady3p binding

The initial point that led to the investigation of Ssp1p was based on the finding that Ady3p and Ssp1p appeared to be two hybrid interaction partners (Ito et al., 2000; Uetz et al., 2000). For the full length proteins, this interaction was confirmed with the two hybrid system routinely used in our lab (Moreno-Borchart et al., 2001). As I had already started to assort certain functions of the protein to specific regions of Ssp1p, I also wanted to map the association sites required for interaction with Ady3p. The same subdomains of Ssp1p (Figure 16A) were cloned into the corresponding two hybrid vectors to produce fusion proteins with either the lexA DNA binding domain or the Gal1 transcriptional activation domain. All of these constructs and the full length ADY3 clones were tested against each other. Concerning the two hybrid interaction between full length Ady3p and full length Ssp1p the interaction could be confirmed in one direction, while the second *ADY3* clone is apparently not active (Figure 20). Additionally week interactions between Z-*SSP1*, C-*SSP1*, N+Z-*SSP1*, Z+C-*SSP1* and full length *ADY3* could be observed.

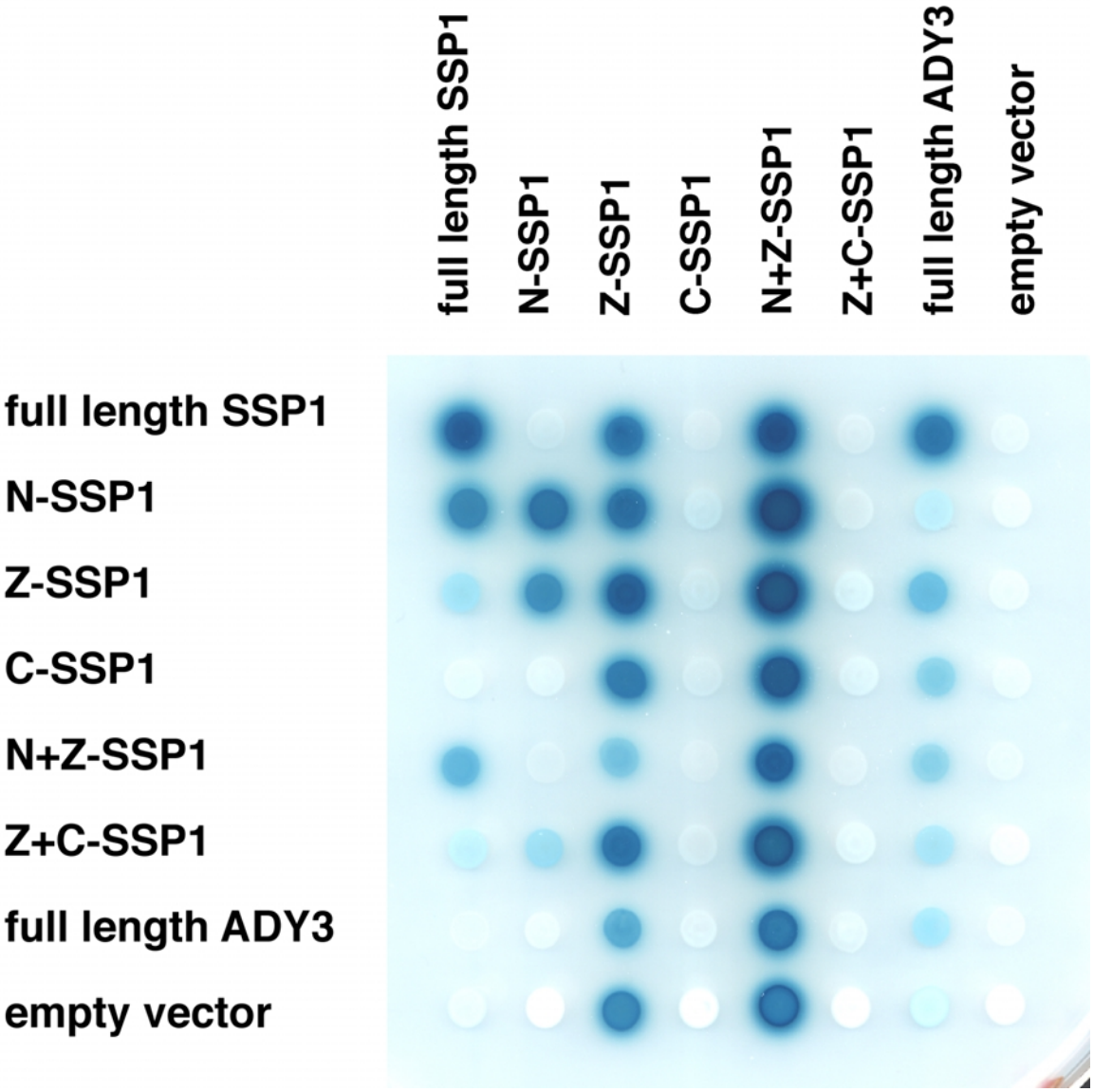

Figure 20:
Yeast two hybrid interactions between Ssp1p subdomains and Ady3p. For this assay the corresponding domains of *SSP1* (Figure 16A) were cloned into pMM5 (fusion with lexA-BD, vertical row) and pMM6 (fusion with Gal4-AD, horizontal row). *SSP1* constructs are full length (aa's 1-572), N (aa's 1-269), Z (aa's 217-455), C (aa's 428-572), N+Z (aa's 1-455), Z+C (aa's 217-572) and NN (aa's 1-131), NC+Z (aa's 131-455) and NC (aa'a 131-269). Two hybrid reporter yeast strains (SGY37 and YPH500) were transformed with one plasmid (either from pMM5 or pMM6) and then mated with the strain containing the other plasmid and plated on double selection media. βGalactosidase activity was measured in an overlay assay

These two hybrid interactions are weak but clearly above background threshold, whereas N-*SSP1* and *ADY3* do not show interaction. With all the appropriate precaution this points to an interaction between Ady3p and Ssp1p which is mediated by the central and C-terminal parts of Ssp1p, while the N-terminus is not required.

When we look at the autointeractions of the *SSP1* clones we can confirm association between the two full length *SSP1* clones. Furthermore, strong interactions are found between N-*SSP1* and full length *SSP1*, between N-*SSP1* and N-*SSP1* as well as N-*SSP1* and Z-*SSP1*. Albeit weaker, there is also an interaction between N+Z-*SSP1* and full length *SSP1*. The clone C-*SSP1* is not involved in any autointeraction activity of *SSP1*. Unfortunately, this assay also gives some unclear results: the clones Z-*SSP1* and N+Z-*SSP1* display autoactivity in one direction, they also interact with empty vector. Some of the interactions, e.g. N-SSP1 with full length SSP1, work only in one direction and are inactive in the other.

But still this data, which can be considered preliminary and should be reconfirmed, supports a model for the assembly of the LEP coat on the prospore membrane. Autointeraction of Ssp1p is mediated by its N-terminal part, which is also the region required for membrane association and lipid binding. Interaction of Ssp1p with Ady3p requires the C-terminal part of Ssp1p instead. This could be interpreted in a way that Ssp1p forms the first, prospore membrane most proximal layer of the LEP coat. Parallel arrangements of Ssp1p cover the membrane, where they are associated to lipids with their N-termini. The C-termini pointing from the membrane are then the sites for interaction with Ady3p. Ady3p would then form the second protein layer of the LEP coat on the prospore membrane

2.6 Analysis of mutant phenotypes for Δ*spo70* and Δ*spo71* strains

Numerous genes required for sporulation have been identified in genetic screens. For most of them the exact function in the process of spore formation remains unclear. As we have identified a protein structure on the prospore membrane we now possess a novel tool to analyse these mutants. In immunofluorescence experiments with an antibody against Ady3p it can be seen whether the structure of the LEP coat is altered, either in size and shape or in the timing it appears and dissociates. This approach was succesfully applied to link the sporulation defect of two genes, *SMA1* and *SMA2*, to a malfunction of prospore membrane formation (Rabitsch et al., 2001). For two other deletion mutants, Δ*spo70* and Δ*spo71*, we find that the donut structures formed by the LEP coat accumulate to a much higher extent and persist much longer then in the wild type situation (M. Knop, unpublished data). This indicates a phenotype in closure of the prospore membrane and/or disassembly of the LEP coat.

Both mutants, Δ*spo70* and Δ*spo71*, display a complete block in spore formation. *SPO70* is a meiosis specific homolog of *CDC20*, a regulatory subunit of the anaphase promoting complex (APC) (Chu et al., 1998). A proposed function of *SPO70* in meiosis is the modulation of the ubiquitin ligase activity of the APC in a way that allows the exit from meiosis, analogous to the situation at the end of mitosis (Cooper et al., 2000). *SPO70* is required for the degradation of B-type cyclins in meiosis and the deletion phenotype also reveals a block in expression of late meiotic genes (Figure 14). *SPO70* is likely to be necessary to overcome a meiotic cell cycle checkpoint and therefore the deletion mutant arrests at the end of meiosis.

Much less is known about the function of *SPO71*. It is a sporulation specific protein and might localize to the spore wall (Chu et al., 1998). Like for a deletion of *SSP1*, the Δ*spo71* mutant undergoes both meiotic divisions but afterwards fails to form spores. Interestingly, *SPO71* is predicted to encode a transmembrane protein.

Epistasis experiments combining mutants of *ssp1*, *spo70* and *spo71*

To explain the setup for these experiments, it has to be noticed that they were performed before we knew about the role of *SSP1* in recruiting other proteins to the LEP coat and shaping the PSM, described in the preceding chapters. Our knowledge at that time was that *spo70* and *spo71* mutants are probably delayed in PSM closure while the *ssp1* mutant was reported to close PSMs prematurely (Nag et al., 1997). The question was, what would happen if we combined these mutations. A hypothesis was that then the PSM would again close at the right time and spores would form at wild type level. Therefore strains with deletions of two genes, Δ*spo70* Δ*ssp1* and Δ*spo71* Δ*ssp1*, respectively, were constructed. The idea was to visualize the LEP coat using the antibody against Ady3p. The observation was that in all strains containing a deletion for *ssp1*, either Δ*ssp1* alone or Δ*spo70* Δ*ssp1* and Δ*spo71* Δ*ssp1*, Ady3p was no longer detectable at the PSM. However, in the double mutants as well as in all single mutants, spore formation was still completely blocked. The observation that in Δ*spo70* and Δ*spo71* single mutant strains LEP coats accumulated to a higher extent compared to wild type was confirmed (data not shown).

Furthermore, it was also checked if and at what time the *spo70* and *spo71* mutants would lose their viability. To this end the strains were induced to sporulate and after a certain time dilutions were replated on rich media and the forming colonies were counted. The mutant strains lose viability over time, this starts from 10 hours on. After 24 hours viability for both is below 10 %, after 48 hours below 1 % (Figure 21A). These kinetics and the proportions are very similar to the loss of viability detected for the Δ*ssp1* mutant (Figure 14A). The values for Δ*spo71* are virtually idential to those for Δ*ssp1*, Δ*spo70* seems to lose viability even a bit faster.

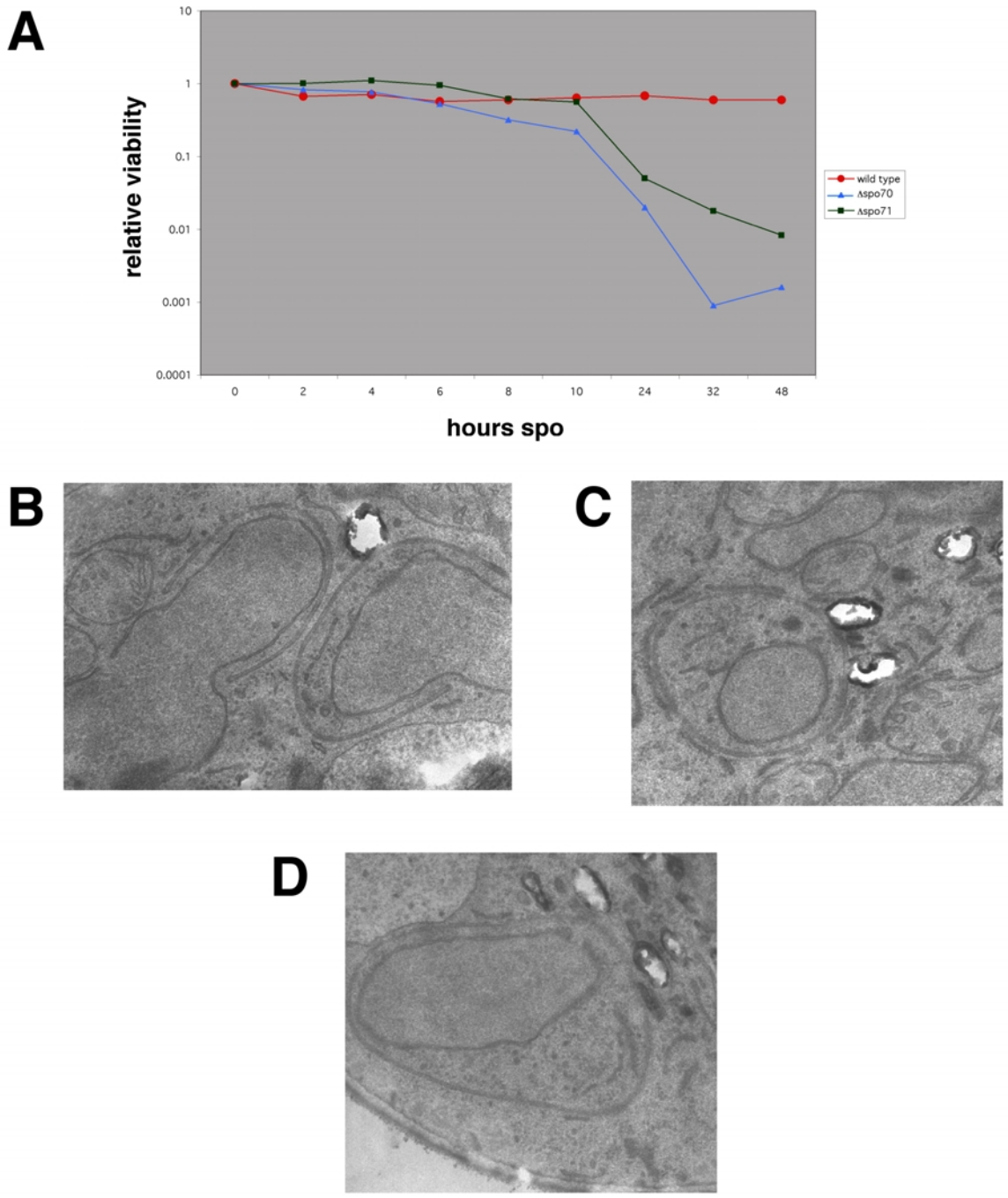

Figure 21:
Mutant phenotypes of Δ*spo70* and Δ*spo71* strains in sporulation. (A) Relative viability of strains wild type (YKS32), Δ*spo70* (KN3279) and Δ*spo71* (YMK527-1) plotted against time. (B) and (C) Transmission electron microscopy pictures of sections from sporulating yeast after 6 hours of sporulation from strain Δ*spo70* (KN3279) and (D) Δ*spo71* (YMK527-1).

In addition, the loss of viability was measured for the double mutant strains Δ*spo70* Δ*ssp1* and Δ*spo71* Δ*ssp1*. Again a pattern very similar to the one observed for the *ssp1* mutant was found (data not shown). Taken together, the mutants of *spo70* and *spo71* appear to have a phenotype very similar to *ssp1* concerning the defect in spore formation and the loss of viability. The double mutants again behave like the single mutants for the features tested here.

Immature spores containing cytoplasm are still formed In the Δ*spo70* and Δ*spo71* deletion mutants

As the phenotypes for the deletion mutants of *spo70* and *spo71* resemble the phenotype of a *ssp1* deletion, I wanted to see whether they also have an effect on the shaping of the PSM. To this end samples for electron microscopy were prepared to visualize the PSMs. In the Δ*spo70* mutant prospore membranes surrounding the nuclear lobes could still be detected (Figure 21B). The shaping of the membranes appears normal, they grow at a distance to the nuclear envelope and the opening at the tip is wide (compare with Figure 12B and Figure 13A-C). The same holds true for the Δ*spo71* mutant (data not shown). Both mutant strains are still able to form immature spores which contain cytoplasm, which is a striking difference to the *ssp1* deletion phenotype (Figure 21C and D, compare with Figure 12D and Figure 13D-F). Whether the PSMs in these immature spores have already closed cannot be judged as the pictures represent only single sections through the yeast cells. However, it is apparent from these micrographs that the mutant strains reach a stage in sporulation which is never reached by the Δ*ssp1* strain. On the other hand, mature spores with a spore wall (Figure 12F) are never observed in the Δ*spo70* or Δ*spo71* deletion strains. These results suggest that *SSP1* probably acts before *SPO70* and *SPO71* in the pathway of spore formation. *SSP1* clearly has an effect on the shaping of the PSM whereas this feature seems to be normal in the absence of *SPO70* or *SPO71*.

Chapter 3

Materials and Methods

3.1 General considerations

Standard molecular biology techniques are described in several general laboratory manuals (Ausubel et al., 1994; Sambrook et al., 1989). Standard yeast methods including recipes for yeast media are described elsewhere (Guthrie and Fink, 1991). In the following parts only methods applied in this work which might not be common are explained in detail.
Standard chemicals were ordered from Sigma Aldrich, Fluka or Merck. Qiagen kits were used for all DNA preparations. Sources for special chemicals and other materials are mentioned in the corresponding chapters.

3.2 Gene tagging and disruption with a PCR based cassette mechanism

All chromosomal tags at the C-terminus and all gene disruptions (deletion mutants) were induced with a cassette mechanism (Knop et al., 1999). This tagging protocol relies on homologous recombination of the yeast genome with a transformed PCR fragment. Tag and marker are amplified in a PCR from cassette modules, the annealing sites are conserved between the modules. Additionally, the primers contain homologous sequences (55 base pairs) from the region before and after the Stop codon (gene tagging) or before the Start codon and after the Stop codon (gene disruption) of a certain gene. So one set of three primers for each gene allows tagging with several tags (ProA, GFP, HA, Myc), use of a combination of markers (kanMX, his3MX, klTRP1) and deletion of the entire coding region. This technique has an enormous versatility and allows reliable and fast genomic manipulations. Problems occur only when the tag interferes with the functionality for a certain gene, as it is the case for *SSP1*.
The genomic manipulation can be verified by preparing yeast genomic DNA and testing for the presence of the cassette at the right place in a control PCR reaction.

3.3 Sporulation conditions

All sporulation experiments described here were performed in liquid culture at 30 °C. In general, sporulation is induced by starving diploid yeast for nitrogen in the presence of a nonfermentable carbon source (Schwacha and Kleckner, 1994). Starvation is induced after all yeast have finished the last cell cycle, this ensures that they sporulate synchronously. Before inoculation of the preculture the yeast are streaked to single colonies first on YP-Glycerol, then on YPD (Guthrie and Fink, 1991). For the results described here two different sporulation protocols were applied: a slower but more synchronous one (Figure 7A and B) and a faster one (all other experiments).

Sporulation Protocol 1 (slower)

Inoculate a preculture in YPD, shake 24 hours. Use this preculture to inoculate a prespo-culture on YPA (1 % yeast extract, 2 % bacto peptone, 2 % potassium acetate) at a density 0.2 OD 600/ml (around 1:50), shake 16 hours. Centrifuge the yeast at 2000 g for 5 min at room temperature, wash the pellet once with water and resuspend it in twice the volume of sporulation medium (2 % potassium acetate), this is time point zero. Shake at high speed with good aeration.

Sporulation Protocol 2 (faster)

Same as above except prespo-culture is in YPAc (1 % yeast extract, 2 % bacto peptone, 1 % potassium acetate) and shakes for only 14 hours. Sporulation medium contains now only 0.3 % potassium acetate plus 0.02 % raffinose.

3.4 Two hybrid assay

Genes to be tested for interaction were first cloned into two hybrid vectors (pMM5 and pMM6), what results in fusion to the lexA-BD or the Gal4-AD, respectively. Those vectors were transformed into yeast two hybrid tester strains of opposite mating type (SGY37 and YPH500) and mated.

From the resulting strains, which were tested on double selection medium without Histidine and Leucine for presence of both vectors, a dilution was spotted on double selection plates containing Raffinose and Galactose (induction of protein expression). The plates were incubated at 30 ° C for two days. Two hybrid activity was tested in a X-Gal agarose overlay assay.
For the overlay assay the following solution (100 ml) was prepared:
Mix 50 ml 0.5 M $NaHPO_4$ pH 7.0 with 1 ml 10 % SDS, 1 ml 1 M KCl and 0.1 ml 1 M $MgCl_2$, heat solution to about 40 °C. Melt 600 mg low melting point agarose (Biorad) in 30 ml water for 10 s in the microwave, add to the solution while stirring, add 0.2 ml 14.4 M βMercaptoethanol, make up to 100 ml with water. Add 1 ml 40 mg/ml X Gal in DMF to the solution and pour onto the plates, leave on the bench until the agarose has polymerized. Incubate plates at 30 °C. Strong signals are visible after one hour, weaker signals after incubation over night.

3.5 Two hybrid screen

The two hybrid system used including plasmids and construction of the library is described in (James et al., 1996).
The bait plasmid pMF30 containing C-Zip1p (aa's 746-876) fused to the GAL4-BD of pGBDU-C1 was transformed in the two hybrid tester strain PJ69-4A. Transformants were selected on selective medium without Uracil and competent cells of the resulting strain YMF108 were made. The three yeast genomic libraries in the vectors pGAD-C1, -C2 and -C3 were cotransformed and in test transformations the efficiency was monitored by selecting for both plasmid markers in medium withour Uracil (pGBDU-C1) and Leucine (pGAD-CX). The screen was performed with 2.6 to 4.1 millions of independent transformants what should screen more then 99.9% of the library clones (James et al., 1996). The screen was first plated on selective medium without Histidine and after 14 days it was replica plated on selective medium without Adenine.

Positive clones were plated on SC medium without Leucine containing 5'FOA (Sigma) to force them to loose the bait plasmid pMF30 and again tested for the reporters on SC without Histidine and Adenine. The remaining library plasmid was isolated by the yeast plasmid rescued method and sequenced.

3.6 Yeast plasmid rescue

In order to rescue plasmids which were transformed into yeast (from the library in the two hybrid screen) the following protocol was used (yeast mini prep):
Yeast (approximately one match head) were scraped from a plate and resuspended in 0.5 ml water. They were briefly spun down and resuspended in 250 µl S buffer (10 mM K_2HPO_4 pH 7.2, 10 mM EDTA, 50 mM βMercaptoethanol, 50 µg/ml Zymoliase 100 T (Seikagaku Brewery, Japan)). Samples were incubated at 37 °C for 30 min, then 55 µl lysis solution (25 mM Tris pH 7.5, 25 mM EDTA, 2.5 % SDS) was added. The samples were incubated at 65 °C for 30 min with gentle shaking, then 83 µl 3 M KAcetate pH 5.5 was added. They were chilled on ice for 10 min and centrifuged at 12000 g for 10 min at 4 °C. The supernatant was poured off into a new tube and 400 µl ice cold ethanol was added. After incubation on ice for 10 min the DNA was pelleted by centrifugation at 12000 g for 10 min at 4 °C. The DNA pellet was washed with 70 % ice cold ethanol and recentrifuged. DNA was resolved in 20 µl water.

3.7 Protein overexpression in yeast

To overexpress certain genes in yeast they were generally cloned in front of the Gal1 promoter and this promoter was induced. This can be achieved by changing the nutritional conditions: the promoter is repressed when cells grow on glucose (normal growth conditions), it is moderately activated on raffinose and it is strongly induced in the presence of galactose.

Yeast grown on plates were simply transferred from glucose plates to plates containing raffinose and galactose (two hybrid assay, overexpression toxicity assay). When the gene was to be overexpressed in liquid culture (overexpression of GFP fusions for fluorescence microscopy, mitotic overexpression for gel filtration) the following protocol was applied:
A preculture was grown on selective SC medium containing 2 % glucose for 24 hours at 30 °C. Three cultures with different optical densities (0.015; 0.03 and 0.06 OD600/ml) were inoculated on SC medium containing 2 % raffinose. These cultures were shaken for 15 hours at 30 °C and then the cultures with an OD600 between 0.2 and 0.4 were used for induction. Galactose was added to a concentration of 2 % and the cells were again shaken at 30 °C. Expression of the overexpressed GFP fusions was detectable from two hours on, the expression peak was around 4-6 hours.

3.8 Preparing TCA extracts from yeast

The most simple method to prepare yeast extracts for western blot analysis is the TCA method.
Yeast corresponding to 1-5 OD 600 are pelleted and resuspended in 1 ml of cold water. Add 150 µl of lysis solution (1,85 M NaOH, 7.5 % Mercaptoethanol) and incubate on ice for 15 min. Then add 150 µl trichloracetic acid (TCA) and incubate on ice for 10 min. Centifuge at 12000 g for 10 min at 4 °C and decant the supernatant. The pellet is resuspended in 100 µl HU buffer/OD and shaken at 65 °C for 10 min.
HU buffer (gel loading buffer):
8 M Urea, 5 % SDS, 200 mM $NaPO_4$ pH 6.8, 0.1 M EDTA, 15 mg DTT/ml, traces bromphenole blue

3.9 Glass bead lysis

This protocol to prepare yeast extract was used for complex purification, dephosphorylation and gel filtration experiments.
Lysis buffer is 1 x TBS (tris buffered saline) with all protease inhibitors. The yeast pellet was dissolved in approximately the same volume lysis buffer. An equal volume acid washed glass beads (Sigma) was added to the solution. The mixture was vortexed 15 times 30 s at maximum speed. Cooling is essential to avoid protein degradation and so all the samples were cooled on ice for 30 s after vortexing 30 s. The supernatant was poured off, Triton X was added to a concentration of 1 % and the samples were stirred on ice for 15 min. This gives the fraction raw extract.

3.10 Fractionation of yeast extracts

Input for the fractionation is the raw extract from the glass bead lysis, in this procedure the extract was cleared from cellular debris and the solubility of the protein was tested. In the low speed centrifugation the raw extract was spun at 500 g for 5 min at 4 °C. The supernatant was subjected to a high speed centrifugation at 20000 g for 15 min. Samples from all the fractions were taken for gel analysis.

3.11 Protease Inhibitors

Preparing yeast extracts by glass bead lysis we had massive problems with protein degradation, numerous proteases are upregulated during sporulation. Therefore we used an extensive cocktail of protease inhibitors when we prepared extracts.
Before harvesting, PMSF (phenylmethylsulfonylfluoride, Sigma) at a concentration of 2 mM was directly added to the medium (10 min prior to harvest).

Protease inhibitors in the lysis buffer:
2 x Complete (Boehringer Mannheim)
Pepstatin (Biomol) 5 μg/ml
Chymostatin (Biomol) 6 μg/ml
Antipain (Sigma) 2.4 μg/ml
Leupeptin (Biomol) 1.72 μg/ml
Aprotinin (Sigma) 1.8 μg/ml
Trypsin Inhibitor (Sigma) 2 μg/ml
Benzamidin (Sigma) 5 mM
PMSF (Sigma) 2 mM

3.12 Purification of protein complexes using the ProA tag

The rationale for this experiment was to identify interacting proteins when Zip1p-ProA was purified with magnetic beads coupled to IgGs. Large quantities of sporulating yeast (1.5 liters) were harvested after 6 hours sporulation. Extracts were made with glass bead lysis and after fractionation the high speed supernatant and pellet were subjected to incubation with IgG beads. Extraction of the pellet was done by stirring the sample on ice for 30 min in extraction buffer (50 mM Tris pH 7.5, 300 mM NaCl, 10 mM EDTA, 2 mM EGTA). Incubation with IgG coupled magnetic beads (Dianova) was performed in the equivalent buffer containing 200 mM NaCl over night at 4 °C on a roller. Washing of the beads was performed in magnetic racks (Dianova) 4 times with the same buffer, 2 times with 5 mM NH_4Acetate. The beads were first eluted by shaking in 1% SDS at 65 °C for 10 min, the eluates were mixed with HU loading buffer. The second elution was performed by shaking the beads directly in HU buffer for 10 min at 65 °C. Eluates were then loaded on an SDS gel. After Coomassie staining the interesting bands were cut out and sequenced by mass spectrometry (in cooperation).

3.13 Dephosphorylation assay

For this dephosphorylation assay we needed purified Ssp1p expressed in yeast. First we tried to immunoprecipitate Ssp1p with the antibody but apparently only the unphosphorylated form of Ssp1p is precipitated efficiently. Therefore we decided to isolate phosphorylated Ssp1p out of an SDS gel.
Glass bead extract was prepared in the presence of phosphatase inhibitors (NaF 25 mM and NaOrthovanadate 5 mM) and the low speed supernatant was loaded on a preparative SDS gel. At the expected height (70-80 KD) the band was cut out. The piece of gel was shrinked in acetonitrile and rehydrated in alkaline phosphatase buffer (Boehringer Mannheim). The gel was homogenized by pressing it through a syringe, an equal volume of 1 % SDS was added and the sample was shaken at maximum speed at 95 °C for 10 min. The gel slurry was loaded on the membrane of a Spin X tube (Costar) and centrifuged at maximum speed for 5 min. A clear solution was collected below the membrane and this was split into three reactions. To the first reaction nothing was added, 20 U calf intestine phosphatase (CIP, Boehringer Mannheim) were added to the second reaction and the third reaction was brougth to a concentration of 25 mM NaF and 5 mM NaOrthovanadate before addition of 20 U CIP. All reactions were incubated at 30 °C for one hour.

3.14 Gel filtration

Sporulating wild type yeast or strains overexpressing Ssp1p after galactose induction were harvested and subjected to glass bead lysis in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT). After the lysis the buffer was adjusted to 0.1 % Triton X. The high speed supernatant was loaded on a Superose 6 column in the SMART System (Pharmacia). Eluates from the column were collected in 26 fractions of 50 µl.

The protein was precipitated by addition of 8 µl TCA, incubated on ice for 15 min and centrifuged at 13000 g for 15 min at 4°C. The pellets were dissolved in HU buffer and loaded on a SDS gel. To normalize the column several marker proteins were loaded and their elution peaks were noted.

3.15 Protein-lipid overlay assay

This assay was described in (Dowler et al., 2000; Kanai et al., 2001). One microliter of lipid solution containing 400 – 6.25 pmol of the corresponding lipid (concentration 400 – 6.25 pmol/µl) solved in chloroform : methanol : water in the ratio 1 : 2 : 0.8 was spotted on a nitrocellulose membrane (Hybond C-extra, Amersham Pharmacia) and dried at room temperature for one hour. The membrane was blocked in TBS 0.1 % Tween 20 containing 3 % fatty acid free BSA (Sigma) over night at 4 °C. Then the protein solution with a protein concentration of 0.25 µg/ml was added and incubated for two hours at room temperature. The membrane was washed 6 times for 5 min in TBS 0.1 % Tween 20 (TBST). The first antibody was incubated for one hour in blocking solution (TBST 3 % fatty acid free BSA), the membrane was washed 6 times for 5 min in TBST. The secondary antibody (coupled to horseradish peroxidase) was incubated for two hours in blocking solution at room temperature, the membrane was washed 12 times for 5 min in TBS. Then the membrane was exposed to film.

3.16 Preparation and purification of polyclonal rabbit antibodies

The two self made antibodies described here were raised against the N-terminus of Zip1p (pMM3, GST-N-Zip1p) and against full length Ssp1p (pKS54, 6His-Ssp1p). The two fusions proteins were affinity purified and loaded on a preparative SDS gel. The blotted protein was stained with Ponceau S and the corresponding bands were cut out from the blot.

The bands were dried in the speedvac, chopped into small pieces and suspended in 500 µl DMSO, this mixture was incubated at 50 °C over night shaking. 250 µl of this solution were mixed with 250 µl water plus 500 µl adjuvans and injected into the rabbit. After two further boosts test sera from the rabbit recognized the proteins in extracts. The two antibodies were affinity purified by binding to the same proteins with a different tag, trpE-N-Zip1p (pMM1) and GST-Ssp1p (pKS53). Purified protein was coupled to columns of CNBr sepharose (Pharmacia). Coupling of the proteins (1-10 mg) to the column (approximately 2 ml) was done in coupling buffer (100 mM $NaHCO_3$ pH 8.3, 0,5 M NaCl) over night at 4°C. Efficiency of the coupling can be determined by measuring the protein concentrations of the supernatant before coupling and after. Free sites were blocked with 0.2 M Glycine pH 8.0 for 2 hours at room temperature. Columns were washed with PBS 1% Triton X, then the serum was applied to the column. The columns were washed with PBS 1% Triton X and eluted with Glycine buffer (0.2 M Glycine, 1 mM EGTA pH 2.5) and with Guanidinium buffer (4 M Guanidinium HCl pH 7.0). Eluates were tested for protein concentration and antibody activity.

3.17 Immunofluorescence microscopy

Immunofluorescence experiments were always performed with cultures of sporulating yeast. To 40 ml of sporulating culture 5 ml fixation buffer (1 M KH_2PO_4 and 0.5 mM $MgCl_2$) and 5 ml formaldehyde 37 % were added. Fixation was done on a roller at room temperature for one hour. Cells were washed (centrifugation 2000 g for 3 min, decant supernatant) three times with buffer SP (1.2 M Sorbitol, 100 mM KH_2PO_4 and 0.5 mM $MgCl_2$). The cell wall was digested during incubation in SPβ (SP with 14 µl βMercaptoethanol 14.4 M/ml buffer and 200 µg/ml buffer Zymoliase 100 T (Seikagaku Brewery, Japan)) for one hour on a roller at 30 °C. Cells were washed three times with SP. Then the cells were resuspended in 0.5 ml SP with 2 mM PMSF. These suspension could be used directly for immunofluorescence.

Immunofluorescence samples were prepared on multiwell slides (ICN Biomedicals) . First the wells were coated with 20 µl 0.1 % Polylysine (Sigma) for 5 min, they were washed five times with water and then 20 µl of the cell suspension was pipetted on each well and incubated for 20 min in a humid chamber. The wells were washed with PBS (phosphate buffered saline) 1% Triton X for 5 min, then three times in PBS 1 % BSA (bovine serum albumine) and blocked in the same buffer for 20 min in the humid chamber. The first antibody in PBS 1 % BSA was incubated in a humid chamber for 60 min, the wells were washed 5 times in PBS 1 % BSA. In the same buffer the secondary antibody was incubated for 60 min, washed 8 times and dried for 2-4 min. 3 µl DAPI mounting (0.5 µg/ml DAPI in 50% Glycerol) was added to each well and the slide was sealed with a cover slip and nail polish.
Microscopy was performed on a Leica DM IRBE inverse microscope with a Coolsnap Camera (Invitro Systems). The microscope software Metamorph (Universal Imaging Cooperation) allows scanning through the cells in multiple layers and calculation of the best signal to noise ratio with the deconvolution process.

3.18 Meiotic spreads

Whereas in immunofluorescence experiments whole cells are subjected to incubation with antibodies, in meiotic spreads only the surface spread DNA is analysed. This means that only DNA binding or associated proteins can be localized in this way, the classical application for meiotic spreads is to detect synaptonemal complex proteins on pachytene DNA (Figure 7).
This method is described in detail in (Loidl et al., 1998). 20 ml of a sporulating yeast culture was pelleted for 5 min at 2500 g, the pellet was resuspended in 1 ml sporulation media containing 0.8 M Sorbitol. 10 µl DTT and 5 µl Zymoliase 100 T 25 mg/ml (Seikagaku Brewery, Japan) was added per ml. This mixture was incubated for 30 min at 37 °C to digest the cell walls.

10 ml chilled stop solution (0.1 M MES (N-morpholino-ethan sulfonic acid), 1 mM EDTA, 0.5 mM $MgCl_2$, 1 M Sorbitol) was added, the yeast were centrifuged at 2500 g for 5 min. The pellet was resuspended in 1 ml stop solution, PMSF was added to a concentration of 2 mM. This solution can be directly used for spreading.
20 µl of cell suspension was pipetted on a clean glass slide, then 40 µl fixative (4 % Paraformaldehyde, 3.4 % Sucrose) was added, the liquids were mixed by tilting the slide. 80 µl detergent (1 % Lipsol, LIP Ltd.) were added, then again 80 µl fixative, after each addition the slide was tilted to mix the solutions. A glass rod was passed lightly over the surface of the slide (for approximately half a minute) to spread the DNA. These slides were then dried over night in the hood.
The spreads were washed twice for 5 min in a Coplin jar filled with PBS. 100 µl blocking buffer (0.5 w/v % BSA, 0.2 w/v % gelatine in PBS) was pipetted on the slide and incubated under a cover slip for 10 min. The cover slip was rinsed off under PBS and 40 µl of primary antibody in blocking solution was added, incubation again under a cover slip for one hour in a humid chamber at room temperature. Spreads were washed 5 times in PBS, 40 µl of secondary antibody in blocking solution was incubated under a cover slip for two hours at room temperature. Slides were washed twice for 5 min in PBS, 30 µl DAPI mounting (0.25 µg/ml DAPI in 50 % glycerol) was added, a cover slip was sealed with nail polish. Microscopy was performed like in immunofluorescence experiments, deconvolution was never applied because the structures detected were already in one plane.

3.19 Electron microscopy

This method is optimized for staining cellular membranes and is described in detail (Neiman, 1998). 45 ml of sporulating yeast culture were mixed with 5 ml 25 % glutaraldehyde (biological grade, Polysciences Inc.). The suspension was incubated at room temperature for one hour and on ice over night.

Cells were pelleted at 2000 g for 4 min and washed twice with water. The pellet was resuspended in 1 ml 4 % $KMnO_4$ and incubated at room temperature for 30 min with occasional mixing. Cells were washed twice with water. The pellet was resuspended in 500 µl saturated Uranlylacetate (7 % in water) and incubated at room temperature for 30 min in the dark, then it was washed once with water. Cells were dehydrated by washing them twice in each of the following solutions: 30 % EtOH, 50 % EtOH, 70 % EtOH, 85 % EtOH, 95 % EtOH, 100 % EtOH, 100 % EtOH dry, Propylenoxide. Then the cells were washed three times in EPON mix (5 ml Agar 100 Resin, 1.5 ml DDSA (dodecenyl succinic anhydride), 3.5 ml MNA (methyl nadic anhydride); all chemicals from Agar Scientific), incubation twice for one hour and once over night. Cells were washed once with EPON mix containing DMP 30 (EPON mix plus 0.15 ml DMP 30 (2,4,6-tri-(dimethylaminomethyl)-phenol)) and transferred to BEEM capsules (better equipment for electron microscopy, Plano). The suspension was incubated one day at room temperature in the dark, then the cells were spun down in a vertical (swing out) centrifuge at 6000 g for 20 min and again incubated for one day. The samples were then incubated at 60 °C for 24 hours. Contrasting and cutting of these samples was performed in cooperation.

3.20 Overexpression of yeast proteins in bacteria and purification

The vector system we used (pET) and the expression conditions are described in detail on the Novagen homepage (www.novagen.com). Plasmids with the pET-28c backbone and corresponding inserts were transformed into the *E. coli* strain BL21-DE3-RIL, selection on kanamycin and chloramphenicol. A preculture was inoculated from a single colony and shaken over night at 30 °C. The next morning, a main culture was inoculated to a density of 0.1 OD 600/ml, it was shaken at 30 °C until it reached approximately 0.6 OD 600/ml, then the expression of the T7 polymerase is induced by adding IPTG to a concentration of 0.4 mM.

Further incubation at 30 °C for 3-4 hours, then the flask was cooled down on ice for 5 min and the cells were pelleted.
Purification of the 6-His-tagged protein on a Nickel NTA column (Qiagen) under native conditions was performed exactly like described in the Qiagen manual.

3.21 Northern blot experiments

Preparation of yeast RNA was performed with reagents and after a protocol of Molecular Research Center Inc. (MRC, homepage www.mrcgene.com). 10 OD 600 sporulating yeast culture were harvested and resuspended in 300 µl TRI reagent (MRC), 250 µl acid washed glass beads (Sigma) were added, cell lysis by vortexing in bead mill for 3 min at 4 °C. 700 µl TRI reagent was added, the mixture was shaken at low speed at 55 °C for 30 min, glass beads and debris were pelleted by centrifugation at 10000 g for 10 min at 4 °C. The supernatant was mixed with 100 µl BCP (1-bromo-3-chlorpropane, MRC), vortexed 20 s at full speed and incubated at room temperature for 5-10 min. The two phases were separated by centrifugation at 12000 g for 15 min at 4 °C, the upper, aqueous phase contained the RNA and was transferred to a new tube. 5 µl polyacryl carrier (MRC) and 250 µl isopropanole were added, the solution was mixed and then 250 µl high salt precipitation solution (MRC) were added, mixed, incubated at room temperature for 10 min. RNA was precipitated by centrifugation at 12000 g for 8 min at 4 °C, the pellet was washed once with 75 % EtOH and resuspended in 20-50 µl Formazol (MRC). RNA was dissolved by shaking 15 min at 50 °C, then the RNA concentration was measured photometrically.
A large agarose gel with 1 % agarose, 1 x MOPS running buffer (40 mM MOPS (3-(N-morpholino)-propanesulfonic acid) pH 7.0, 10 mM NaAcetate, 1 mM EDTA) and 1.5 M formaldehyde was poured (in the hood). RNA was mixed with an equal volume of loading buffer (1 mM EDTA, 0,25 % w/v bromphenol blue, 50 % w/v glycerol) and 1.5 µl of a diluted ethidium bromide solution (4 µg/ml) was added before loading.

Bands from rRNA were checked in the gel (degradation!), gel was intensively rinsed in water and incubated for 15 min in 50 mM NaOH/1.5 M NaCl. Gel was incubated 15 min in 20 x SSC (3 M NaCl, 0.4 M NaCitrat pH 7.0) and transferred to nylon membrane (Zeta Probe, Biorad) by capillary blot in the same buffer.
RNA was crosslinked to the membrane in a stratalinker. The membrane was wettened in 6 x SSC and put in a hybridisation glass tube with 20 ml formaldehyde prehybridisation/hybridisation solution (5 x SSC, 5 x Denhardts solution, 50 % formamide, 1 % SDS, 100 µg/ml single stranded salmon sperm DNA). Prehybridisation for two hours at 42 °C. Radiolabelling of a DNA probe was made with the High Prime kit (Roche) following exactly the kit protocol. Probe was added to the prehybridisation solution, hybridisation at 42 °C over night. The blot was washed twice 5 min with 2 x SSC/0.1 % SDS at room temperature, twice for 15 min in 0.2 x SSC/0.1 % SDS at room temperature and twice in 0.2 x SSC/0.1 % SDS at 42 °C. Then the membrane was exposed to film.

3.22 List of antibodies

Primary antibodies:
Rabbit αN-Zip1p
Rabbit αSsp1p
Mouse αtubulin (WA3)
Sheep αGFP
MouseαGFP (Boehringer Mannheim)
Secondary antibodies:
GoatαRabbit-HRP
IgG-HRP
DonkeyαSheep-Alexa488 (Jackson Immuno Research Laboratories)
DonkeyαRabbit-Cy5 (Jackson Immuno Research Laboratories)
GoatαRabbit-Alexa546 (Jackson Immuno Research Laboratories)
GoatαMouse-Alexa488 (Jackson Immuno Research Laboratories)

3.23 Table of plasmids

p415-Gal1-GFP	yeast expression vector introducing GFP tag, centromere –leu (Sikorski and Hieter, 1989)
p426-Gal1	yeast expression vector 2μm -ura (Sikorski and Hieter, 1989)
pATH1	bacterial expression vector introducing trpE tag
pET-3a	bacterial expression vector (Novagen)
pET-28c	bacterial expression vector introducing 6His and T7 tag (Novagen)
pGAD-CX	expression vector for yeast genomic DNA libraries, fusion to *GAL4*-AD, 2 μm –leu (James et al., 1996)
pGBDU-C1	expression vector for cloning of two hybrid baits, fusion to *GAL4*-BD, 2μm –ura (James et al., 1996)
pGEX-5X1	bacterial expression vector introducing GST tag (Pharmacia)
pKS54	full length *SSP1* (aa's 1-572) cloned into pET-28c
pKS89	p416-Gal1-full length *SSP1*
pMF3	full length *ZIP1*(aa's 1-876) cloned into pMM5
pMF4	N-*ZIP1*(aa's 1-184) cloned into pMM5
pMF5	C-*ZIP1*(aa's 746-876) cloned into pMM5
pMF6	N+cc-*ZIP1*(aa's 1-746) cloned into pMM5
pMF7	C+cc-*ZIP1*(aa's 186-876) cloned into pMM5
pMF8	cc-*ZIP1*(aa's 166-746) cloned into pMM5
pMF9	full length *ZIP1*(aa's 1-876) cloned into pMM6
pMF10	N-*ZIP1*(aa's 1-184) cloned into pMM6
pMF11	C-*ZIP1*(aa's 746-876) cloned into pMM6
pMF12	N+cc-*ZIP1*(aa's 1-746) cloned into pMM6
pMF13	C+cc-*ZIP1*(aa's 186-876) cloned into pMM6
pMF14	cc-*ZIP1*(aa's 166-746) cloned into pMM6
pMF30	C-*ZIP1* (aa's 746-876) cloned into pGBDU-C1
pMF43	full length *SSP1* (aa's 1-572) cloned into pET-3a
pMF51	N-*SSP1* (aa's 1-269) cloned into pET-28c
pMF52	NN-*SSP1* (aa's 1-131) cloned into pET-28c
pMF53	C-*SSP1* (aa's 428-572) cloned into pET-28c
pMF54	N+Z-*SSP1* (aa's 1-455) cloned into pET-28c
pMF55	Z+C-*SSP1* (aa's 217-572) cloned into pET-28c
pMF56	Z-*SSP1* (aa's 217-455) cloned into pET-28c
pMF57	NC+Z-*SSP1* (aa's 131-455) cloned into pET-28c
pMF58	full length *SSP1* (aa's 1-572) cloned into p426-Gal1
pMF59	N-*SSP1* (aa's 1-269) cloned into p426-Gal1
pMF60	Z-*SSP1* (aa's 217-455) cloned into p426-Gal1
pMF61	C-*SSP1* (aa's 428-572) cloned into p426-Gal1
pMF62	N+Z-*SSP1* (aa's 1-455) cloned into p426-Gal1
pMF63	Z+C-*SSP1* (aa's 217-572) cloned into p426-Gal1

pMF64	NN-*SSP1* (aa's 1-131) cloned into p426-Gal1
pMF65	NC+Z-*SSP1* (aa's 131-455) cloned into p426-Gal1
pMF66	full length *SSP1* (aa's 1-572) cloned into p415-Gal1-GFP
pMF67	N-*SSP1* (aa's 1-269) cloned into p415-Gal1-GFP
pMF68	Z-*SSP1* (aa's 217-455) cloned into p415-Gal1-GFP
pMF69	C-*SSP1* (aa's 428-572) cloned into p415-Gal1-GFP
pMF70	N+Z-*SSP1* (aa's 1-455) cloned into p415-Gal1-GFP
pMF71	Z+C-*SSP1* (aa's 217-572) cloned into p415-Gal1-GFP
pMF72	NN-*SSP1* (aa's 1-131) cloned into p415-Gal1-GFP
pMF73	NC+Z-*SSP1* (aa's 131-455) cloned into p415-Gal1-GFP
pMF74	NC-*SSP1* (aa's 131-269) cloned into p426-Gal1
pMF75	NC-*SSP1* (aa's 131-269) cloned into p415-Gal1-GFP
pMF76	NC-*SSP1* (aa's 131-269) cloned into pET-28c
pMF78	full length *SSP1* (aa's 1-572) cloned into pMM5
pMF79	N-*SSP1* (aa's 1-269) cloned into pMM5
pMF80	Z-*SSP1* (aa's 217-455) cloned into pMM5
pMF81	C-*SSP1* (aa's 428-572) cloned into pMM5
pMF82	N+Z-*SSP1* (aa's 1-455) cloned into pMM5
pMF83	Z+C-*SSP1* (aa's 217-572) cloned into pMM5
pMF84	full length *SSP1* (aa's 1-572) cloned into pMM6
pMF85	N-*SSP1* (aa's 1-269) cloned into pMM6
pMF86	Z-*SSP1* (aa's 217-455) cloned into pMM6
pMF87	C-*SSP1* (aa's 428-572) cloned into pMM6
pMF88	N+Z-*SSP1* (aa's 1-455) cloned into pMM6
pMF89	Z+C-*SSP1* (aa's 217-572) cloned into pMM6
pMK163	N-*NUF1* in pET-28c
pMM1	N-Zip1 (aa's 1-185) cloned into pATH1
pMM3	N-Zip1 (aa's 1-185) cloned in pGEX-5X
pMM5	p423-Gal1-lexA-Myc –his (Schramm et al., 2000)
pMM6	p425-Gal1-Gal4-HA –leu (Schramm et al., 2000)
pUK27	*MDM1*-PX in pET-28c

3.24 Table of yeast strains

ESM356-1	MAT**a** ura3-52 lys2-801 trp1Δ63 his3Δ200 leu2Δ1GAL+
KN3279	K8409 Δspo70::his3MX4/“ (Rabitsch et al., 2001)
LH175	MAT**a** ho::hisG lys2 ura3 leu2 his3 trp1ΔFA
LH176	MAT**α** ho::hisG lys2 ura3 leu2 his3 trp1ΔFA
NKY289	MAT**a** ura3 lys2 ho::hisG (Alani et al., 1987)
NKY292	MAT**α** ura3 lys2 leu2::hisG ho::LYS2 (Alani et al., 1987)
PJ69-4A	MAT**a** trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ (James et al., 1996)
SGY37	MAT**a** two hybrid tester strain
YAM37-12	MAT**a**/MAT**α** Δady3::kanMX4/“ DON1-eGFP::kanMX4/“ in NKY
YKS32	MAT**a**/MAT**α** NKY289-1 x NKY292-1 (diploid wild type NKY)
YKS53	MAT**a**/MAT**α** DON1-eGFP::kanMX4/“ in NKY
YKS65	MAT**a**/MAT**α** Δmpc54::kanMX4/“ Δmpc70::kanMX4/“ DON1-eGFP::kanMX4/“ in NKY
YKS127	MAT**a**/MAT**α** Δssp1::his3MX4/“ in NKY
YKS136-1	ESM356-1 with p416-Gal1-full length *SSP1*
YKS178-1	ESM356-1 with p426-Gal1-full length *SSP1*
YMF13-1	MAT**a**/MAT**α** NKY289-1 x NKY292-1 (diploid wild type NKY)
YMF45-2-1	MAT**a**/MAT**α** Δzip2::kanMX4/“ in LH
YMF108-1	PJ69-4A with pMF30 (C-*ZIP1* in pGBDU-C1)
YMF129-1	MAT**a**/MAT**α** Δssp1::his3MX4/“ and Δspo70::hphMX/“ in NKY
YMF130-1	MAT**a**/MAT**α** Δssp1::his3MX4/“ and Δspo71::hphMX/“ in NKY
YMK314-1	MAT**a**/MAT**α** *ZIP1*-ProA::kanMX4/“ in NKY
YMK367-1	MAT**a**/MAT**α** his4-N met4 ura3 leu2 trp1 lys2 ho::LYS2
YMK527-1	MAT**a**/MAT**α** Δspo71::kanMX/“ in NKY
YPH500	MAT**α** two hybrid tester strain

Chapter 4

Discussion

4.1 Ssp1p is a novel leading edge protein coat component

One of the most important results of the work presented here is the identification of Ssp1p as a novel component of the LEP coat. Starting from a two hybrid interaction between Ady3p and Ssp1p in a whole genome screen I could confirm the connection between the two proteins by colocalization in meiotic spread experiments. The LEP coat, a structure which was identified in the lab of M. Knop, consists of a protein complex that is associated with a membrane subdomain while the membrane is growing. Ssp1p is the most important component of this protein complex as it is required for the correct localization of the other two components, Ady3p and Don1p (Moreno-Borchart et al., 2001). Therefore the general idea is that Ssp1p recruits the other proteins to the LEP coat via protein protein interactions: Ssp1p binds to Ady3p, Ady3p then probably binds to Don1p.

I have demonstrated that Ssp1p localizes not only to the LEP coat but all along the prospore membrane. This is a striking difference to the other LEP coat components. However, in some cases a weak staining for Don1p and Ady3p along the PSM was also observed. But still immunofluorescence microscopy experiments detecting either Ssp1p or Ady3p/Don1p lead to completely different results. A technical explanation for this could be that Ssp1p is inaccessable to the antibody because it is buried by the protein complex on the one side and the PSM on the other side. Ssp1p in the LEP coat would then be visible only if the membrane structure is disturbed in meiotic spreads.

Another explanation for this paradox is that not all Ssp1p protein molecules interact with Ady3p. This could be achieved by modification of only a fraction of the Ssp1p protein pool. I have shown that Ssp1p is phosphorylated and this modification influences the solubility of the protein (Figure 9). So it would be possible that only the phosphorylated form of Ssp1p is bound to Ady3p in the LEP coat.

This fraction could be relatively insoluble while the unmodified form of the protein on the prospore membrane is better soluble. Following this direction one would need to know more about the phosphorylation of Ssp1p. Searching databases for possible phosphorylation sites in the Ssp1p sequence resulted in numerous serine-, threonine- and tyrosine residues with a high prediction for phosphorylation. As Ssp1p is probably phosphorylated at several sites, a mutagenesis experiment appeared undoable. A more promising approach would be to identify the kinase responsible for Ssp1p phosphorylation, either as an interaction partner or genetically.

4.2 Deletion of *SSP1* results in misshaping of the prospore membrane

In this study I have shown that deletion of *SSP1* leads to a dramatical change in the architecture of the prospore membrane. The PSM, which stays apart from the nuclear envelope in the wild type strain, sticks to the NE very tightly in the Δ*ssp1* mutant (Figures 12 and 13). This leads to a closure of the PSM before it has incorporated cytoplasm. It also seems that in the Δ*ssp1* mutant only part of the nucleus is encapsulated. It has been known that deletion of *SSP1* results in a block of spore formation and in loss of viability in the course of sporulation (Nag et al., 1997). This phenotype can now be explained with the aberrant PSM shaping.

This leads to the question which function the LEP coat has during the formation of the PSM. It is not distinguished here between the function of the LEP coat and the function of *SSP1* because in the deletion strain all known LEP coat components are mislocalized. From my results I can propose a model in which the LEP coat resides on the tip of the growing prospore membrane like a stiff ring functioning as a scaffold for the structure of the PSM (Figure 22). In the absence of the LEP coat the prospore membrane would collapse and eventually stick to the NE due to the lack of rigidity. Still it is an open question how the forming PSM curves around the nuclear envelope. In theory, a newly formed membrane at the SPB could move to all directions. However, a regulatory mechanism must force the membrane to bend around the nuclear lobe. The nature of this process is still unknown and it would significantly enhance our understanding of the *ssp1* phenotype.

The size of the LEP coat ring seen in time lapse fluorescence microscopy does not change significantly during meiosis II as it moves inward along the spindle (S. Reber and M. Knop, unpublished data).

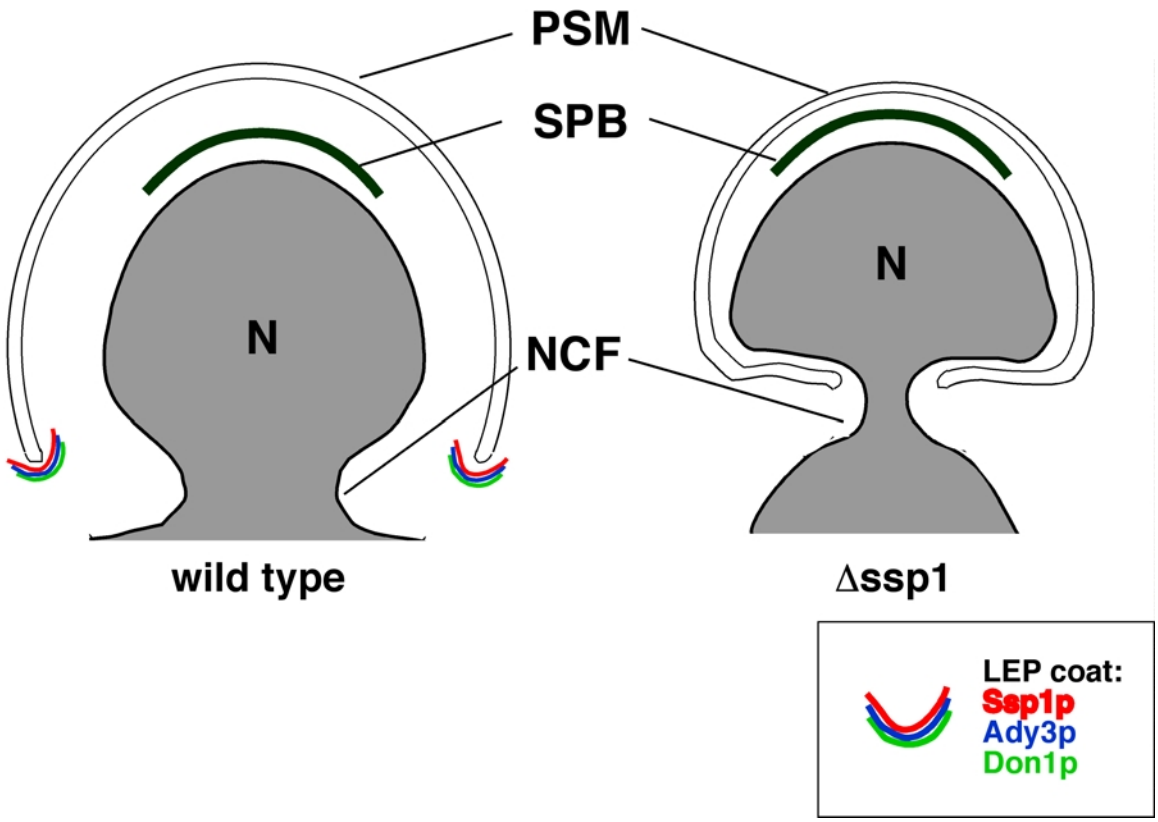

Figure 22:
Model for the role of the LEP coat in shaping the prospore membrane. The LEP coat consists out of three proteins, Ssp1p, Ady3p and Don1p. Ssp1p anchors the LEP coat in the membrane through lipid binding. In the absence of *SSP1* the structure of the prospore membrane is dramatically changed (N=nucleus, PSM=prospore membrane, SPB=spindle pole body, NCF=nuclear cleavage furrow).

This could mean that after the LEP coat has assembled the complex itself is not changed during membrane growth. The process of LEP coat assembly is not understood, but after it has formed the enlarging membrane would simply enforce the movement of the protein ring. However, the idea that the structure of the complex would be an intrinsic feature of the protein components is challenged by the finding that the Δ*sma2* mutant forms enlarged giant LEP coats (Rabitsch et al., 2001).

Two important questions for the understanding of the morphological process remain open: how is the LEP coat disassembled before PSM closure and what forces drive nuclear division?

Probably LEP coat dissociation from the membrane is a prerequisite for PSM closure. An interesting, though purely speculative model links specific protein degradation to the disassembly of the LEP coat. A checkpoint might ensure that the PSMs don not close prior to incorporation of all the proper contents. Only after this has been verified the LEP coat might disassemble. This process might require the ubiquitin ligase activity of the APC. The proteasome could therefore mediate, either direct or indirect, the degradation of the LEP coat proteins. This would be a parallel to the situation in mitosis where the APC is required for exit of mitosis. Degradation of B-type cyclins mediated by the APC probably is required for exit from mitosis and meiosis (Cooper et al., 2000). This idea is supported by the late meiotic deletion phenotype of *SPO70* which encodes for an APC activator. In this mutant the LEP coat appears to be retained much longer on the PSM than in the wild type preventing the closure of the PSMs (Figure 21).

The second question is how the constriction and ultimately division of the nucleus after meiosis II is achieved. One idea was that this is mediated by a contractile actinomyosin ring. But so far our results argue against such an involvement (S. Reber and M. Knop, unpublished data). There was also the idea that the LEP coat itself as a contractile ring would be responsible for the division. Strong arguments against this model are the findings that nuclear division takes place in the *ssp1* mutant without a LEP coat (Figure 13) as well as in the *sma2* mutant with its giant LEP coats far away from the site of constriction (P. Maier and M. Knop, unpublished data). Possibly septins are involved in this process, but until now this remains elusive. The nuclear cleavage furrow (NCF) appears to have a reduced diameter in the *ssp1* mutant compared to wild type. The reason for this is not clear. If the PSM itself would induce nuclear constriction then again the wild type situation would be enigmatic.

4.3 Lipid binding of Ssp1p anchors the LEP coat in the membrane

I could show that Ssp1p is a lipid binding protein and this finding is very important for an understanding of the LEP coat. For a long time it was unknown how the LEP coat proteins could be associated to the membrane without being transmembrane proteins. This problem was solved by discovering the lipid binding activity of Ssp1p, which is the protein responsible for anchoring the complex in the membrane. I have shown that Ssp1p specifically binds to all PIPs and to Phosphatidylserine and Cardiolipin (Figure 15).

The observation that Ssp1p contains a lipid binding domain is very fascinating as more and more important cellular functions are linked to proteins with such properties. Especially the PX (phox homology) domains are found in many proteins involved in vesicle targeting (Sato et al., 2001). The three dimensional structure of a PX domain, which was solved recently, gave futher insights into the mechanism of protein-lipid interaction (Hiroaki et al., 2001). However, the part of Ssp1p with the highest sequence similarity to a PX domain, the central Z-domain, clearly has no PIP binding activity. I was able to demonstrate that it is the N-terminus of Ssp1p which contains the lipid binding activity (Figure 17). However, this part of the protein does not show any similarity to known lipid binding domains. We therefore plan to investigate whether two stretches of 3 and 4 Lysins in the N-terminus, interestingly one in the NN part and the other in the NC part, play a role in PIP binding. As lipid binding domains usually contain large folding motifs, which are conserved in many proteins, Ssp1p might represent a member of a new lipid binding protein family. This would make the N-terminus of Ssp1p a promising target for structural analysis.

Two hybrid data indicating that it might be the C-terminal part of Ssp1p which interacts with Ady3p while the N-terminus is also responsible for autointeraction (Figure 20) lead to the following model: Ssp1p builds the membrane proximal layer of the LEP coat with all the N-termini anchored in the membrane and the C-termini bound by Ady3p, which forms the next layer of the LEP coat (Figure 22). This model for sure needs further verification. One important experiment which is still missing is the expression of Ssp1p with a deleted N-terminus in meiotic yeast. So far technical problems prevented us from doing this.

4.4 Ssp1p might be involved in membrane vesicle trafficking

The most critical open question for an understanding of the whole process concerns the targeting of membrane vesicles to the spindle pole body. During vegetative growth membrane vesicles at the SPB would be at the wrong place whereas in meiosis a massive accumulation is required exactly there. This switch, which redirects membrane vesicles to the SPB, is for sure tightly regulated. However, until now we have no clue how this could work.
An idea about the origin of these vesicles emerged from several mutants of the late secretory pathway, which usually regulate the vesicle transport from the Golgi to the plasma membrane. Interestingly, these mutants are also defective in prospore membrane formation (Neiman, 1998). This could mean that in meiosis post-Golgi vesicles are redirected to the SPB. However, the molecular components of this switch still remain unidentified.

I have demonstrated that Ssp1p mitotically overexpressed localizes to the plasma membrane of the bud (Figure 18). For vegetatively growing yeast this is the site of secretory vesicle docking. Of course this finding is no evidence for a role in meiosis because Ssp1p usually is not expressed in vegetative growing cells. Nevertheless, it allows some comparisons: Ssp1p is a lipid binding protein and we propose that lipid binding mediates the localization to the PSM. The PSM in meiosis is the site of secretory vesicle fusion. In mitosis, Ssp1p again localizes to the site of secretory vesicle fusion, however this is now the plasma membrane of the bud. One explanation would be that the lipid composition of these membrane substructures is responsible for this behaviour.

In this study I have also presented data indicating that the overexpression of Ssp1p is toxic to mitotic cells. A clue how this could be exerted comes from electron microscopy experiments from yeast mitotically overexpressing Ssp1p. In these strains an accumulation of membrane vesicles at the bud site compared to wild type can be observed (R. Haguenauer-Tsapis and M. Knop, unpublished data). This could be the result of an inhibitory effect of Ssp1p on vesicle fusion at the bud membrane. Ssp1p, which is not expressed endogenously in mitotic cells, might interfere with the secretory pathway machinery when it is associated with the target membranes. However, that does not mean that Ssp1p in meiosis also has an inhibitory effect on vesicle fusion because the membrane fusion machinery might be different in meiosis.

When we look at the results which domains of Ssp1p are responsible for the three effects described, namely PIP binding, membrane localization and overexpression toxicity, a functional link between these phenomena can be assumed (Table 1). Full length *SSP1*, N-terminus and the construct N+Z are active in all three assays, while the Z-domain and C-terminus are not. This is no direct evidence but it gives a strong hint that PIP binding of Ssp1p results in membrane localization of the protein, which is toxic to the cells when the protein is overexpressed in vegetatively growing yeast. At least the link between lipid binding and membrane localization makes perfect sense and confirms that the observations we made in *in vitro* expreriments have a relevance *in vivo*.

The N-terminus of Ssp1p appears to be the minimal domain required for activity in all the assays used. As the N-terminus comprises a large part of the protein we wanted to narrow down the responsible region. Therefore we chopped the N-terminus into two parts, the extreme N-terminus NN and the C-terminal half of the N-terminus, NC. When we used these constructs in the assays the result is ambiguous. The construct NN still binds to the bud membrane but is inactive for PIP binding. The construct NC alone neither binds PIPs nor does it localize to membranes. But the domain NC+Z shows activity in PIP binding and this is probably not due to the Z region.

That could mean that the NC construct cannot be properly folded when it is expressed alone but it still contains activity. So we cannot allocate the activity of the N-terminus to either of the two constructs, NN and NC, entirely. Probably both parts participate in the activity of the N-terminus.

	PIP binding	Membrane localization	Overexpression Toxicity
Full length	4	4	4
N	4	4	4
Z	-	-	-
C	-	-	-
N+Z	4	4	4
Z+C	-	-	-
NN	-	4	-
NC+Z	4	**?**	-
NC	-	-	-

Table 1:
Activity of the Ssp1p domains for the three assays PIP binding, membrane localization and overexpression toxicity (summary of Figures 17, 18 and 19)
(4 **)** = active, **(-)** = inactive, ? = doubtable

Ssp1p is certainly not the only regulator responsible for redirecting the secretory pathway. First, when *SSP1* is deleted, in meiosis the PSM is still formed. This requires fusion of secretory vesicles at the SPB (Figure 5). Second, when *SSP1* is mitotically overexpressed, secretory vesicles still go to the bud membrane, they do not accumulate at the SPB (R. Haguenauer-Tsapis and M. Knop, unpublished data). On the other hand, Ssp1p has an effect on both processes.

If *SSP1 i*s deleted in meiosis the prospore membrane is misshaped, which could also be an effect of improper vesicle fusion. Again the same domains of Ssp1p are required for these effects, no matter whether it is in mitosis or meiosis. Therefore, although we do not completely understand the mechanism, we would propose a role for *SSP1* in vesicle trafficking. The localization of mitotically overexpressed Ssp1p resembles that of several components of the secretory pathway, which also accumulate at the target membrane. Therefore, an interaction with components of the secretory pathway is likely and finding such an interactor of Ssp1p could allow to gain new insights into the process of vesicle targeting.

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