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Coordination of endoplasmic reticulum and mRNA localization in *Saccharomyces cerevisiae*

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Erklärung

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Ehrenwörtliche Versicherung

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

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Maria Schmid

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1. Introduction

1.1. mRNA localization, a conserved process essential for somatic cell polarity and embryonic development.

Cytoplasmic mRNA localization is a powerful mechanism for generating spatially restricted protein function in a variety of organisms and cell types. Virtually every cell that demonstrates some form of asymmetry will almost certainly contain some unevenly distributed RNAs. Such localized mRNAs are not instantly subjected to translation but first are targeted to specific destinations for local translation. Thereby gene expression can be locally regulated on a post-transcriptional level. After description of the signal peptide and other protein resident sorting signals (von Heijne, 1990) it was thought for some time that protein targeting to specific subcellular locations was solely performed following translation. However it soon became clear that localization of mRNAs can serve as an important tool to create the basis for local protein expression prior to translation (Bassell *et al.*, 1999; Jansen, 2001; Shav-Tal *et al.*, 2005).

One major advantage of localizing an mRNA rather than the encoded protein is that on a single transported message several rounds of translation can occur at a specific subcellular location. From an energetic point of view this is therefore more favourable since it helps cutting the cell's energy cost for transport. Secondly, the local distribution of a transcript helps to prevent the unwanted and perhaps even deleterious expression of a protein in other regions of the cell. Finally, mRNA targeting and the resulting local translational control enables a fast and independent regulation of protein expression in different parts of the cell. In the nervous system, for instance, this allows for a quick response to synaptic stimuli in peripheral regions of neurons far from translational events in the cell body.

Until recently, estimations had envisaged that in *Drosophila* only 1-10% of all transcripts are distributed to specific sites prior to translation (Palacios *et al.*, 2001). However, last year a milestone publication in *Cell* (Lecuyer et al., 2007) made clear that the importance of mRNA localization seems to be far higher than expected until then. Lecuyer and co-workers reported that the majority - that is to say 71% - of the embryonically expressed mRNAs in *Drosophila* are targeted to specific sites and the authors propose that the process of mRNA localization is involved in the majority of cellular processes.

In general, mRNA localization is an important contributor to cell polarity in both somatic cells and oocytes. Within somatic cells this process is crucial for the establishment and maintenance of cell morphology and motility. In the case of oocytes and embryos, mRNA trafficking is the basis for patterning during embryonic development.

1.1.1. mRNA localization in polarized somatic cells

1.1.1.1. Migrating fibroblasts

As mentioned above, mRNA sorting is also crucial in somatic cells. In asymmetric cells like migrating fibroblasts this process is important for establishment and maintenance of cell polarity. One of the best characterized examples is ß-actin mRNA. In several motile cell types, β-actin mRNA is targeted to the leading edge of lamellipodia (Condeelis & Singer, 2005). These flattened cytoplasmic extensions are actin rich and polymerizing actin filaments provide the protrusive force for the extension of lamellipodia during cell motility. In case of the ß-actin transcript, mRNA targeting is thought to facilitate the compartmentalized assembly of a multifactor complex, i.e. ß-actin filaments. RNA localization apparently creates a microenvironment in which the newly synthesized actin monomers are available at much higher concentration. As ß-actin translation occurs in a restricted cell compartment like the thin lamellipodium (Rodriguez *et al.*, 2006), elevated local levels of ß-actin monomers can considerably enhance the dynamics of actin polymerization. Additionally, mRNA localization easily allows for sorting of specific actin isoforms. Since only ß-actin, but not α- or γ-actin mRNA, is targeted to the cell periphery in moving cells (Kislauskis *et al.*, 1993), its local translation probably prevents formation of unwanted isoform heteromers. The importance of mRNA localization in migrating cells becomes clear upon their failure to correctly target ß-actin mRNA which leads to an impaired cell morphology and motility (Kislauskis *et al.*, 1997). Even more severe, the loss of cell polarization due to mistargeting of ß-actin mRNA can have deleterious effects such as the gain of metastatic potential in tumour cells (CondeelisSinger *et al.*, 2005; Shestakova *et al.*, 1999).

1.1.1.2. Neurons

Another somatic cell structure in which cellular asymmetry is especially critical is the nervous system. Neurons transmit electrical and chemical signals and they use their intrinsic polarity to split signal input and output domains. For this purpose, they develop two types of processes, axons and dendrites, and to make it even more challenging, these have further specialized functional areas such as growth cones and synapses. To establish and maintain this polarity the different domains must vary in their protein composition. One way to achieve this is by selected mRNA targeting and localized translation within dendrites and axons (Job *et al.*, 2001). Therefore, RNA localization is a widespread phenomenon in neurons.
To list only a few examples: messages coding for microtubule-associated protein MAP2 (Garner *et al.*, 1988), Arc (activity-related cytoskeletal protein) (Steward *et al.*, 1998), the α-

subunit of Calcium/Calmodulin dependent Kinase II (CaMKIIα) (Mayford *et al.*, 1996), ß-actin mRNA (Tiruchinapalli et al., 2003) localize to dendrites whereas tau mRNA (Aronov *et al.*, 2002) and again ß-actin mRNA (Bassell et al., 1998) are known to travel along axons.

For many candidates detailed knowledge about the functional significance of their localization has not yet been derived. Nevertheless, there are more and more indications that mRNA localization is essential for the establishment and maintenance of neuronal and synaptic morphology. mRNA targeting obviously contributes to the growth of developing axons and enhances growth cone dynamics and consequently axon guidance (Steward, 2002). In addition, there is growing evidence that mRNA targeting and local translation are crucial for the formation of synaptic plasticity which is involved in higher brain functions as learning and memory (Martin, 2004; Sutton *et al.*, 2006).

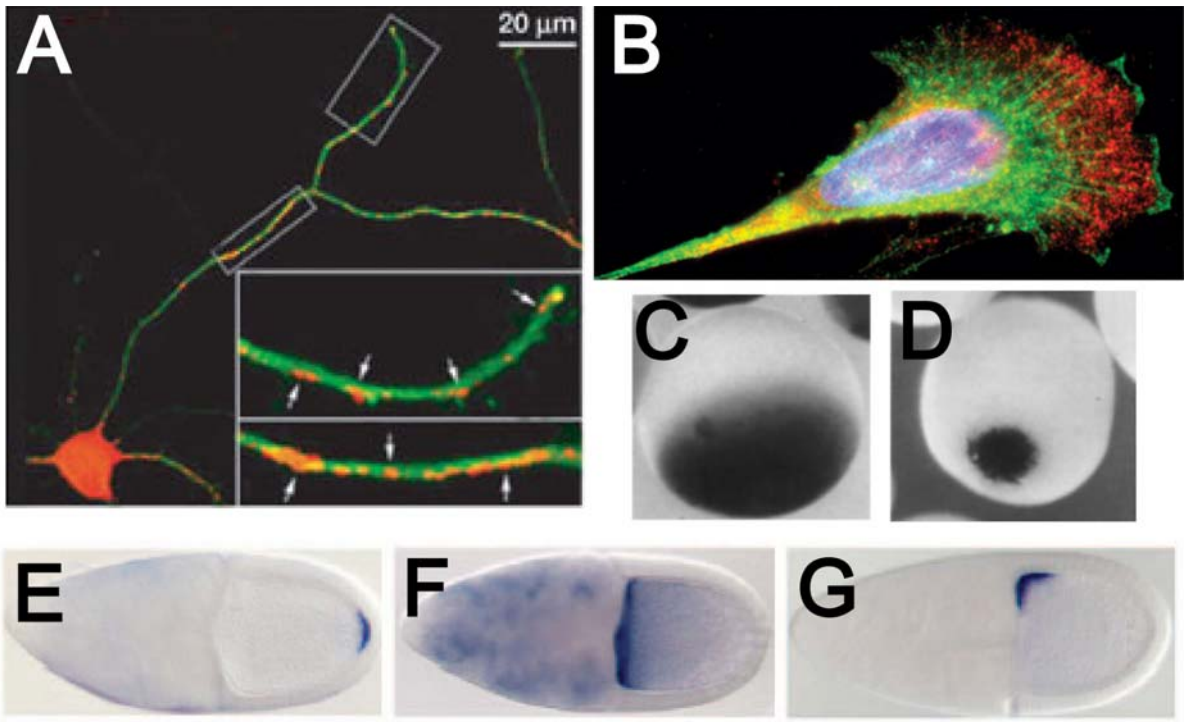


Figure 1: mRNA localization in higher eukaryotes (pictures taken from (Dahm *et al.*, 2005; St Johnston, 1995, 2005))
(A) In cultured hippocampal neurons, β-actin mRNA granules (red) localize to developing axons. An axonal marker is shown in green, arrows indicate β-actin mRNA particles.
(B) ß-actin mRNA is targeted to the leading edge of migrating fibroblasts.
(C and D) Localization of Vg1 and Xcat2 mRNA, respectively, to the vegetal pole of *Xenopus* occytes.
(E, F and G) *Drosophila* embryos with specifically sorted mRNAs: *oskar, bicoid and gurken.*

1.1.2. mRNA localization in oocytes and developing embryos

1.1.2.1. *Drosophila melanogaster*

From what is known so far, mRNA localization in non somatic cells is primarily involved in developmental processes. For instance in the *Drosophila* embryo, proper development is dependent on regionalization of both proteins and RNAs (Palacios *et al.*, 2001). The large number of localized mRNAs in *Drosophila* mentioned earlier (Lecuyer et al., 2007) indicates that mRNA sorting is important for a vast number of cellular processes. However, the best characterized mRNAs that are localized asymmetrically within the developing egg or syncytial embryo are the ones involved in determination of the oocyte, specification of embryonic axes, and establishment of germ cells in *Drosophila*. Amongst them are for example maternal mRNAs encoding anterior and posterior determinants like *oskar (osk)*, *bicoid (bcd)* or *nanos (nos)* (Lasko, 1999).

One of the first RNAs to localize during oogenesis is *osk* mRNA. The targeting of *osk* message to the posterior pole initiates assembly of the germ plasm (Lasko, 1999). Two other transcripts, *nos* and *bcd* are localized to opposite poles of the oocyte. *nos* accumulates at the posterior pole at the end of oogenesis. Its mRNA localization requires Oskar protein and thus depends on the prior posterior targeting of *osk* mRNA (Johnstone *et al.*, 2001). *bcd* mRNA becomes localized to the anterior pole during late stages of oogenesis. The encoded protein, a transcription factor, then forms a morphogenic gradient along the embryo by diffusion from the anterior pole (Ephrussi *et al.*, 2004).

Another localized transcript, *gurken (grk)* mRNA, encodes the *Drosophila* homolog of TGFα. Its localization to the antero-dorsal region of the oocyte is not only crucial for establishment of the antero-posterior axis but also for outlining the dorso-ventral axis (Johnstone *et al.*, 2001).

The aforementioned mRNAs represent only a minute fraction of all localized transcripts but they illustrate that mRNA sorting in *Drosophila* is crucial for normal establishment of the embryonic body plan.

1.1.2.2. *Xenopus laevis*

Also in another organism, *Xenopus laevis*, localization of numerous mRNAs to either the animal or the vegetal hemisphere of the oocyte coincides with polarity along the animal/vegetal axis (Kloc *et al.*, 2005). Oogenesis in *Xenopus* is divided into six stages and during this time period the animal/vegetal axis is developed. This axis then determines the

fate map of the three primary germ layers in the future embryo: endo-, meso- and ectoderm. The animal hemisphere of the oocyte later on gives rise to ectodermal components like the skin and the nervous system. The progeny of the vegetal pole follow endodermal fates primarily forming the gut. Finally, cells in the equatorial or marginal zone will build the mesoderm and thus elements like muscles, blood and bones. Amongst the transcripts localized to the animal hemisphere are for instance An1 (a ubiquitin like fusion protein), An2 (a mitochondrial ATPase subunit) and An3 (a DEAD box RNA helicase). Examples for vegetally sorted messages are Xcat2 (Zn finger protein), Xdazl (RNA binding protein), VegT (T-box transcription factor) and Vg1 (a TGF-ß family member) (King *et al.*, 2005).

One interesting feature about mRNA localization to the vegetal cortex in *Xenopus* oocytes is that vegetally localized RNAs follow two distinct sorting pathways and arrive at the cortex during different phases of oogenesis. These two chronologically different mechanisms are termed “early” and “late” pathway (Figure 2) (Kloc *et al.*, 1995).

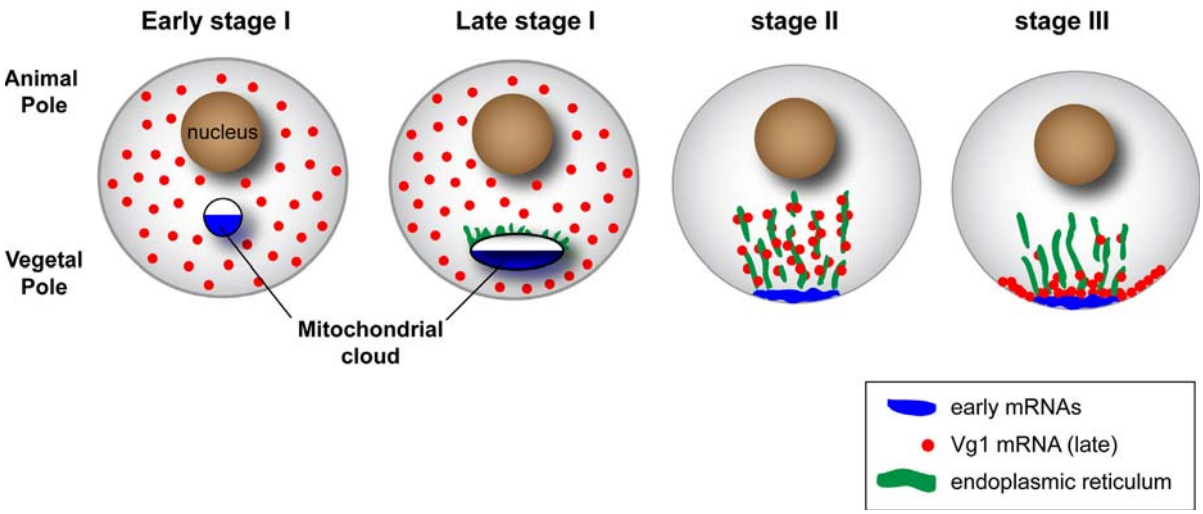


Figure 2: Early and late mRNA localization pathway in *Xenopus*
Early stage I: The Vg1 mRNA (red dots) is homogenously distributed throughout the cytoplasm while the early mRNAs are located within the METRO (messenger transport organizer) of the MC (Mitochondrial cloud) (blue). Late stage I: A cap of ER forms at the nuclear side of the MC and the METRO including the early mRNAs migrates towards the vegetal pole. Stage II: Formation of a wedge shaped ER structure (green) when the early mRNAs are located as an apical disk at the vegetal pole (blue). Vg1 mRNA co-localizes with this ER subdomain. Stage III: Translocation of Vg1 mRNA to the vegetal cortex, a process probably mediated by the ER.

The early pathway or METRO pathway localizes mRNAs such as Xcat2 and Xdazl in stage I and II oocytes using a specialized structure referred to as the METRO (messenger transport organizer). In a first step, early mRNAs localize to a macroscopic structure called the mitochondrial cloud (MC) in late stage I. The MC is an accumulation of mitochondria enclosed by electron-dense material that lies on the presumed vegetal side of the oocyte nucleus. The fraction of the MC that contains early localizing transcripts is the METRO. Via

this structure, the messages are then translocated to a dense, disk shaped region at the apex of the vegetal pole in stage II (Mowry *et al.*, 1999).

The late or Vg1 pathway localizes mRNAs such as VegT and Vg1. During stage I and early stage II, when METRO RNAs are localized, late mRNAs like Vg1 are uniformly distributed throughout the cytoplasm and excluded from the MC. Vg1 starts to localize only when the MC arrives at the vegetal cortex, breaks down and the early RNAs become associated with the vegetal cortex. Concomitantly to this events a unique ER structure forms in a wedge shaped zone between the oocyte nucleus and the vegetal pole. Vg1 then accumulates and co-localizes with this specialized ER subdomain. During stages III and IV, Vg1 translocates to the vegetal cortex where, in contrast to the early transcripts, it becomes broadly distributed. By stages V to VI, Vg1 mRNA inhabits a thin cortical layer reaching from the vegetal pole up to equatorial zone (King *et al.*, 2005).

Although occurring during different stages of oogenesis, both pathways are indispensable for normal development and differentiation of the *Xenopus* embryo.

1.2. Mechanisms of mRNA localization

There are a few mechanistic options on how asymmetric sorting of newly synthesized transcripts can occur.

First, the probably easiest way to achieve regionalization of mRNAs is their local synthesis. This is the case for example in mammalian myofibres, large multinucleated, syncytial cells. The mRNAs for δ- and ε-subunits of the acetylcholine receptor are exclusively transcribed in the nuclei which are directly adjacent to the neuromuscular junctions. This local transcription process generates the mRNAs for the receptor subunits right at the synapse, their future site of function (St Johnston, 2005).

Secondly, non-uniform distribution of messages can be driven by a vectorial nucleo-cytoplasmic export from one side of a nucleus only. In the biflagellated single cell algae *Chlamydomonas reinhardtii* for instance, ß2-tubulin mRNA is enriched at the posterior region of the cell which contains a high concentration of ribosomes. The basis for this phenomenon is probably the preferred positioning of nuclear pore complexes (NPCs) at the posterior side of the nucleus. Apparently this targets the messages to the translation “hot spots” in order to achieve high levels of protein expression (Palacios, 2007).

Thirdly, mRNA shuttling to specific sub-cellular regions can be achieved by passive diffusion and entrapment at their final destination via a previously localized anchor. Cytoplasmic streaming events probably facilitate movement by diffusion. *nanos* mRNA in *Drosophila* is segregated to the posterior pole of the embryo by such a mechanism (Forrest *et al.*, 2003)

and in *Xenopus*, the same is true for the early pathway transcripts Xcat2 and Xdazl (Palacios, 2007).

An additional way to achieve compartmentalization of transcripts within a cell or embryo is the spatial control of mRNA stability. In case of *hsp83* mRNA in *Drosophila*, generalized degradation combined with local protection creates its asymmetric distribution. In the beginning, *hsp38* transcript is dispersed uniformly throughout the entire embryo. Its levels are then strongly reduced with exception of the pole plasm where a protection factor mediates its posterior stabilization (Lipshitz *et al.*, 2000). For *hunchback* mRNA on the other hand its localized degradation not its localized protection confers asymmetry. The anterior-posterior gradient for hunchback transcript is created by posterior Nanos protein which inhibits *hunchback* translation and thereby causes its instability at the posterior pole (St Johnston, 1995).

Finally, the probably most studied mechanism to establish asymmetric mRNA patterning is directed cytoplasmic transport (St Johnston, 2005). This active translocation process occurs along cytoskeletal elements such as microtubules or actin filaments and is mediated by members of all three motor protein groups i.e. myosin, kinesin or dynein families (Bullock, 2007). In general, such localized mRNAs contain *cis*-acting localization elements or "zipcodes". These specific sequence elements are mainly found in the untranslated regions (UTRs) of the transcripts and form secondary or tertiary structures as recognition sites for RNA-binding proteins (RBPs). RBPs and additional *trans*-acting factors then couple the corresponding transcripts to the locomotion machinery (ChabanonMickleburgh *et al.*, 2004; Hamilton *et al.*, 2007; Jambhekar *et al.*, 2007). In a last step, the mRNAs need to be retained at their final destination by anchoring (Czaplinski *et al.*, 2006). CaMKIIα mRNA in neurons (Mayford *et al.*, 1996), *bcd*, *osk* and *grk* mRNA in *Drosophila* (Johnstone *et al.*, 2001): examples for actively transported messages exist throughout all kinds of organisms and cell types and amongst them also in the yeast *Saccharomyces cerevisiae*.

1.3. mRNA localization in *S. cerevisiae*

The unicellular yeast *Saccharomyces cerevisiae* is a model system for establishment of cell polarity. Yeast cells proliferate by budding, a process involving the development and asymmetric enlargement of the bud which finally pinches off to form a new daughter cell. This asymmetric cell division proceeds in a stem cell like fashion meaning that the division gives rise to two sister cells with different fates. This is achieved by segregation of determinants such as localized proteins and mRNAs which then establish distinct cell fates for the mother and the daughter cell (Horvitz *et al.*, 1992).

In yeast mRNA localization serves as an important mechanism for the polarized inheritance of cell fate determinants during asymmetric cell division and probably the best characterized example so far is the localization of *ASH1* mRNA to the bud tip of the daughter cell.

1.3.1. *ASH1* mRNA - the most prominent localized mRNA in yeast regulates mating type switching

The asymmetric localization of *ASH1* mRNA in yeast serves to control the process of mating type switching. *S. cerevisiae* has the capability to swap between a diploid and a haploid growth form. Upon nutrient deprivation, diploids can partition themselves to four haploid spores by meiosis and in return, the way back to a diploid state is achieved by the fusion of two haploid cells with opposite mating type (a or α). During the vegetative growth phase of haploid cells, mothers and daughters have distinct fates in terms of interconversion between a and α mating types. Only mother cells can undergo mating type switching and never daughter cells. This assures that an isolated haploid spore can again form a diploid cell by fusion with its progeny.

On the molecular level this is achieved by exclusively expressing the *HO* endonuclease in mother not daughter cells. *HO* endonuclease initiates mating type switching by genomic rearrangement of the *MAT* locus which then leads to the alteration of a cell from a to α or *vice versa* (Cosma, 2004). In a daughter cell, expression of the *HO* endonuclease is specifically repressed due to the uneven accumulation of the transcriptional inhibitor Ash1p in daughter but not mother cell nuclei (Bobola *et al.*, 1996b; Jansen *et al.*, 1996; Sil *et al.*, 1996). This non-uniform Ash1p expression pattern results solely from the asymmetric targeting of its mRNA to the bud tip of the daughter cell (Chartrand *et al.*, 2002; Long *et al.*, 1997; Takizawa *et al.*, 1997) (Figure 3).

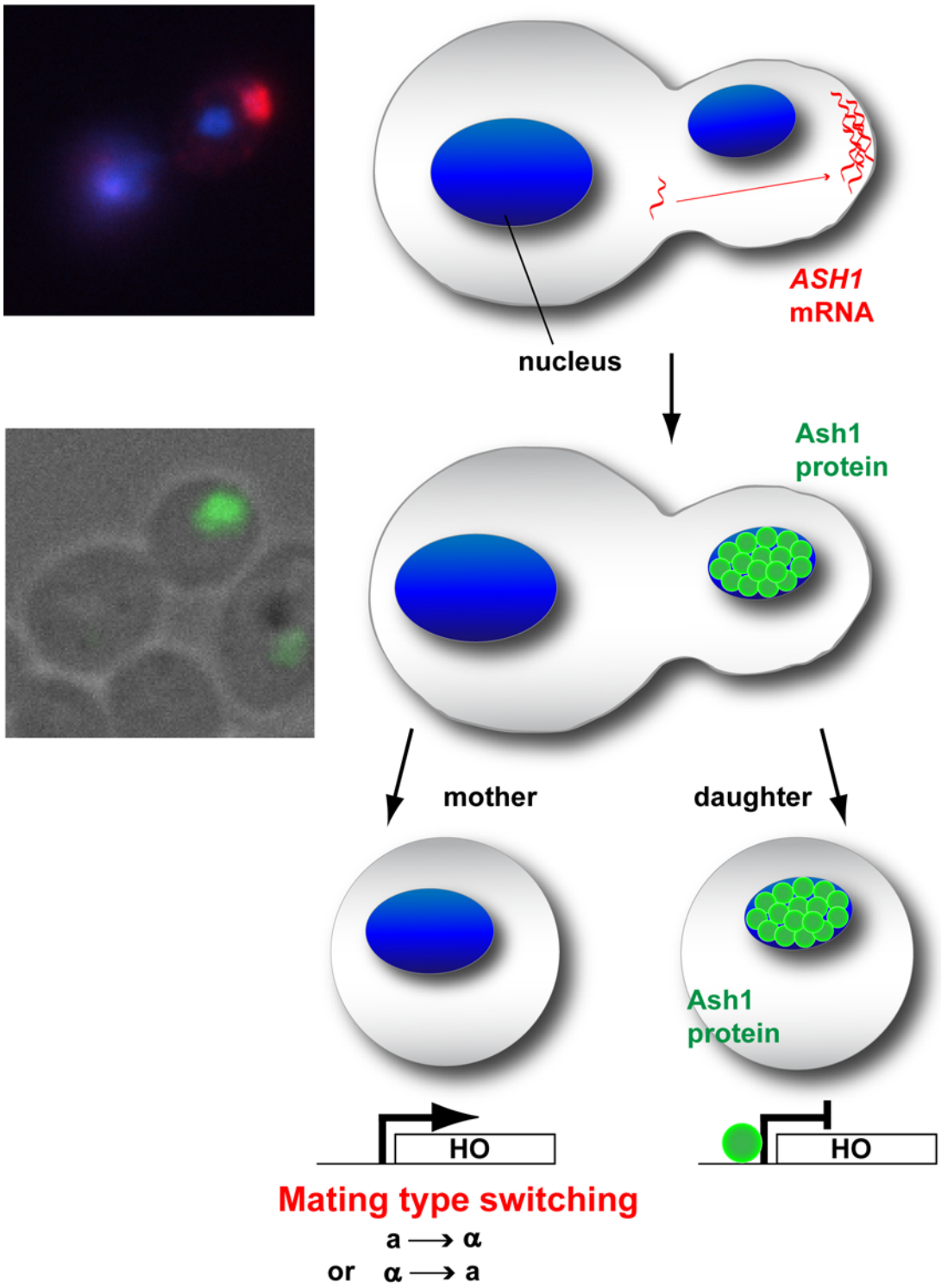


Figure 3: Localization of *ASH1* mRNA regulates mating type switching in *Saccharomyces cerevisiae*: *ASH1* mRNA is targeted to the bud tip during late anaphase. This results in the exclusive expression of the Ash1p transcription repressor in the nucleus of the daughter cell. Therefore only in the daughter cell expression of the *HO* endonuclease and hence mating type interconversion is suppressed.

Within the *ASH1* transcript, four *cis*-acting localization signals, also termed “zipcodes”, are responsible for its targeting to the yeast bud tip (Chartrand *et al.*, 2002; Chartrand *et al.*, 1999; Gonzalez *et al.*, 1999). Three of these localization elements (LEs) lie within the coding sequence of the mRNA: E1 (115 bp), E2A (118 bp) and E2B (250 bp). The fourth zipcode, E3 (118 bp), extends into the 3’UTR and it consists of the last 15 bp of the coding sequence, the stop codon and 100 bp of the 3’UTR (Chartrand *et al.*, 1999). The four LEs are functionally redundant since each of them is sufficient to mediate targeting of a reporter mRNA to the bud tip (Chartrand *et al.*, 2002). Each LE can be bound by the RNA binding protein She2p (Bohl *et al.*, 2000; Long *et al.*, 2000). All four LEs in concert have a synergistic effect and together they increase the frequency and efficiency of the mRNA translocation process (Bertrand *et al.*, 1998; Long *et al.*, 1997; Takizawa *et al.*, 1997).

The comparison of all four *ASH1* LE sequences did not show any obvious sequence homologies implying that rather structure not primary sequence serves as the sorting signal. In addition, the zipcodes were predicted to form RNA secondary structures containing stem-loops (Chartrand *et al.*, 2002; Chartrand *et al.*, 1999; Gonzalez *et al.*, 1999). Recently a conserved RNA motif was identified which is necessary for bud localization of *ASH1* and two other localized yeast mRNAs (*IST2* and *EAR1*). This motif consists of a CGA triplet in a loop combined with a single-stranded cytosine six bases from and on the opposite side of the triplet (Olivier et al., 2005). Another study identified a motif which is applicable to a larger group of localized yeast mRNAs and consists of a conserved, single-stranded, seven base motif containing a CG dinucleotide though the structural context of this motif also seems to be of great relevance (Jambhekar et al., 2005). These data indicate that She2p recognizes very precise, three dimensional structures in the zipcodes of its mRNA targets (Jambhekar *et al.*, 2007).

The *ASH1* zipcodes seem to have an additional role besides mediating the mere transport of mRNAs. Apparently they keep the localizing messages in a translationally-quiescent state while those are on their way to the bud tip. This translational silencing suppresses unintended Ash1p expression in the mother cell (Chartrand *et al.*, 2002). However, as we will see later in this section, translational repression also depends on some cooperating *trans*-acting factors (Section 1.3.2.2.)

1.3.2. *Trans*-acting factors: the mRNA localization machinery

1.3.2.1. The core locasome

In the original genetic screen selecting for mutants defective in asymmetric expression of HO endonuclease, five genes *SHE1-5* were identified (Jansen *et al.*, 1996). Three of them namely *SHE1*, *SHE2* and *SHE3* code for proteins which together form the so called core locasome (Figure 4). This heterotrimeric complex essential for mRNA transport consists of the motor protein Myo4p/She1p, the RNA binding protein She2p, and She3p, the adaptor protein bridging the other two components. All three factors of the core locasome co-localize *in vivo* with each other and with the transported *ASH1* mRNA (Bohl *et al.*, 2000; Gonsalvez *et al.*, 2004; Long *et al.*, 2000; Takizawa & Vale, 2000).

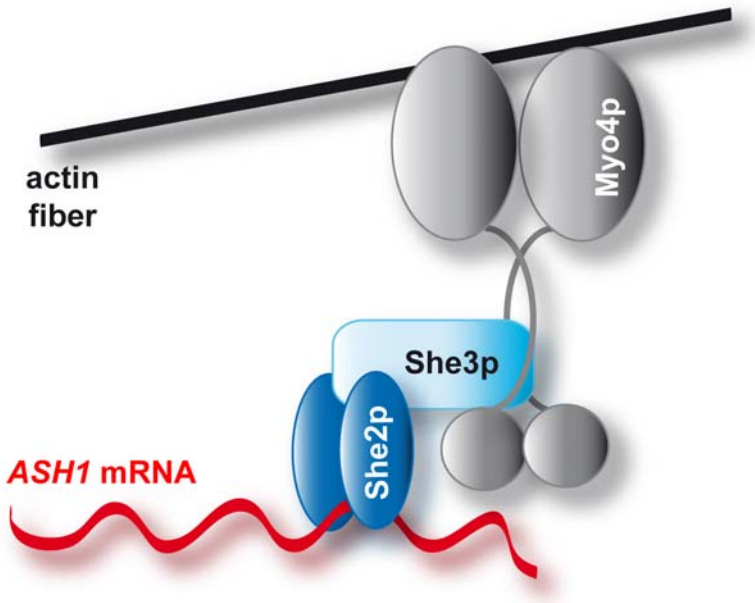


Figure 4: Components of the core locasome
The central machinery of mRNA localization in yeast consists of three components: the mRNA binding protein She2p, the adaptor protein She3p and a motor protein of the type V family of myosins, Myo4p.

1.3.2.1.1. Myo4 (She1p), a motor protein of the myosin V family

mRNA localization in yeast is dependent on the actin cytoskeleton as latrunculin-A, an actin filament disrupting drug or the use of mutants leading to the depolymerization of actin cables both result in the loss of *ASH1* mRNA targeting (Long *et al.*, 1997; Takizawa *et al.*, 1997).
The actin based motor essential for mRNA localization in yeast is She1p, also named Myo4p, a nonessential motor protein of the class V myosin family (Jansen *et al.*, 1996). Studies in living yeast demonstrated that Myo4p is the factor which actively transports the

ASH1 cargo along polarized actin cables (Beach *et al.*, 1999; Bertrand *et al.*, 1998; Munchow *et al.*, 1999).

Besides Myo4p there is a second type V myosin in yeast, Myo2p. In contrast to Myo4p, Myo2p is essential in yeast and it is responsible for the transport of the vacuole, post-Golgi vesicles, the trans-Golgi-network, peroxisomes and mitochondria and for the proper orientation of the mitotic spindle (Altmann *et al.*, 2008; Pruyne *et al.*, 2004). However, Myo4p is the only motor protein in yeast which mediates mRNA localization, but like Myo2p, Myo4p is a motor with very low processivity (Reck-Peterson *et al.*, 2001). Therefore the presence of several zipcodes in a localized mRNA like *ASH1* presumably helps to assure continuous movement of the cargo (Darzacq *et al.*, 2003). In the absence of She3p, Myo4p does not translocate to the bud tip. This suggests that Myo4p must associate with its transport cargo or as a minimum with its adaptor She3p in order to localize to the bud tip (Jansen *et al.*, 1996).

1.3.2.1.2. She3p, the adaptor protein

The adaptor protein She3p is necessary for Myo4p's association with the She2p-mRNA complex. She3p displays the characteristics of a bona fide adaptor: it interacts with the RNA binding protein She2p via its C-terminus while it can bind to the coiled-coil region and the C-terminal tail of Myo4p via its N-terminus thereby linking the motor to its cargo complex (Bohl *et al.*, 2000; Heuck *et al.*, 2007; Long *et al.*, 2000; Takizawa & Vale, 2000).

Interestingly the requirement of She2p for RNA localization can be circumvented by directly tethering the mRNA to She3p. If the MS2 coat protein (MS2-CP) is fused to She3p and a reporter mRNA contains the MS2 loops which are bound by the MS2-CP, this mRNA becomes correctly targeted even in absence of She2p (Long *et al.*, 2000). This confirms that the Myo4 protein is recruited to the mRNA via the adaptor protein She3p which in turn binds to the RNA binding protein She2p.

1.3.2.1.3. She2p, an unconventional RNA-binding protein

Amongst the group of *SHE* genes, only the 28 kDa protein She2p associates specifically with all four LEs, albeit with weak affinity in the nano-molar range (Chartrand *et al.*, 1999; Niessing *et al.*, 2004). It is the key player for mRNA localization as it binds to the zipcodes even if the other *SHE* genes are deleted (Bohl *et al.*, 2000; Takizawa & Vale, 2000). However, She2p-mRNA binding seems to be enhanced in the presence of She3p (Bohl *et al.*, 2000).

By sequence analysis, She2p is an non-canonical RNA-binding protein lacking so far described RNA-binding domains such as RRMs (RNA-recognition motif) or KH-domains (heterogeneous nuclear (hn)RNP K-homology domain) (Lunde *et al.*, 2007).

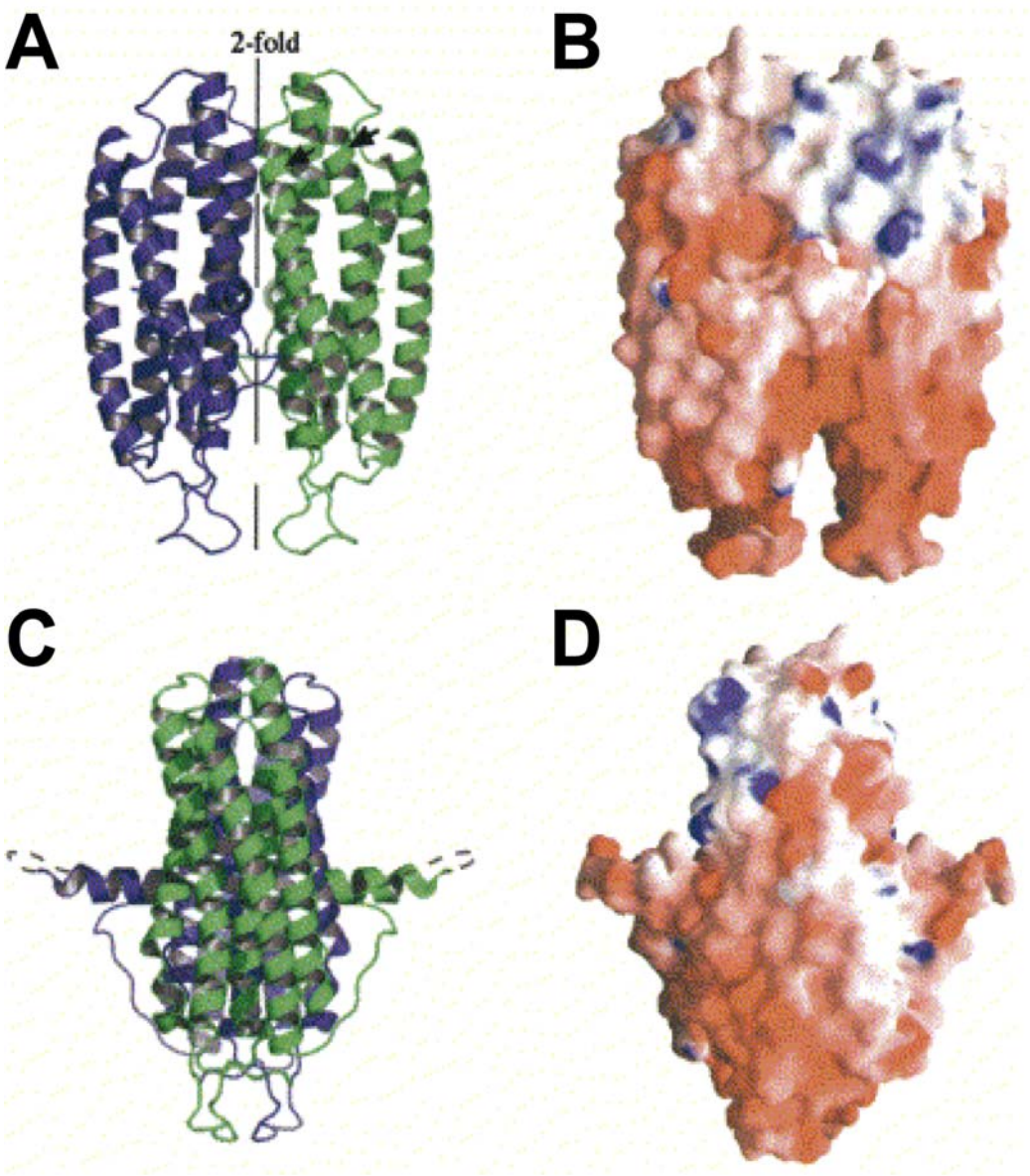


Figure 5: X-ray structure of She2p homodimer (Figure adapted from Niessing *et al.*, 2004).
(A) Stereoview of the She2p homodimer with each monomer in blue or green (PyMOL, DeLano Scientific, CA). Vertical line labeled with "2-fold" indicates the axis of 2-fold non-crystallographic symmetry relating the halves of the homodimer. Arrows on the green subunit denote the two α-helices of the basic helical hairpin, containing residues required for RNA binding. (B) GRASP surface representation of the chemical properties of the solvent-accessible surface of She2p. The surface electrostatic potential is colour coded red and blue, representing electrostatic potentials between < −14 to > +14 k_BT, where k_B is the Boltzmann constant and T is the temperature. Orientation is identical to (A). (C) Stereoview of (A) rotated 90° around the vertical axis. Dotted lines represent an eight amino acid gap in the final refinement model.(D) GRASP surface representation of (B) rotated 90° around the vertical axis.

From X-ray structural analysis (Figure 5) it is known that the She2 polypeptide folds into a single globular domain consisting of a bundle of five antiparallel α-helices with a small additional helix protruding at right angles form the middle of the globular domain. Furthermore, there is an unstructured loop which projects at the bottom of the She2p molecule (Niessing *et al.*, 2004). Two globular She2p monomers form a symmetric homodimer and this pairing is essential for She2p function. If dimerization is disrupted like in the case of the S120Y or C68Y mutant protein, She2p does neither efficiently bind mRNA *in vitro* nor does it correctly localize *ASH1* mRNA in yeast cells (Niessing *et al.*, 2004). The comparison of the She2p structure to other structures available in databases, lead to the conclusion that She2p displays a novel protein fold and is a completely unconventional RNA-binding protein. Apparently one of these She2p homodimers binds to one mRNA zipcode element (Niessing *et al.*, 2004).

From previous studies it is known that She2p's RNA-binding activity lies within the first 70 amino acids since the deletion of this domain disrupts its ability to co-precipitate *ASH1* mRNA (Kruse et al., 2002). Consistent with this, another study identified five amino acid residues involved in mRNA binding within these first 70 amino acids (Asn36, Arg43, Arg44, Arg52 and Arg63). If these specific residues e.g. Asn36 or Arg63 were converted to Ser or Leu respectively (N36S, R63K), these She2p mutants lost the ability to bind mRNA *in vivo* and *in vitro* (Gonsalvez et al., 2003). From the She2p X-ray structure it became clear that these five residues lie within a surface area with positive electrostatic potential. This basic surface patch (Figure 5) is exceptional compared to the remaining exterior of She2p which in large parts is negatively charged. This positively charged surface area consisting of two antiparallel α-helices separated by a loop is now defined as the "basic helical hairpin" RNA binding motif of She2p (Niessing *et al.*, 2004).
In addition to the basic helical hairpin as the primary mRNA binding site, a conserved, uncharged surface patch at the top of the She2p dimer is also important for its function. Mutation of Leu130 to Tyr (L130Y) also leads to a reduced *in vitro* RNA binding activity of She2p (Niessing *et al.*, 2004) which is consistent with data showing that the L130S mutant is impaired in *ASH1* mRNA localization (Gonsalvez et al., 2003).

1.3.2.2. Other *trans*-acting and accessory factors for mRNA localization

Besides the three components of the core locasome, two additional genes, *SHE4* and *SHE5*, were identified in the original genetic screen to have a defect in *ASH1* mRNA localization (Jansen, 2001). She4p is a member of the UCS class of proteins that are involved in the proper folding of myosin motor domains (Yu *et al.*, 2003). She4p was reported to associate with the motor domains of the yeast class V myosin Myo4p and the class I myosin Myo5p through its UCS domain (Toi *et al.*, 2003; Wesche *et al.*, 2003). This indicates that She4p might act as a myosin "chaperone" for Myo4p, assuring its proper folding and function and thus also to guarantee the integrity of the mRNA targeting motor. In *she5*Δ mutants, *ASH1* mRNA mislocalizes to the bud neck (Takizawa *et al.*, 1997). She5p/Bni1p is a formin that acts in the nucleation of actin filament assembly (Evangelista *et al.*, 2003) and mRNA mistargeting probably results from defects in the actin cytoskeleton (Gonsalvez *et al.*, 2004).

In addition to the She proteins, three other *trans*-acting factors essential for *ASH1* mRNA localization have been identified: Khd1p, Puf6p and Loc1p.
Two of them, Khd1p and Puf6p act as translational repressors while the mRNA cargo is *en route* to its final destination (Gu *et al.*, 2004; Irie *et al.*, 2002). Both of them are regulated by yeast kinases at the plasma membrane and their phosphorylation causes their dissociation from the *ASH1* mRNA and finally leads to the release of the message from translational silencing (Deng *et al.*, 2008; Paquin *et al.*, 2007). Khd1p is a protein containing three KH-domains, it binds the E1 element of *ASH1* mRNA (Irie et al., 2002) and the kinase regulating its function is the casein kinase I (Yck1p) (Paquin et al., 2007). Puf6p is a member of the PUF family of highly conserved RNA-binding proteins and it binds to the conserved PUF binding element UUGU in the E3 LE of *ASH1* mRNA (Gu *et al.*, 2004). In analogy to Khd1p, the translational repression by Puf6p is terminated by the casein kinase II (Ykc2p) (Deng *et al.*, 2008).
An exclusively nuclear protein with a role in mRNA localization is Loc1p. Loc1p binds to the *ASH1* E3 element and the transcript is delocalized in *loc1*Δ mutants (Long et al., 2001). However, Loc1p so far was mainly described as a factor involved in ribosome biogenesis (Harnpicharnchai *et al.*, 2001; Urbinati *et al.*, 2006). Recently it was found that Loc1p also influences translational regulation of *ASH1* mRNA as Ash1p levels are increased in *loc1*Δ cells (Komili *et al.*, 2007) and (T. G. Du et al., 2008).

1.3.3. Additional localized mRNAs in *S. cerevisiae*

ASH1 was the first localized mRNA discovered in *S. cerevisiae* but meanwhile 23 additional transcripts were identified to be actively transported to the bud tip. For this, immunoprecipitation experiments with tagged versions of the core locasome proteins (Myo4p, She3p and She2p) were combined with DNA microarray technology from the associated RNAs (Shepard *et al.*, 2003; TakizawaDeRisi *et al.*, 2000). The potential mRNA candidates were then validated by FISH (TakizawaDeRisi *et al.*, 2000) or in a living cell GFP-RNA assay (Shepard et al., 2003). All newly identified 23 mRNAs are localized to the tip of growing buds in a *SHE* dependent manner. However, only 8 of them display asymmetric distribution of the encoded protein, the others are located symmetrically in mother and daughter cells.

One such targeted message is *IST2* mRNA, which encodes an integral plasma membrane protein (Takizawa & Vale, 2000). Ist2p is symmetrically distributed between mother and daughter cells if expressed from its endogenous promoter. In this case, mRNA localization is the prerequisite for Ist2p to be synthesized in the bud. Upon deletion of the She-machinery, Ist2p is mostly excluded from the plasma membrane of daughter cells (Juschke *et al.*, 2004). However, some Ist2p also reaches the daughter cells even in absence of mRNA transport and this is due to a recently identified peptide-sorting signal (Franz *et al.*, 2007).

Unlike Ash1p or Ist2p, the localization of the other proteins encoded by targeted mRNAs is unaltered in *she2Δ* deletion mutants, even if the protein distribution is asymmetric (Shepard et al., 2003). This indicates that in these cases mRNA localization is not the key determinant and might serve as a redundant mechanism in addition to protein resident sorting signals.

Eleven out of the 24 localized mRNAs are expressed only at specific stages of the cell cycle. Though the encoded proteins have diverse functions some of them are involved in common processes belonging to yeast stress signalling and response pathways and the synthesis and remodelling of the plasma membrane and the cell wall (Shepard et al., 2003).

Finally one of the most remarkable features of all targeted mRNAs is that the majority and to be precise 16 out of 24 transcripts encode membrane or membrane associated proteins (Table 1).

	Shepard *et al.*, 2003		SGD (http://www.yeastgenome.org)		
mRNA	**Cell cycle regulation**	**Protein Localization**	**Cellular compartment of the encoded protein**	**signal peptide**	**predicted TMDs**
ASH1	**M**	bud nucleus	nucleus	-	-
BRO1	None	**punctae on vacuole**	cytoplasm, **endosomes, membranes**	-	-
CLB2	**M**	nuclei, spindle pole	nucleus	-	-
CPS1	None	cytoplasmic punctae	**vacuole**	Yes	1 TMD
DNM1	**S**	mitochondrial periphery	**outer mitochondrial membrane**	-	-
EGT2	**M**	**membranes**, large-bud enriched	**cell wall**	Yes	GPI anchor
ERG2	**M**	**ER**	**ER**	Yes	1 TMD
IST2	None	**bud plasma membrane**	**plasma membrane, cell periphery**	-	8 TMDs
MID2	None	**cell periphery**, mother-bud junction	**plasma membrane, cell periphery**	Yes	1 TMD
MMR1	**M**	bud sites & tips, mother-bud junction	**outer mitochondrial membrane**	-	-
SRL1	**G1**	periphery of small buds	**cell wall**	Yes	-
TPO1	**M**	**bud plasma membrane**	**ER, cell periphery, bud**	-	12 TMD
WSC2	**S**	**membranes,** bud enriched	**cell periphery**	Yes	1 TMD
TAM41	None	mitochondria	mitochondria	-	-
IRC8	**M**	**membranes,** bud enriched	no localization data	Yes	4 TMD
YLR434C	None	mitochondria	no localization data	-	-
TCB3	**G2**	**membranes,** bud enriched	**cell periphery**	-	1 TMD
EAR1	None	**ER**	**endosomes**	Yes	1 TMD
TCB2	None	**membranes,** bud enriched	**cell periphery**	-	1 TMD
KSS1	None	not defined	cytoplasm, nucleus	-	-
LCB1	None	**ER**	**ER**	-	-
MET4	None	nuclei	nucleus	-	-
MTL1	None	not defined	no localization data	Yes	1 TMD
YPL066 W	None	not defined	bud neck		

Table 1: Localized mRNAs in *S. cerevisiae*: table 1 gives an overview of yeast localized mRNAs and their important characteristics. Grey cells mark mRNAs encoding membrane or secreted proteins. The acronym “TMD” in the last column stands for transmembrane domain.

1.4. Inheritance of cortical endoplasmic reticulum in *S. cerevisiae*

Interestingly, two components of the yeast mRNA localization machinery, namely the adaptor protein She3p and the motor protein Myo4p, have been identified as crucial players in a second bud directed transport procedure: the segregation of cortical endoplasmic reticulum (ER).

1.4.1. Structure and function of the ER

One hallmark of eukaryotic cells in general is the separation of their cytoplasm into several membrane-bound compartments, i.e. organelles. This functional compartmentalization is beneficial but simultaneously confronts the cell with the task of maintaining its organelle population during each round of cell division (Fagarasanu *et al.*, 2007). The ER is probably one of the more complex of these organelles since it is the key component to a variety of processes vital for the smooth functioning of eukaryotic cells. Morphologically and functionally it is divided into distinct subdomains: rough ER (rER), smooth ER (sER) and transitional ER (tER), which together accomplish a variety of functions (Estrada de Martin *et al.*, 2005; Voeltz *et al.*, 2007).

The rER is characterized by membrane-bound ribosomes and is responsible for all processes linked to the biogenesis of secretory and membrane proteins. Proteins destined for secretion or membrane insertion mostly contain an N-terminal signal peptide and are recruited to the ER via the signal recognition particle (SRP)-pathway at the end of which they become co-translationally transferred through the translocon channel into the lumen or membrane of the ER (Figure 6) (Halic *et al.*, 2005; Rapoport, 2007). Different luminal proteins then operate to assure the proper folding and core glycosylation of these secretory proteins (Kleizen *et al.*, 2004; Ruddock *et al.*, 2006). The sER, defined by the absence of membrane-associated ribosomes, functions in cellular processes like biosynthesis of phospholipids, cholesterol and steroids and detoxification reactions. In addition it can serve as a sequestering and storage site for Ca^{2+} which can then be released for signal transduction purposes. The tER is a particular type of sER where cargo packaging and vesicle budding and fusion take place – a reason why it is also termed ER exit site (Baumann *et al.*, 2001).

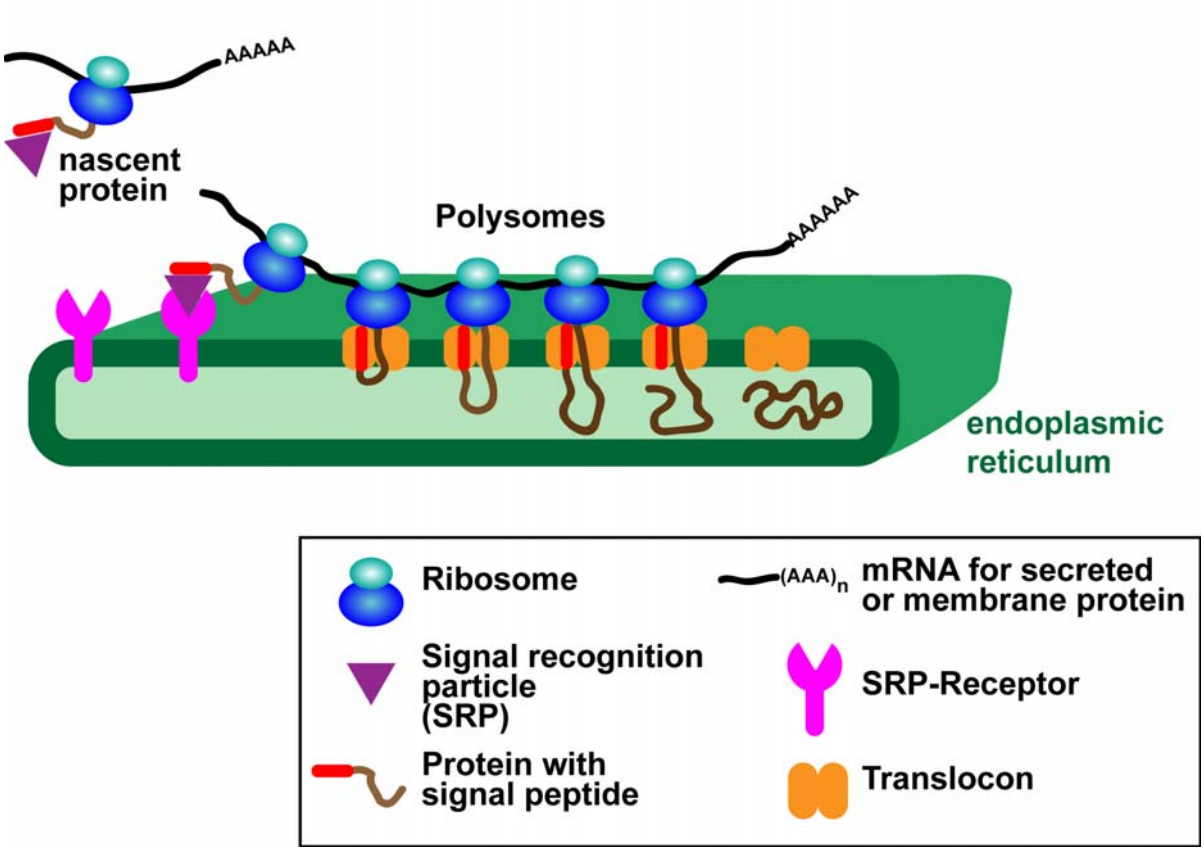


Figure 6: The SRP-pathway: ribosome-mRNA-nascent polypeptide complexes displaying a signal peptide in the nascent chain are bound by the SRP (signal recognition particle) and recruited to the ER membrane via the SRP-receptor. At the ER membrane the proteins are co-translationally translocated across (for secreted proteins) or into (for transmembrane proteins) the ER membrane through the Sec61p translocon channel.

In all cell types the ER builds a system of interconnected membranes with a common intra-luminal space and is composed of sheet like cisternae and arrays of tubules. Frequently these ER structures even reach the outmost extensions of the cell (Voeltz *et al.*, 2007). The ER can be further categorized into two classes: the perinuclear and the peripheral ER. The perinuclear ER consists of membrane sheets surrounding the nucleus and is contiguous with the nuclear envelope (NE). Except for minor differences in structural organization the peripheral ER in yeast resembles the one in higher eukaryotic cells. In those it takes up almost the complete cytoplasmic compartment. In budding yeast, the peripheral ER forms a highly dynamic network of interconnected tubules – similar to that of higher eukaryotes – but is positioned right underneath the cell cortex (Prinz et al., 2000). Therefore the peripheral ER is also termed “cortical ER” in *S. cerevisiae*. Only a few individual large tubules span the cytoplasm in order to connect the cortical ER and its perinuclear counterpart (Voeltz *et al.*, 2002).

1.4.2. Inheritance of the ER

The ER is an organelle that cannot be simply synthesized *de novo* upon cell division but has to be passed on from the mother to the daughter cell (Y. Du *et al.*, 2004). The fates of cortical and perinuclear ER during cell division however are quite different.

Budding yeast undergoes closed mitosis meaning that the nuclear envelope and the surrounding ER remain intact. During M-phase, the perinuclear ER is therefore partitioned to the daughter cell along with the nucleus and the microtubule cytoskeleton is necessary for this transport process (Fagarasanu *et al.*, 2007; Lowe *et al.*, 2007).

The inheritance of cortical ER precedes the segregation of perinuclear ER and it is dependent on actin cables as demonstrated by latrunculin A treatment, an actin filament disrupting drug (Estrada et al., 2003). From a mechanistic point of view, cortical ER inheritance is a multi-step procedure and is performed in a strictly ordered, cell cycle dependent manner (Figure 7).

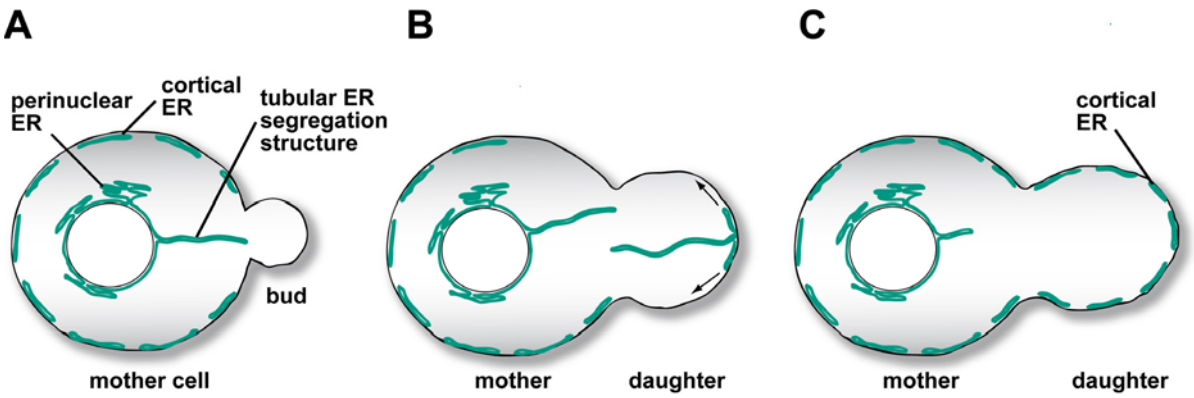


Figure 7: Inheritance of cortical ER in *S. cerevisiae* proceeds in several steps. (A) ER segregation structures i.e. ER tubules emanating from the perinuclear region of the mother cell move into the daughter cell along the mother-bud axis. (B) The tubules become anchored at the cortex of the bud tip. (C) These anchored, first cortical ER elements expand and finally fill in the whole cortex of the daughter cell building a reticular network like in the mother cell.

The first step happens during early S-phase when a small bud has just emerged. Cytoplasmic ER tubules derived from the perinuclear region of the mother cell align along the mother-bud axis and migrate over this distance into the daughter cell (Estrada et al., 2003). These tubules, also termed “ER segregation structures” are the first ER elements appearing in the newly forming daughter cell (Estrada de Martin *et al.*, 2005). This occurs shortly after the first secretory vesicles have reached the bud, but long before the nucleus starts to divide (Y. Du *et al.*, 2001; Preuss *et al.*, 1991). Secondly, the tubular elements become anchored to the plasma membrane at the bud tip and form the first building block of cortical ER. In a last

step, this first cortical domain extends into the whole bud forming a polygonal ER network like the one in the mother cell (Y. Du *et al.*, 2004; Fehrenbacher *et al.*, 2002).

In order to identify components of the cortical ER inheritance machinery, several genetic screens have been performed in yeast.
Some factors have been found whose exact role in ER segregation has not been unravelled so far. Amongst them is Ice2p, an integral membrane protein which is supposed to span the ER membrane multiple times. In cells lacking Ice2p, not only the transport of cortical ER into the daughter cell is impaired but also the structure of cortical ER network in the mother cell is strongly affected. Though the exact role of Ice2p still has to be determined, it seems to be important for morphology and segregation of cortical ER (Estrada de Martin *et al.*, 2004). This contrasts the phenotype of other mutants in which only ER inheritance itself is impaired. Such a mutant is the *aux1* deletion mutant. Aux1p (also called Swa2p) was previously described as a J-Domain-containing co-chaperone involved in the uncoating of clathrin-coated vesicles. However it was shown recently that, independently from this function, it fulfils a second task in the process of cortical ER inheritance. Cells with a disruption of the *AUX1* gene are defective in ER inheritance whereas the overall integrity of ER in the mother cell is maintained. Obviously Aux1p localizes to ER membranes but its role in ER inheritance remains elusive (Y. Du *et al.*, 2001).
In addition it has been reported that factors of the exocyst such as Sec3p, Sec5p and Sec8p are also involved in ER inheritance (Reinke *et al.*, 2004; Wiederkehr *et al.*, 2004; Wiederkehr *et al.*, 2003). The exocyst is an octameric complex which is necessary for tethering secretory vesicles to the plasma membrane prior to their docking and fusion (TerBush *et al.*, 1996). Sec3p is a non essential component since without it cells can still grow and undergo secretion. It is supposed to act as a spatial landmark for polarized secretion and in *sec3Δ* cells vesicles are not targeted to the appropriate fusion sites. Additionally, cells lacking Sec3p have a defect in ER inheritance most likely because they fail to retain migrating ER tubules at the bud tip during the anchoring step (Wiederkehr *et al.*, 2003). For the tubule anchoring process Sec3p might indirectly collaborate with members of the reticulon family (Rtn1p and Rtn2p) and Yop1 which are all conserved transmembrane proteins involved in generation and maintenance of ER tubules (De Craene *et al.*, 2006; Hu *et al.*, 2008; Voeltz *et al.*, 2006). The three proteins are exclusively distributed to tubular ER structures and if they are all simultaneously deleted peripheral tubular ER is disrupted (Voeltz *et al.*, 2006). Interestingly Rtn1p interacts with Sec6p, another exocyst component and this interaction might facilitate the attachment of ER tubules at the tip of the growing bud (De Craene et al., 2006). In cells with a deficiency in anchoring, the process of cortical ER inheritance is considerably disturbed (De Craene *et al.*, 2006; Wiederkehr *et al.*, 2003).

Furthermore, another screen searching for cortical ER inheritance mutants identified genes involved in ER-to-Golgi transport like *SEC21* and *SEC23* and genes acting in the SRP-dependent ER translocation pathway (Prinz et al., 2000). Amongst the second group are genes coding for the α- and β- subunit of the SRP receptor, *SRP101* and *SRP102*. This receptor, a heterodimeric complex located in the ER membrane, acts together with its SRP (signal recognition particle) ligand to target ribosome-nascent polypeptide complexes to the ER membrane and to mediate co-translational protein translocation (Figure 6) (Halic *et al.*, 2005; Keenan *et al.*, 2001). At restrictive temperature, *srp101-47* and *srp102-510* temperature-sensitive (ts) strains, display abnormalities in the cortical ER structure of the mother cell and a defect in cortical ER inheritance (Prinz et al., 2000). The mechanistic details of this deficiency however are still unclear.
Finally, as shortly mentioned at the beginning of section 1.4., the nonessential myosin V family motor protein Myo4 and the adaptor protein She3p were recently identified to be essential factors for segregation of cortial ER into daughter cells (Estrada et al., 2003). Previously, both proteins were described to be involved in the asymmetric localization of mRNA to the bud (Section 1.3.). However, in either a *MYO4* or a *SHE3* deletion strain also the process of ER inheritance is impaired (Estrada et al., 2003) and this is the only case so far where there is a clear explanation for this phenotype. A mutation in the ATP-binding region of the motor domain of Myo4 (*myo4-1*) had the same effect than the disruption of the whole *MYO4* gene implying that the inheritance of peripheral ER in yeast is dependent on the motor activity of Myo4p. In contrast to the adaptor protein She3p, the mRNA binding protein She2p is not necessary for this process. Additionally, She3p and Myo4p both co-fractionate with ER marker proteins in subcellular fractionation experiments indicating that the She3p/Myo4p motor complex is associated with ER. The ER is the only organelle whose partitioning is dependent on Myo4p (Estrada et al., 2003). Other organelles, like the vacuole, post-Golgi vesicles, the trans-Golgi-network, peroxisomes and mitochondria are transported by another type V myosin, Myo2p (Altmann *et al.*, 2008; Pruyne *et al.*, 2004). Thus, Myo4p and She3p seem to have a direct role in the inheritance of the cortical ER in yeast. Myo4p probably represents the motor driving the whole process and She3p might act as an adaptor which tethers Myo4p to the ER (Estrada et al., 2003).

Even though it was claimed that the two processes of mRNA localization and ER inheritance are uncoupled from each other, the use of a common machinery still raises the question whether the two transport routes are coordinated.

In previous chapters (1.1.1 and 1.1.2) we have seen that in higher eukaryotes, mRNA localization is a widespread mechanism to spatially control protein function. On the other

hand, as mentioned above, they also possess peripheral ER suggesting that this is not a yeast specific phenomenon. The major difference to yeast is, that those cells undergo an open mitosis in which the NE breaks down and according to the current model is concomitantly absorbed by the peripheral ER network (Estrada de Martin *et al.*, 2005). Moreover, microtubular tracks and associated motors are most likely responsible for its segregation to daughter cells (Estrada de Martin *et al.*, 2005; Lowe *et al.*, 2007). Even in somatic cells forming cellular protrusions one can observe that – in analogy to yeast – the first elements moving into regions of asymmetric cellular growth are single ER tubules. These tubules then form the interconnected network of the peripheral ER in distant areas such as neuronal growth cones or fibroblast leading edges (Y. Du *et al.*, 2004).
As ER inheritance proceeds in a highly organized predictable manner in yeast, *S. cerevisiae* serves as a perfect model organism to study ER segregation and its relationship to other processes important for polarized growth like asymmetric mRNA localization.

1.5. First indications for a link between mRNA localization and ER inheritance in *S. cerevisiae*

The aforementioned discovery that two major players of the mRNA localization pathway, She3p and Myo4p are also involved in the process of ER inheritance raised the question, whether the two transport pathways could be linked.
In a first attempt to analyze whether transport of tubular ER segregation structures and cytoplasmic mRNPs occur independently or in a coordinated way, mRNP and ER trafficking was followed simultaneously *in vivo* by Andreas Jaedicke in (Schmid *et al.*, 2006). ER tubules were visualized by means of a constitutively expressed fusion of GFP with Hmg1p (hydroxymethylglutaryl-coenzyme A reductase), an ER-resident enzyme that catalyzes the production of mevalonate, a precursor to ergosterol and nonsterol isoprenoid compounds. The fusion protein is present in perinuclear and cortical ER as well as in motile ER tubules (Estrada et al., 2003).
ASH1 mRNA containing six MS2 binding sites in its 3'UTR was expressed from a *GAL1* promoter. Visualization occurs via the MS2 coat protein (MS2-CP) which binds to the MS2 loops and is fused to RedStar fluorescence protein (Figure 8). A nuclear localization signal in this fusion protein allows export only if mRNA is bound as a substrate.
Although *ASH1* mRNA is usually transcribed only during mitosis when buds have reached their mature size, we chose this mRNA as model RNA because it can be effectively localized to the bud at any stage of the cell cycle (Long et al., 1997).

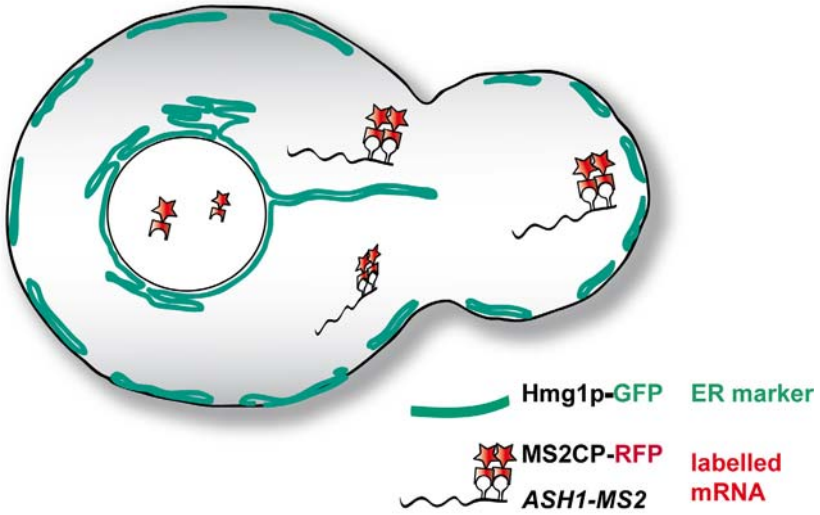


Figure 8: *In vivo* co-imaging of migrating ER tubules and mRNPs.
Movement of ER tubules and mRNAs can be simultaneously visualized by GFP-tagging of an ER marker protein and use of the MS-system for the mRNA of choice. Six MS2 loops are fused to the 3'UTR of the *ASH1* mRNA. These loops are bound by the MS-coat protein (MS2-CP) which in turn is fused to RFP.

For the analysis, larger buds (with a volume between 10% and 25% of the mother cell were not included as they already contain tubular and cortical ER structures. In small- to medium-sized buds however, the tubular ER segregation structures which are just about to move from the mother to the daughter cells can be detected.

In these cells a co-localization of *ASH1*-MS2 RNP particles with tubular ER structures in the bud and in the mother cell was observed. Particles were visible decorating ER tubules along the entire length, but frequently a particle was found at the tip of a moving ER tubule (Figures 9 A and B). Co-localization of mRNP particles and ER tubules was detectable over time spans up to 5 min (Figure 9 C and also movie S1 and S2 in (Schmid *et al.*, 2006)). This suggests that ER tubules and *ASH1*-MS2 mRNPs move in a coordinated manner.

In order to test whether the Myo4p/She3p motor protein complex is needed for the association of mRNPs and ER tubules, the co-localization in cells lacking Myo4p was examined. In more than 80% of *myo4Δ* cells observed, ER tubules and mRNPs do not move into small-sized buds. In the remaining cells (<20%), ER tubules can be detected in the bud which are not associated with *ASH1*-MS2 mRNPs (Figure 9 D). This indicates that a fraction of ER tubules is able to move into the bud independently of Myo4p and is consistent with a study that suggested that Myo4p-dependent transport might not be the sole mechanism for movement of ER tubules into the bud (Reinke *et al.*, 2004).

Strikingly, tubules remaining in the mother cell were still associated with mRNP particles, and both tubules and mRNP particles showed coordinated yet random movement (Figure 1D and movie S3 in (Schmid *et al.*, 2006)), suggesting that co-localization of *ASH1*-MS2 mRNPs and ER tubules is independent of the Myo4/She3p complex.

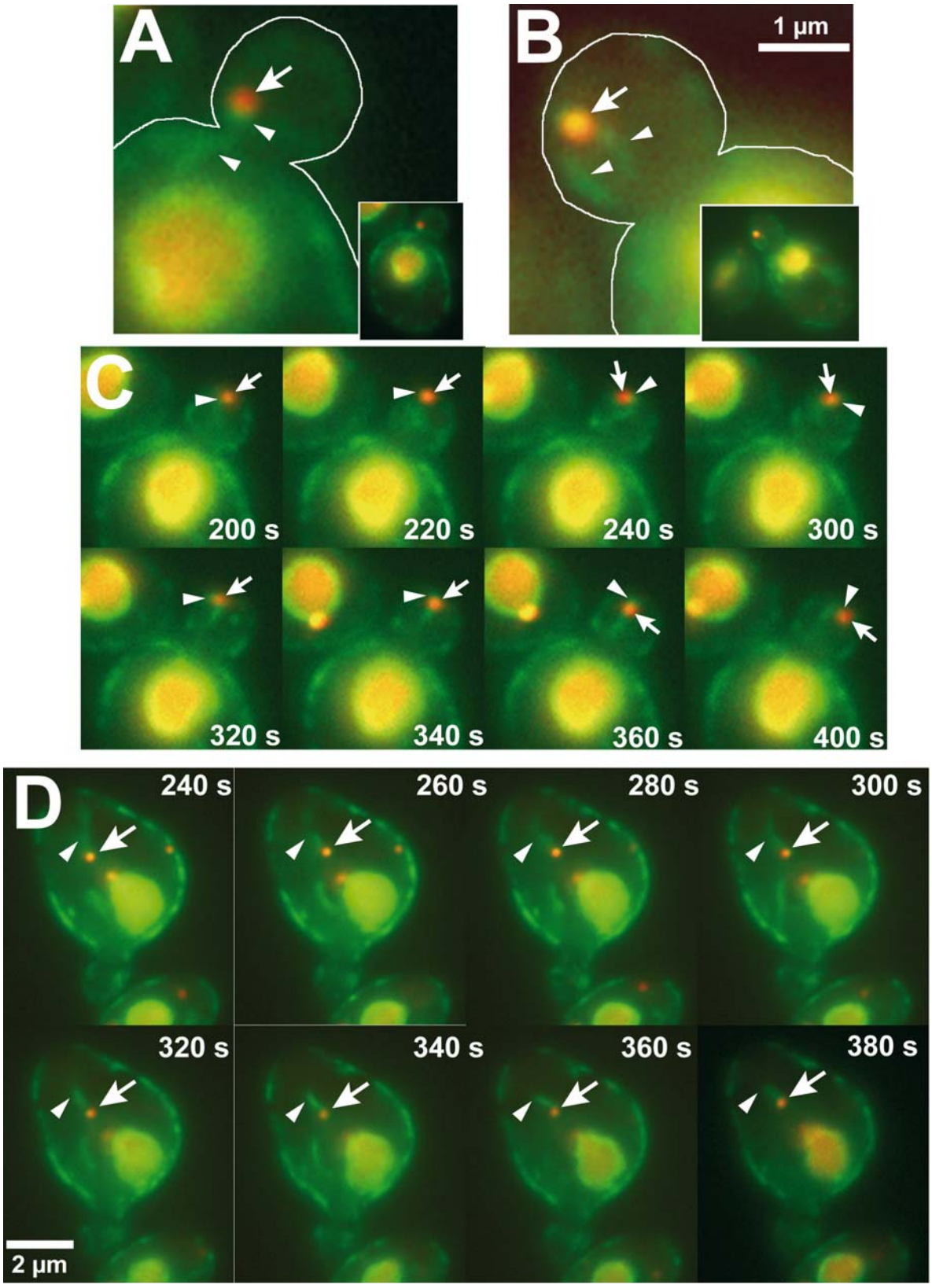


Figure 9: Legend see next page

Figure 9: Co-localization of *ASH1-MS2* mRNP particles with tubular ER in the bud. (adapted from Schmid *et al.*, 2006) (A and B) Representative examples of cells from strain RJY2339 with *ASH1*-MS2 mRNP particles (arrows) labelled by MS2-RedStar fusion protein and ER tubules (arrowheads) labelled by Hmg1p-GFP fusion protein. *ASH1*-MS2 particles (red) co-localize with the tip of ER tubules (green) in the bud. Individual frames from a time-lapse series of the cell shown in (A). The *ASH1*-MS2 mRNP (red, depicted by an arrow) stays associated with an ER tubule (green) for more than 3 min. Arrowhead marks the tip of the ER tubule. Time point of each image is indicated. (D) Co-localization of *ASH1*-MS2 mRNP with ER tubules in the absence of Myo4p. Individual images from a time-lapse series (Movie S3) of strain RJY2372 showing MS2-Red- Star-labelled *ASH1*-MS2 RNP particle (red, arrow) associated with tubular ER structures (green, arrowhead) in the mother cell. Note that in contrast to wild-type cells (C), the marked tubule does not show directional movement to the bud and that no *ASH1*-MS2 particles are visible in the bud (at the bottom of the cell).

1.6. Aim of this work

Cortical ER inheritance and mRNA localization are both highly coordinated transport routes and are important for the regular course of proliferation in *S. cerevisia*e. First, two major players of the mRNA localization machinery, the myosin motor Myo4p and its adaptor protein She3p were discovered to be additionally crucial for cortical ER inheritance (Estrada et al., 2003). Thus the two transport pathways use a common machinery as driving force, indicating for the first time that there might be a connection between the two processes. Furthermore *in vivo* co-imaging data revealed that ER tubules and *ASH1* mRNPs co-localize and even migrate together to the yeast bud (Section 1.5.), an observation which strongly supports the notion that the transport of ER and mRNA might occur in a coordinated manner. From a logistic point of view this would make sense. As it was already mentioned earlier, the majority of localized mRNAs in *S. cerevisiae* encode secreted or membrane proteins (Section 1.3.3.). Therefore it would be even more efficient if they were preassembled and transported together with the structure where they are translated and further processed in the end. Indications for a linkage between mRNA and ER co-transport do not only exist in yeast. In *Xenopus*, Vg1 mRNA is bound by the Vera protein (VgLE binding and endoplasmic reticulum association), an RNA protein also associated with ER membranes and the ER was proposed to have a role in the localization of Vg1 mRNA (Deshler *et al.*, 1997). As another example, *HrPEM* and *macho 1* mRNAs bind to and move with rough ER at the cell cortex of ascidien eggs (Sardet *et al.*, 2003).

One aim of this work was therefore to investigate whether there is a functional link between mRNA localization and ER inheritance in *S. cerevisiae*. Another aspect was the attempt to unravel - via a biochemical approach - the molecular basis of the mRNP and ER tubule co-localization observed in double live imaging. This included various subcellular fractionation methods to narrow down the group of potential mRNA-ER linkers to a specific candidate and finally, the attempt to investigate the molecular mechanism for its membrane association.

2. Results

2.1. Loss of *ASH1-MS2* RNP localization in cells defective for ER inheritance

The co-localization data from live microscopy using the MS2 system described in section 1.5. suggest that segregation of ER and RNA localization in yeast are coupled.

In order to recapitulate these experiments I determined the distribution of both ER tubules and *ASH1*-MS2 RNPs in *aux1Δ*, *myo4 Δ*, *srp101-47^{ts}* and wild type cells with small to medium sized buds. Aux1p, previously implicated in clathrin-mediated membrane trafficking, was recently identified as an essential factor for cortical ER inheritance. *AUX1* deletion specifically causes a delay in the transport of cortical ER elements into the daughter cell, whereas the inheritance of perinuclear ER and the general morphology of the ER are unaffected (Y. Du *et al.*, 2001). *SRP101* encodes a subunit of the signal recognition particle receptor, a heterodimeric protein in the ER membrane. At restrictive temperature, strains carrying a *srp101-47^{ts}* mutation show similar ER segregation defects like *aux1Δ* cells (Prinz et al., 2000).

ER tubules were visualized with a GFP-tagged ER marker Hmg1p-GFP. *ASH1-MS2* mRNPs were labelled by co-expression of the MS2 coat protein (MS2-CP) fused to the RedStar fluorescence protein.

In wild-type cells, the majority of buds contain ER tubules (72%) and *ASH1*-MS2 mRNPs (92%) (Figure 10 A). In contrast, only 40% of *srp101-47^{ts}* cells, 27% of *aux1Δ* cells, and 14% of *myo4 Δ* cells show bud-specific cortical ER staining or ER tubules that have moved into small- to medium-sized buds (Figure 10 B, black bars). In accordance with the observed defects in ER segregation, all three mutants also affect *ASH1*-MS2 mRNP particle localization to small- or medium-sized buds. This effect is more pronounced in *myo4 Δ* cells (0% buds with a RNP particle) than in *aux1Δ* (RNP particle in 24% of buds) or *srp101-47^{ts}* (43% buds with RNP particles) cells (Figure 10 B, white bars). In addition, I observed that *ASH1-MS2* RNP signals in *aux1Δ* mother cells are generally weaker than in wild-type or *myo4 Δ* cells, possibly due to a defect in RNP assembly or particle composition.

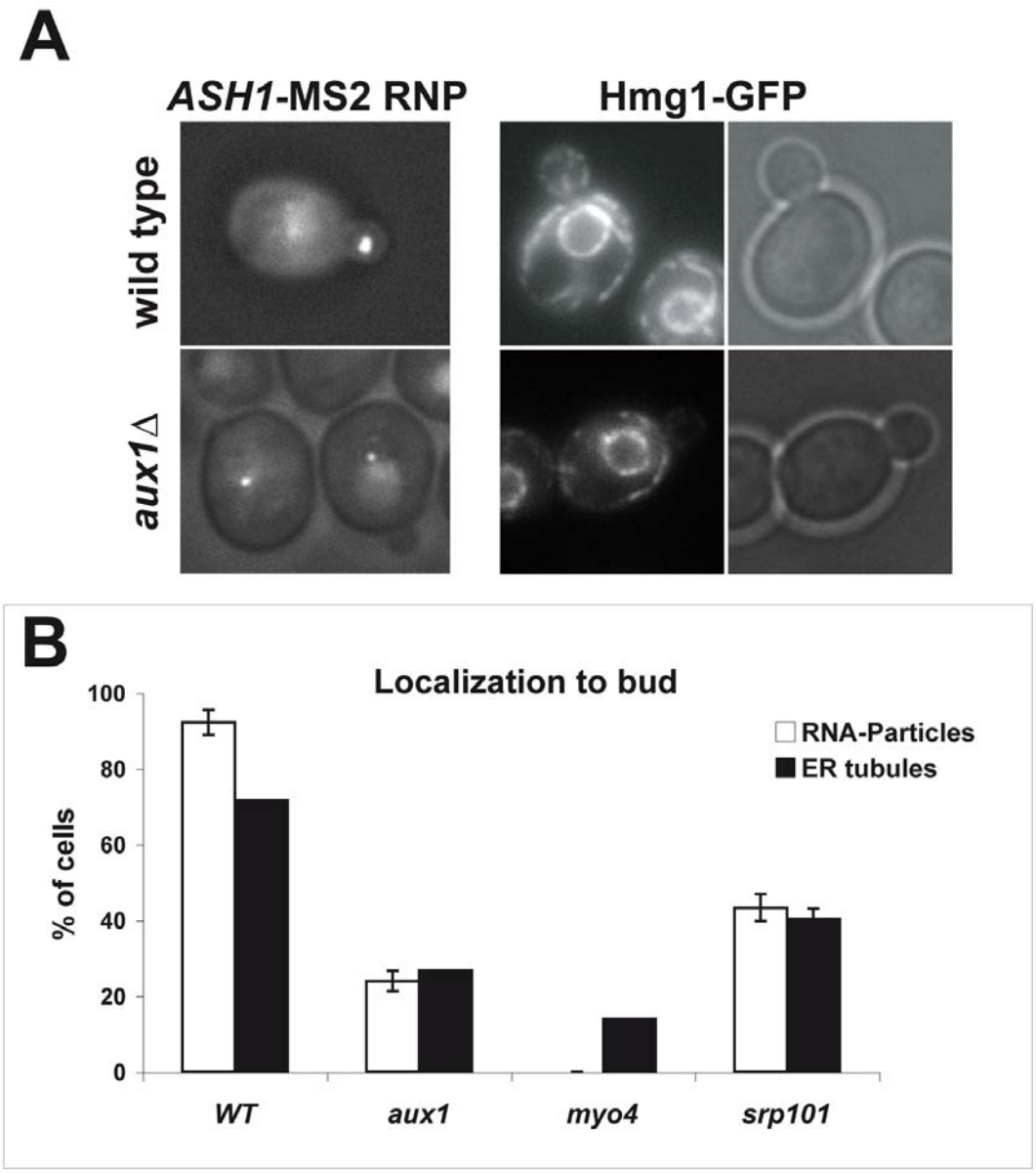


Figure 10: Localization of *ASH1*-MS2 RNP particles to the bud is impaired in mutants affecting ER segregation
(A) Top: Representative images of wild-type cells (RJY2339) with small- or medium-sized buds containing *ASH1-MS2* mRNPs (left) or cortical ER and ER tubules (middle) in the bud. Bottom: Representative images of *aux1Δ* mutant (RJY2794) cells showing absence of *ASH1-MS2* RNPs or cortical ER from the bud. (B) Quantitative analysis of *ASH1*-MS2 RNPs (white bars) and cortical ER (Hmg1p-GFP; black bars) localization to small- or medium-sized buds of wild-type, *aux1Δ, myo4Δ* (RYJ2372), and *srp101-4* ^{ts} (RYJ2858/2859) cells. 164 cells (wild-type), 243 cells (*aux1Δ*), 340 cells (*srp101-47*), or 101 cells (*myo4Δ*) were scored in three independent experiments. Error bars indicate the standard error of the mean (SEM).

The observations of a parallel loss of RNP particle and cortical ER localization in *aux1Δ*, *myo4 Δ* and *srp101-47*^{ts} mutants support the notion that transport of ER tubules and of RNP particles are coordinated and not independent events.

2.2. *IST2-MS2* does not localize as efficiently as *ASH1-MS2* mRNA

Besides *ASH1* there are at least 23 additionally localized mRNAs in *S. cerevisiae* (Shepard et al., 2003). Consequently I wanted to asses whether the aforementioned functional link between mRNA localization and transport of ER tubules was also true for another localized mRNA. In analogy to the *ASH1-MS2* system the *IST2* ORF was cloned under control of the *GAL1* promoter (pRJ1399) and the localization of MS2 RNPs was analyzed in wild type and *myo4Δ* cells.

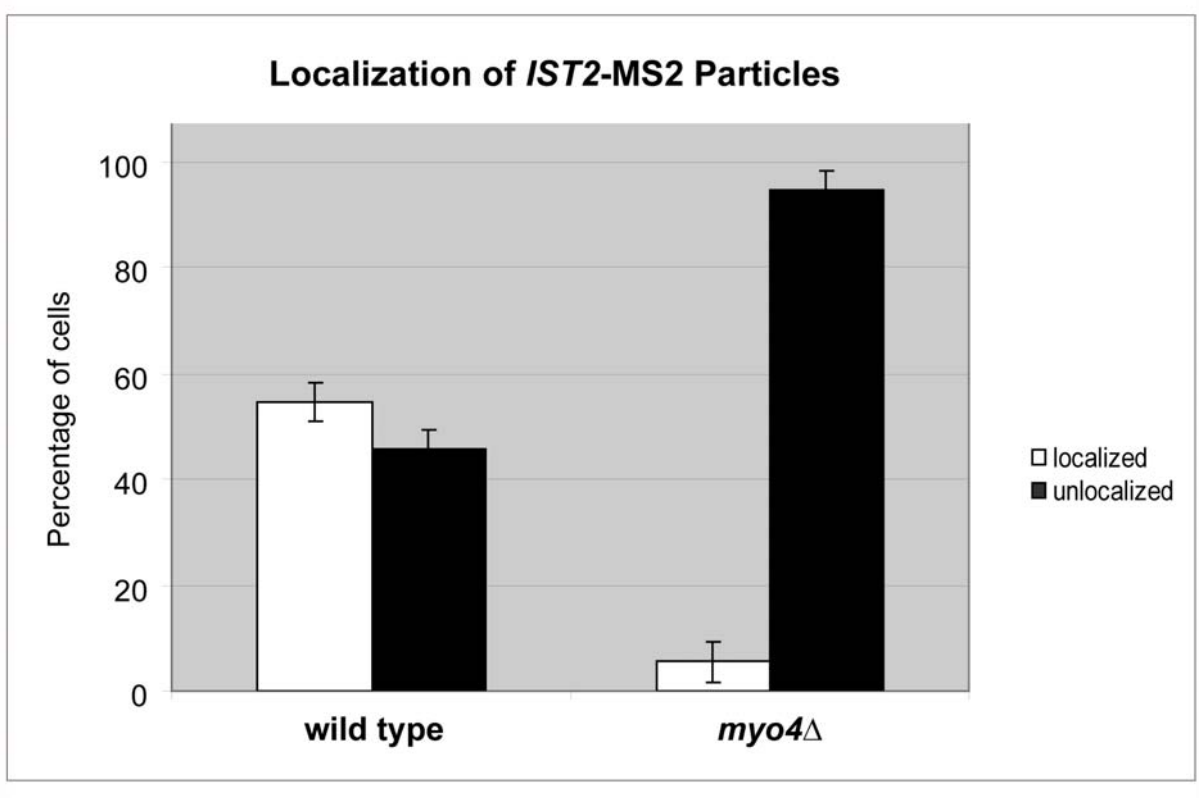


Figure 11: *IST2-MS2* is not suited for live cell imaging
The plasmid encoding IST2-6xMS2 (pRJ1399) was co-transformed with (pRJ741) into wt (RJY2049) and *myo4Δ* (RJY2299) cells and analyzed for localization of mRNPs to the yeast bud. 413 cells (wild-type) and 338 cells (*myo4Δ*) were scored in four and three independent experiments respectively. Error bars indicate the standard error of the mean (SEM).

Consistent with the results obtained for *ASH1* as model mRNA, the vast majority of *IST2* particles (95 %) did not localize to daughter cells in a *myo4Δ* mutant (Figure 11, right panel). Unexpectedly though, the localization in wild type cells amounted to about 54 % only (Figure 11, left columns). Due to this already low degree of localized particles in wild type cells, the MS2 system using *IST2* as a localized mRNA was not suited to further analyse ER segregation mutants for defects in transport of *IST2-MS2* particles.

2.3. *WSC2* mRNA can be used as a model mRNA which is expressed earlier in cell cycle than *ASH1* mRNA

So far, the mRNA which was primarily used to investigate mRNA localization in yeast was *ASH1* mRNA. However, the latter is endogenously expressed only for a very short time-span during late anaphase at the end of the yeast cell cycle (Bobola *et al.*, 1996a). To circumvent the problem of this short time window, *ASH1* was expressed from an inducible *GAL* promoter in the *in vivo* microscopy experiments described earlier. For future *in vivo* microscopy experiments, *ASH1* mRNA should be expressed from its own promoter. It would then be interesting to assess whether a functional correlation between ER and mRNA transport could also be seen with *ASH1* expressed from its endogenous promoter and with a localized mRNA which is *per se* expressed much earlier in cell cycle.

In the long term it is therefore advisable to have another localized model mRNA which is expressed far earlier during cell cycle than *ASH1* mRNA. Different groups conducted genome-wide transcriptional analysis in the yeast *S. cerevisiae* (R. J. Cho et al., 1998; Spellman et al., 1998). According to these studies, *WSC2* is expressed early in cell cycle during S-phase. This localized mRNA (Shepard et al., 2003) encodes a predicted plasma membrane protein involved in maintenance of *S. cerevisiae* cell wall integrity and stress response (Ng, 2001; Verna *et al.*, 1997). To verify the transcriptional analysis data I performed fluorescent *in situ* hybridization with Cy3-labelled oligonucleotides to probe for *ASH1* mRNA and DIG-labelled oligonucleotides followed by anti-DIG and Alexa®488-labelled antibodies to probe for *WSC2* mRNA.

As already described (Long et al., 1997), endogenous *ASH1* mRNA was detected in the daughter cells of binucleate, large budded cells. Characteristically, the nucleus is just about to divide or has already divided and been separated to the daughter and mother cell (Figure 12, upper panel). In sharp contrast to this, *WSC2* mRNA was only detected in newly emerging and small buds of dividing cells (Figure 12, lower panel).

Hence *WSC2* mRNA would be well suited as a localized mRNA expressed early in cell cycle. However, it will be more appropriate to establish a live cell imaging system in order to evaluate temporal differences in mechanisms of mRNA localization at different cell cycle stages. Due to time limitations at the end of this study, these experiments unfortunately could not be performed within the scope of my thesis.

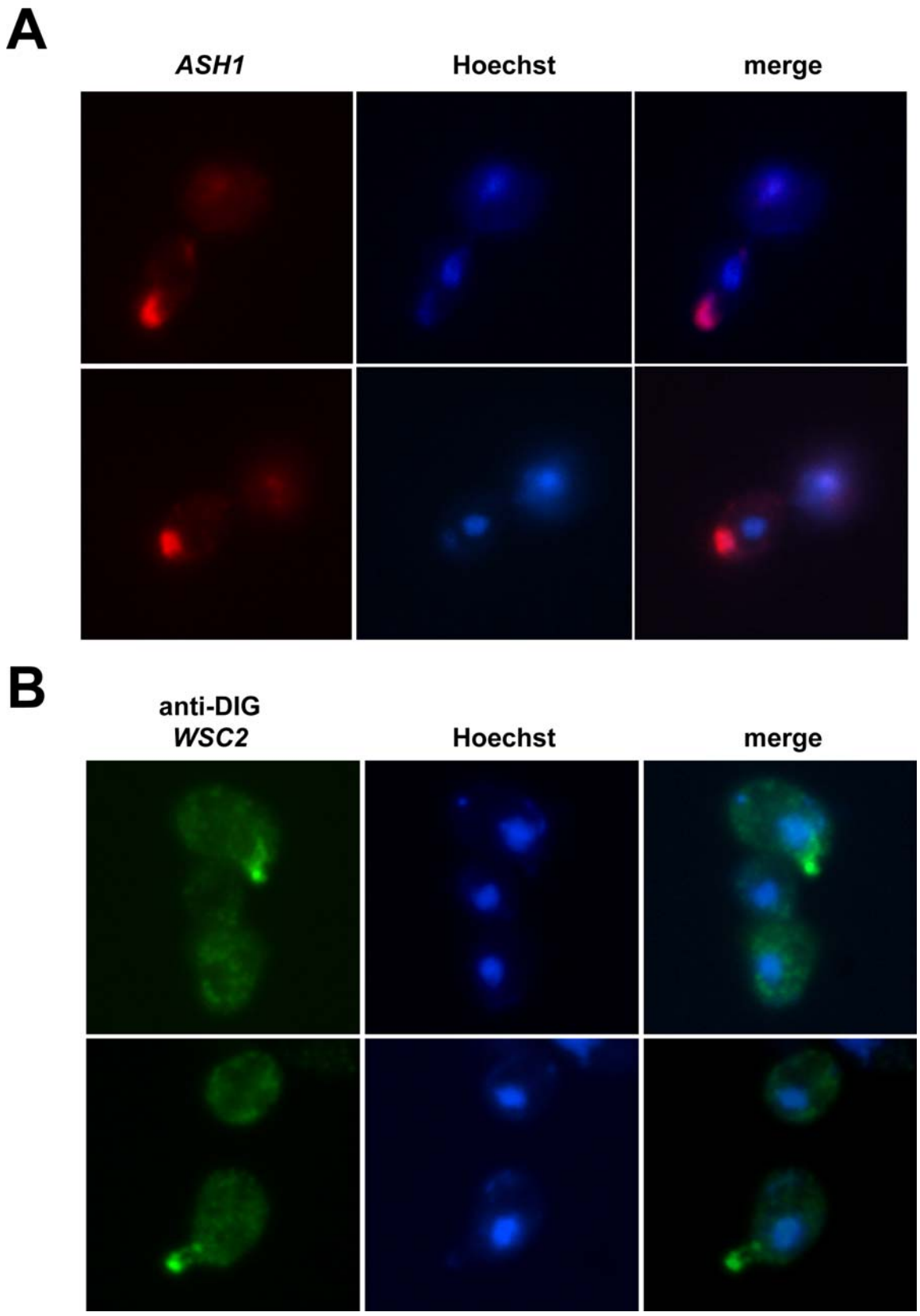


Figure 12: *WSC2* can serve as an additional localized model mRNA expressed earlier in cell cycle than *ASH1*. (A) Cy3 labelled DNA oligonucleotides were used to detect endogenous *ASH1* mRNA expressed from its own promoter in FISH experiments. (B) *WSC2* mRNA was visualized by FISH with DIG-labelled probes.

2.4. Biochemical analysis: co-migration of ER and the *ASH1* mRNA binding protein She2p during subcellular fractionation

Both, co-localization of RNP particles and ER structures in live cell microscopy and defects in RNA localization observed in ER segregation mutants described in sections 1.5. and 2.1. strongly hint to an association of ER structures and RNP particles. Due to limitations in spatial resolution, however, non-associated structures moving close to each other with identical vectors cannot easily be distinguished from associated structures moving as one unit. In order to test for an association of ER and RNPs that contain localized mRNAs, I investigated if the RNA-binding protein She2p co-fractionates with ER in different subcellular fractionation experiments. She2p is part of the *ASH1*-MS2 RNP (Bertrand et al., 1998) and it binds to all described mRNAs that localize to the bud tip, including *ASH1* mRNA (Bohl *et al.*, 2000; Long *et al.*, 2000; Shepard *et al.*, 2003).
For all subcellular fractionation methods described, gentle cell rupture methods like spheroplasting combined with douncing were applied to maintain the integrity of membranous structures.

2.4.1. She2p co-migrates with ER markers in a discontinuous velocity sucrose gradient

In a first approach, cell extracts were prepared and separated on an 18 – 60% velocity sucrose gradient. 12 fractions were collected and the distribution of She2p relative to ER marker proteins was determined by Western blot analysis (Figure 13). Hmg1p-GFP, the marker for ER, migrates close to the bottom of the gradient in the high density fractions. Similar migration behaviour is seen for two other ER marker proteins, Dpm1p (dolichol phosphate mannose synthase, figure 24) and Sec61p (an essential subunit of the yeast translocon, figure 20). As previously shown (Estrada et al., 2003), an HA-tagged version of Myo4p co-fractionates with the ER marker protein (Figure 13). Interestingly, like Myo4p, the RNA-binding protein She2p also fractionates with the ER marker (Figure 13) to the bottom of the gradient thus providing first evidence for a physical association between She2p and ER.

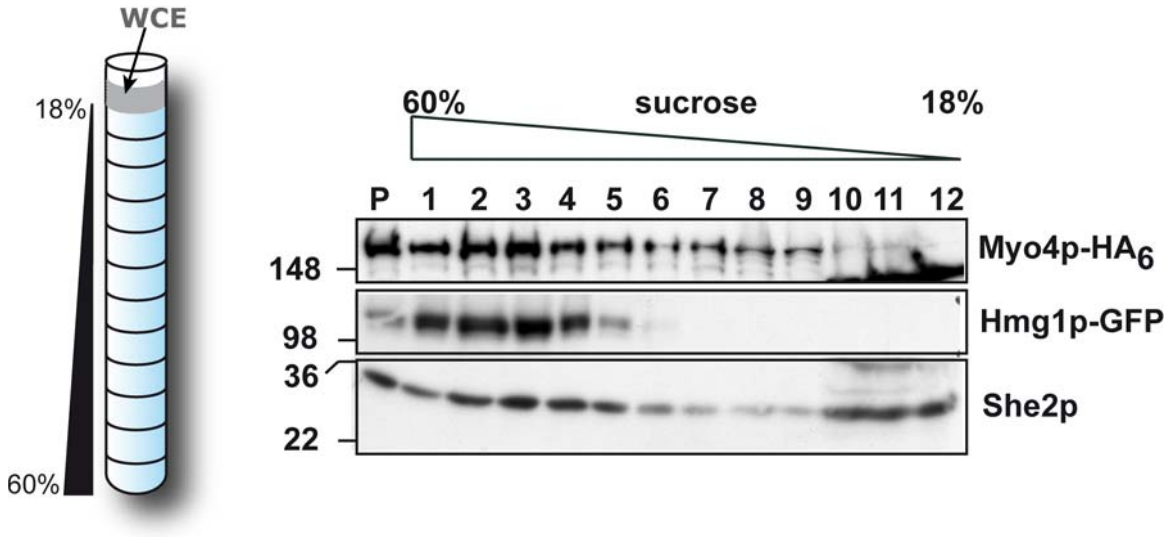


Figure 13: She2p co-migrates with ER marker proteins during velocity sucrose gradient centrifugation.
Cell extract from strain RJY2479 (*MYO4-HA3, URA3::HMG1-GFP*) was separated on a linear 18%–60% sucrose gradient as described in the methods section 5.5.2.. Aliquots of 12 fractions and the pellet were analyzed by Western blotting against HA, GFP, or She2p. She2p and Myo4p co-migrate with the ER marker protein Hmg1p-GFP to the dense fractions of the gradient (fractions 2-4).

2.4.2. She2p is present in the fraction of purified ER microsomes

Since She2p can shuttle between nuclei und cytoplasm (Kruse et al., 2002) and the ER membrane is continuous with the outer leaflet of the nucleus, I wanted to make sure that the heavy fractions containing an ER marker and She2p observed in the section above are not nuclei. Therefore, a crude membrane fraction was prepared from lysed yeast spheroplasts and the resuspended membranes were separated on a two-step sucrose gradient. The method used was originally developed for isolating functional ER membranes from yeast (Wuestehube *et al.*, 1992).

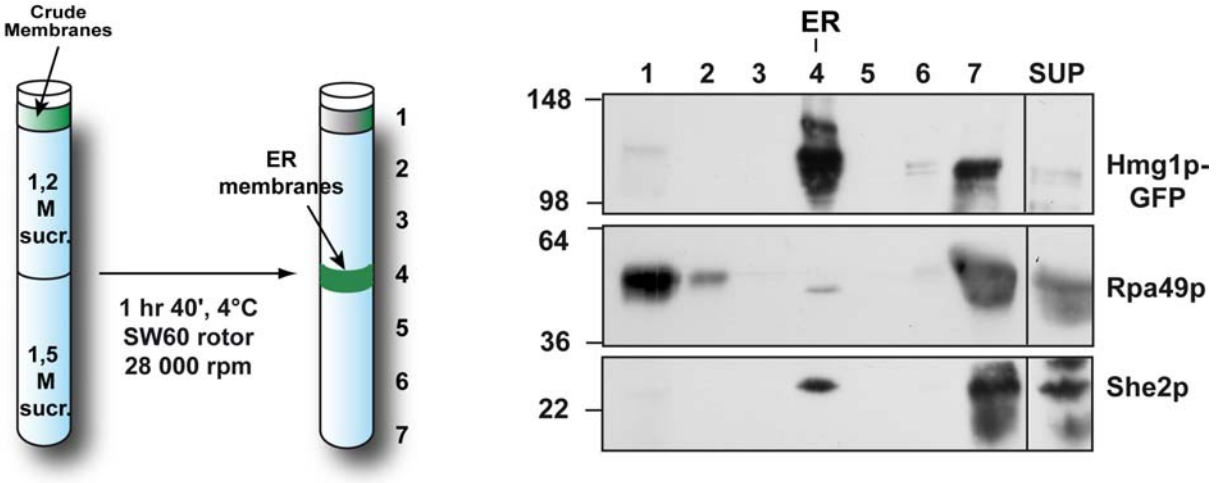


Figure 14: She2p can be detected in a fraction of purified ER microsomes
Yeast ER membranes were purified from a crude membrane pellet by centrifugation through a sucrose cushion. Numbers correspond to fractions from top to bottom. F4, the interphase between 1.2 M and 1.5 M sucrose cushions, contains ER. She2p is also enriched in F4, but the nuclear marker protein Rpa49p is mainly found in F1 (broken nuclei) and F7 (nuclei).

Whereas ER membranes accumulate at the interphase of the two sucrose cushions (Figure 14, fraction 4), a marker for soluble nuclear proteins (Rpa49p, a subunit of RNA polymerase I) is found at the bottom of the gradient (fraction 7) or at the top (fraction 1). Rpa49p in the ”light” fraction 1 appears to correspond to protein released from broken nuclei whereas the Rpa49p signal in the pellet represents nuclear protein in intact nuclei. Only little Rpa49p is detected in fraction 4. In contrast, a significant amount of She2p is present in this fraction, suggesting a co-fractionation of She2p and ER.

2.4.3. Flotation of ER membranes by equilibrium density centrifugation: She2p floats along

In order to rule out the possibility that the heavy She2p fractions correspond to very large RNPs that might accidentally co-migrate with ER in sucrose gradients, I performed flotation experiments as described for neuronal RNP complexes (Kanai *et al.*, 2004). Neuronal RNP marker proteins did not float together with membrane marker proteins to a sucrose cushion with lower density (interphase between 0 and 40% sucrose) but stayed in the 50% sucrose cushion (Kanai *et al.*, 2004).

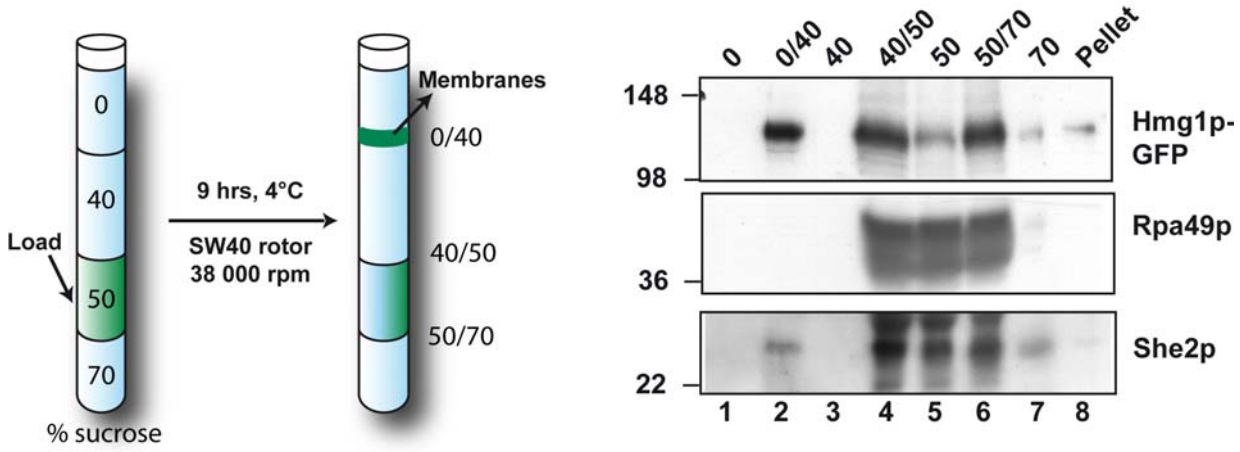


Figure 15: She2p floats to the top of the gradient together with ER membranes
Crude yeast extract equilibrated in 50% sucrose was overlaid with two sucrose cushions containing 0% or 40% sucrose. After equilibrium centrifugation, fractions were taken and analyzed by Western blotting. Numbers on top indicate sucrose concentrations of the corresponding sucrose cushions or interphases between cushions. She2p and Hmg1p-GFP but not Rpa49p float to the 0%/40% sucrose interphase, which indicates a membrane association.

In striking contrast to this, a significant fraction of She2p co-migrates with the ER membrane marker Hmg1p (Figure 15). The floated portion of She2p is apparently smaller than that of Hmg1p. This result is expected since a strong association of an integral membrane protein like Hmg1p with ER membranes is generally not seen for a protein that is peripheral or loosely associated with ER. More important, the nuclear marker Rpa49p does not show flotation suggesting a specific behaviour of She2p.

In summary, the biochemical fractionation experiments demonstrate that a substantial fraction of the RNA-binding protein She2p is co-fractionating with ER markers in several different gradients. These observations strongly substantiate that there is indeed a physical interaction between the RNA binding protein and ER membranes.

2.5. Intact polysomes are not required for She2p-ER association

In light of the data obtained above, the question arose what could constitute the molecular basis of She2p's interaction with ER membranes.
Amongst the group of localized mRNAs in *S. cerevisiae*, many encode membrane or secreted proteins (Table 1). In addition to this we know from other studies (Lange et al., 2008) that different kinds of localized mRNAs are co-assembled and transported together in the same particle. Consequently it seemed possible that localizing mRNPs containing

mRNAs for both soluble and membrane proteins are tethered to the ER via the signal recognition particle (SRP) pathway. This ribonucleoprotein recognizes signal peptides from emerging, newly synthesized membrane or secreted proteins and delivers them to the translocon of ER membranes (Keenan *et al.*, 2001).

In order to test whether association of She2p with ER is mediated by ongoing translation and nascent chain - translocon interaction, I used a common method to disrupt polysomes. By addition of 10mM EDTA to cell extracts ribosomes are split into their 40S and 60S subunits (Frey *et al.*, 2001).

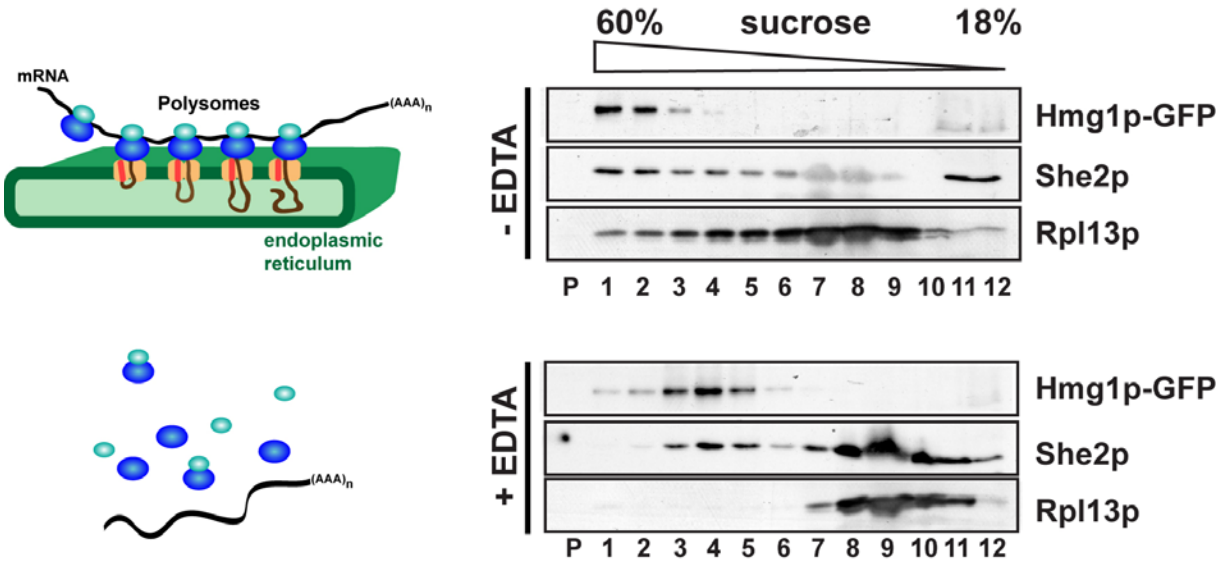


Figure 16: She2p association with ER does not require polysomes
Extracts treated with 10 mM EDTA to disassemble polysomes or mock-treated extracts were separated as described above. A shift of ribosomal protein Rpl13p toward less dense fractions (8–11) of the gradient verifies successful EDTA-mediated polysome disruption. She2p and Hmg1-GFP co-fractionate in both gradients with the peaks of Hmg1p-GFP and She2p shifting from fractions 1–3 to fractions 3–5, indicating a loss of polysomes from the ER.

The EDTA treatment was successful in disrupting polysomes as verified by the disappearance of the ribosomal protein Rpl13p from higher density fractions (Figure 16). Intriguingly, the addition of EDTA had no effect on She2p-ER co-migration into the gradient. ER marker and She2p shifted by two to three fractions towards the top of the gradient, indicating that the now less-dense ER had lost attached ribosomes (Rieder *et al.*, 2000) but in contrast had kept She2p (Figure 16, lower panel).

From this observation we infer that ongoing translation of mRNAs for secreted or membrane proteins is not a prerequisite for interaction of She2p with ER membranes.

2.6. The She2p-ER interaction is not dependent on mRNA

Even if She2p-ER association is not dependent on ongoing translation, it might still be possible that the interaction is mediated indirectly via the mRNAs bound to She2p. Those might act as molecular linkers to yet unknown ER components. Moreover it was recently described that even certain RNAs themselves can bind to phospholipid membranes (Janas *et al.*, 2006).

2.6.1. RNase treatment of whole cell extracts does not disrupt She2-ER association

In a first attempt to test for the mRNA dependence of the She2p-ER co-migration, I treated cell extracts with a combination of Micrococcal Nuclease and RNase A to destroy cellular mRNA before performing velocity gradient centrifugation (Figure 17 A). The efficiency of the treatment was checked by RT-PCR detecting *ASH1* and *IST2* mRNAs (Figure 17 B).
Interestingly, She2p can still be detected in high-density fractions of sucrose gradients after RNase treatment although a substantial amount has moved to less dense fractions (Figure 17 A, lane 11). Even more notably, the She2p portion in the high-density part of the gradient shifts along with ER from the very bottom of the gradient (fractions 1 and 2) to less dense fractions (fractions 3 and 4). This is most likely due to loss of polysomes from the rough ER, in the same way as it was already observed after EDTA treatment. Upon release of macromolecular complexes like polysomes, the mass and also density of a membranous organelle is diminished (Rieder *et al.*, 2000). The issue whether polysomes were indeed disrupted after the RNAse treatment was verified by the complete destruction of the ribosomal RNA *RDN18* which was no longer detectable by RT-PCR (Figure 17 B).
This observation indicates that She2p does not depend on the presence of mRNAs for its interaction with ER.

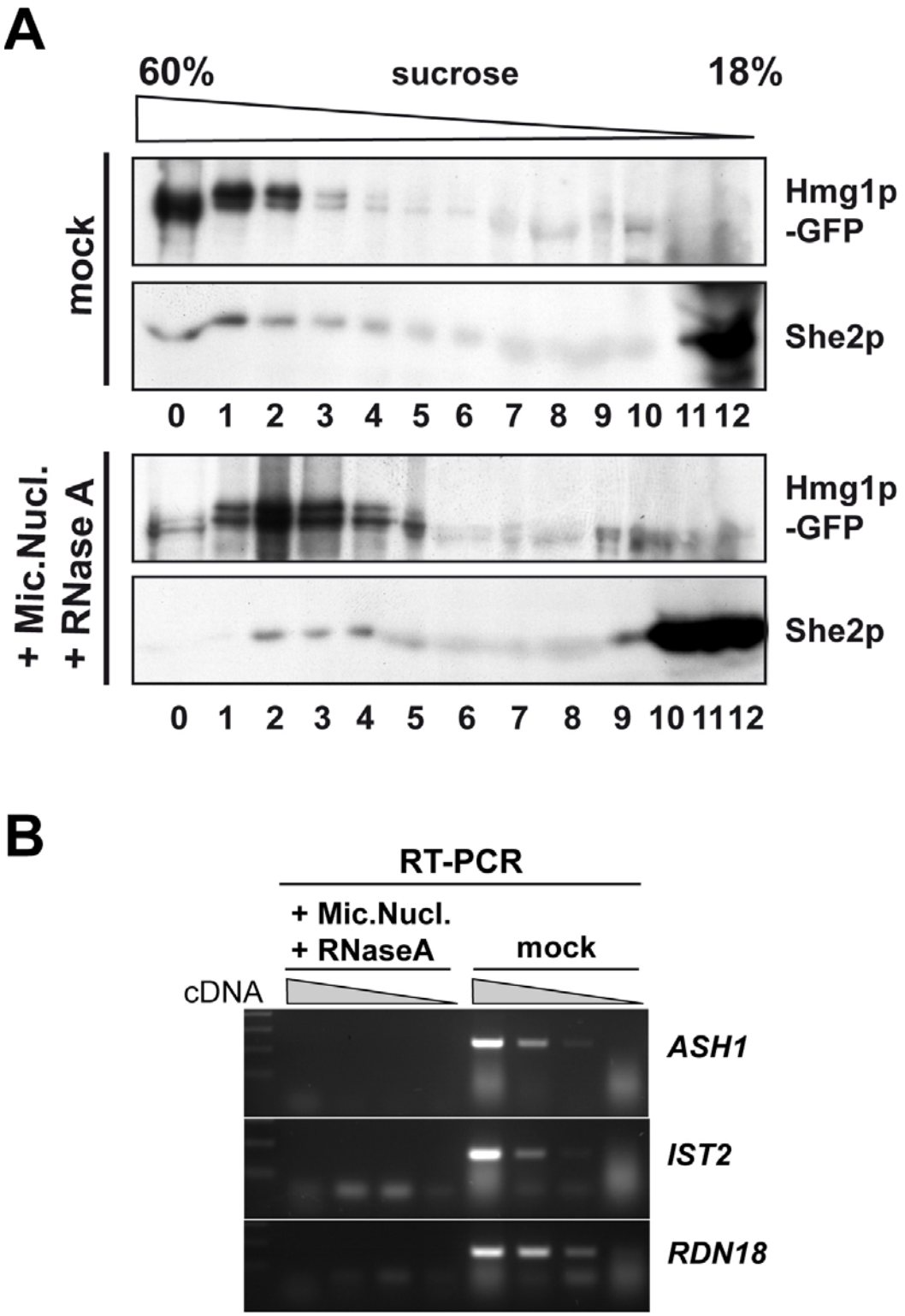


Figure 17: RNase treatment of whole cell extracts
(A) Velocity gradient centrifugation: Extracts treated with a combination of Micrococcal Nuclease and RNase A to completely remove endogenous mRNAs or mock-treated were separated on 18-60% sucrose gradients as described above.
(B) RT-PCR to assess completeness of the RNA-digest: Total RNA was isolated from aliquots of mock and RNase treated cell extracts. After DNase treatment pure RNA was used to generate cDNA and PCR was performed to verify complete RNA digest in RNase treated extracts. Oligonucleotides used were: *ASH1*: RJO 73+74; *IST2*: RJO 2428+2429; ribosomal rRNA *RDN18*: RJO 3158+3159.

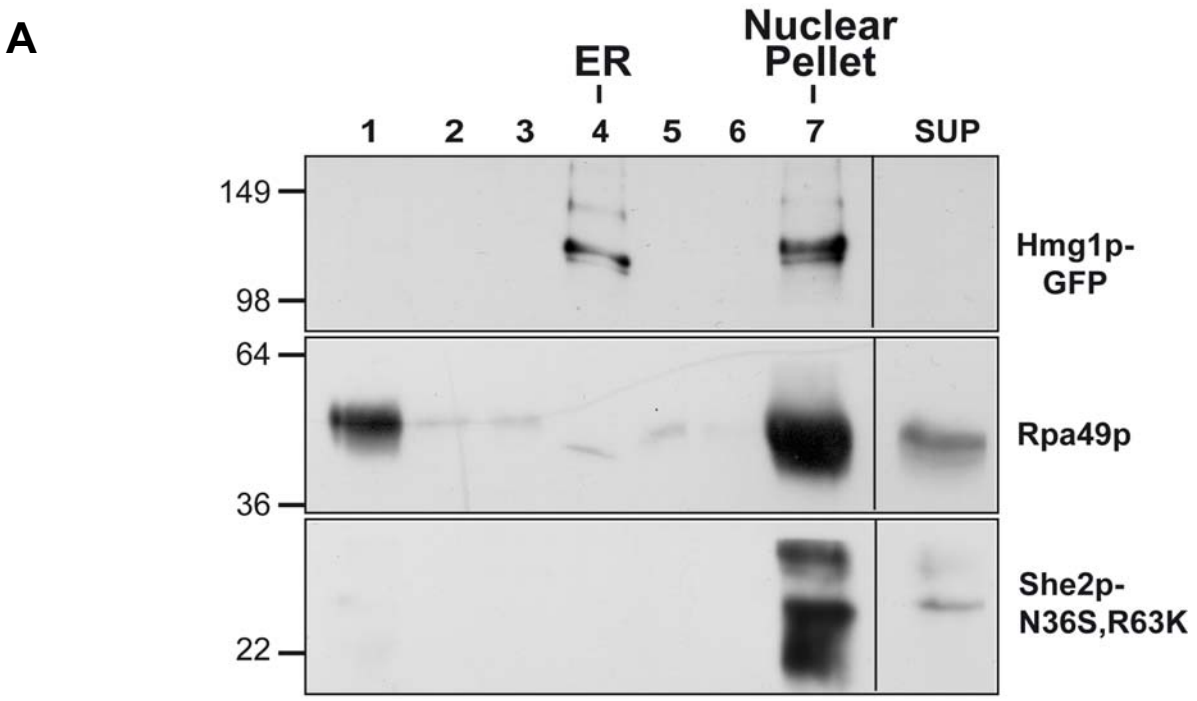
2.6.2. The mRNA binding mutant She2p-N36S,R63K accumulates in the nucleus

To directly assess a possible role of She2p's RNA binding activity in ER association, the most appropriate way is to use a She2p mutant unable to bind *ASH1* and other localized mRNAs. I generated such a mutant by introducing two point mutations into She2p, N36S and R63K, each of them known to disrupt RNA binding (Gonsalvez et al., 2003). Point mutations in the RNA binding site of She2p were reported to not alter the nuclear/cytoplasmic distribution equilibrium of She2p and not to result in nuclear accumulation of She2p (Gonsalvez et al., 2003).

However, when purifying microsomes by 2-step density gradient centrifugation (Section 2.4.2.), the mutant protein is only present in the nuclear pellet but absent from fractions containing ER marker (Figure 18 A, fraction 4).

In order to verify that the She2p-N36S,R63K mutant protein indeed accumulates in nuclei, indirect immunofluorescence against She2p was performed. By microscopy I observed that, in contrast to wild type She2p, the mutant protein is enriched in nuclei (Figure 18 B).

Thus, a She2p mutant deficient in RNA binding cannot be used *in vivo* to directly assess the RNA dependence of a She2p-ER association.



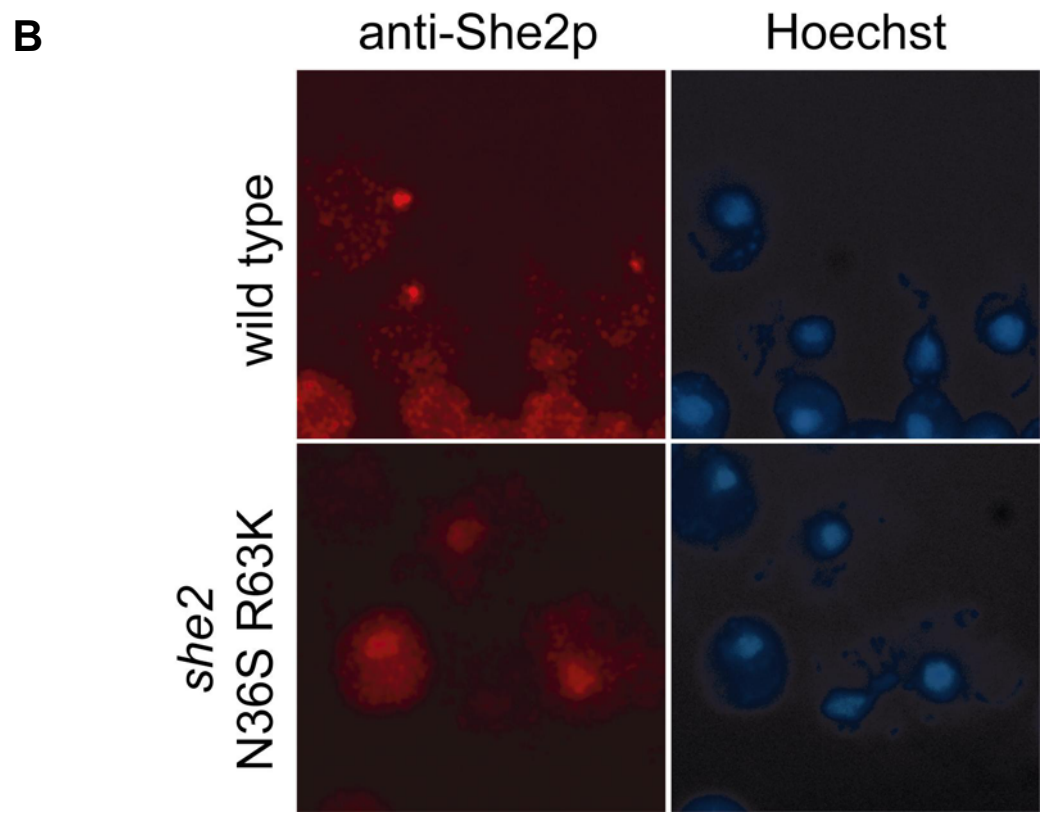


Figure 18: The She2p RNA binding mutant N36S,R63K accumulates in the nucleus
(A) Purification of ER membranes by centrifugation through a two step sucrose gradient: After purification fractions were analyzed by Western Blot and probed for ER (Hmg1-GFP) and nuclear markers (Rpa49p). She2p-N36S,R63K is present only in the nuclear pellet, not in the ER microsomal fraction. (B) Analysis of subcellular localization by indirect immunofluorescence: Indirect immunofluorescence using an a-She2p antibody showed that She2p-N36S,R63K accumulates in the nucleus of yeast cells in contrast to wild type She2p which in turn is enriched at bud tips.

2.7. *In vitro* assay: recombinant She2p co-migrates with ER on a velocity sucrose gradient

As we have seen above, the RNA binding mutant She2p-N36S,R63K cannot be used for subcellular fractionation studies if expressed endogenously in yeast. However, the problem of its nuclear accumulation can be circumvented by adding the protein exogenously. Consequently I set up an *in vitro* system with recombinantly expressed She2p.

2.7.1. Purification of recombinant She2p

As a first premise for the *in vitro* assay it was necessary to generate pure recombinant protein from *E. coli*. For this a GST-She2p fusion protein containing an internal TEV protease cleavage site (Figure 19 A) was expressed in a BL21(DE3) pRIL strain. The fusion protein

was purified via a Glutathione-affinity column and She2 protein was eluted by a TEV cleavage step. Removal of His_6-tagged TEV protease via Ni-NTA beads yielded highly pure recombinant She2 protein (Figure 19 B).

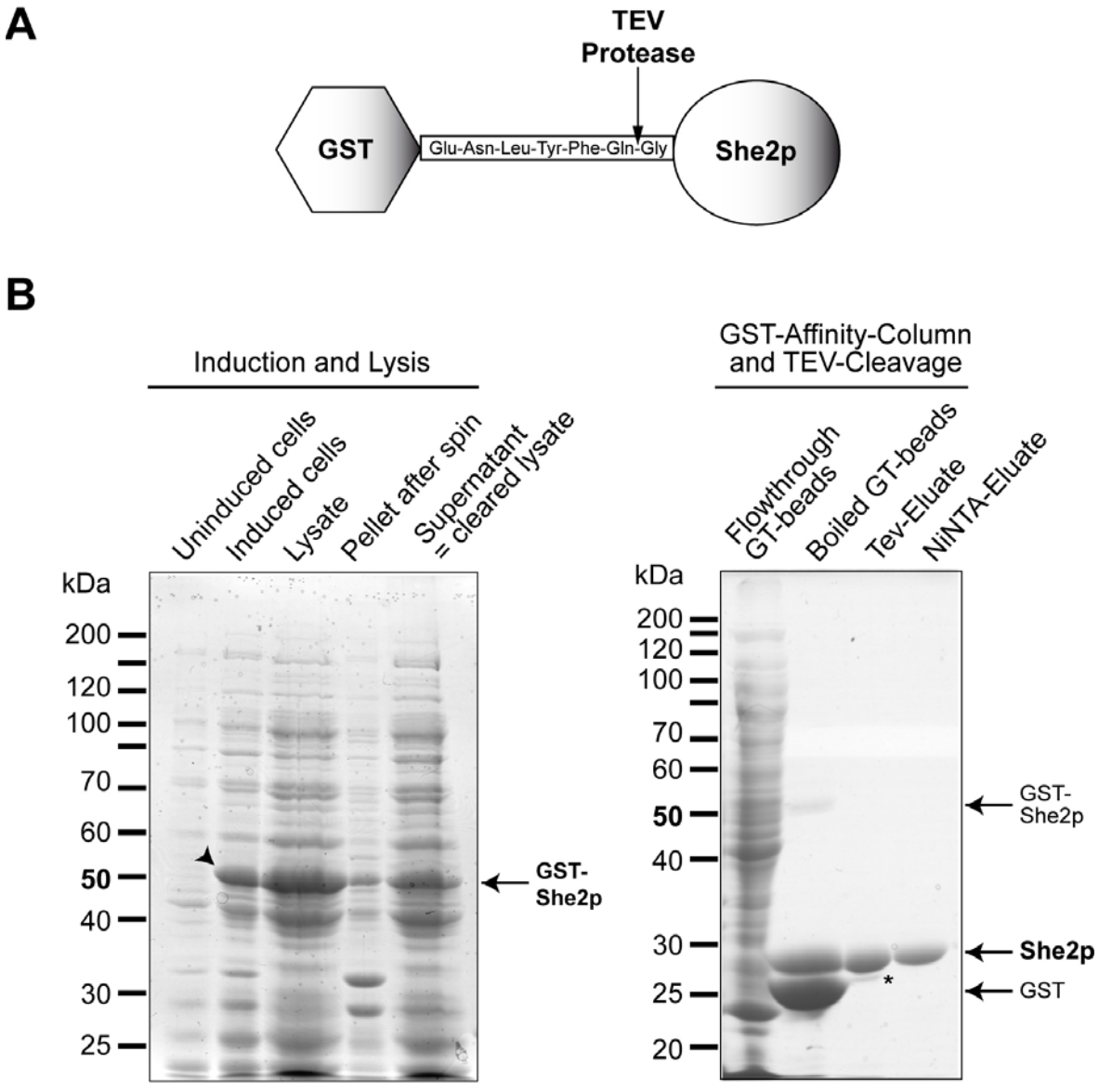
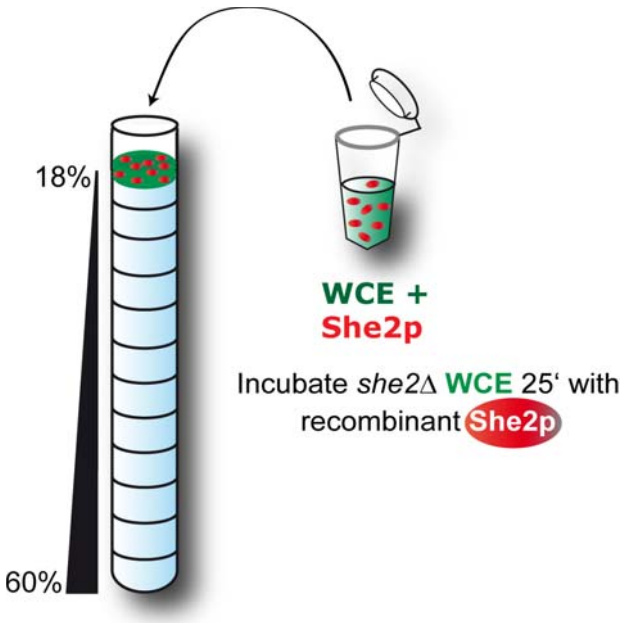


Figure 19: Expression and purification of recombinant She2p from *E. coli*.
(A) Expression vector pRJ20 (pGEX-TEV-*SHE2*) encodes a GST-She2p fusion protein containing an internal cleavage site for TEV-protease.
(B) Expression of GST-She2p (53 kDa) was induced in the BL21(DE3) pRIL strain RJB441 with 1 mM IPTG. The pre-cleared cell lysate was applied to glutathione (GT) beads. She2p was eluted by TEV cleavage (TEV-protease is marked by an asterisk). Pure She2p (28 kDa) was obtained after removal of His_6-tagged TEV with Ni-NTA resin.

2.7.2. Recombinant She2p behaves like endogenous She2p

Assuming that She2p binds to ER membranes it should equally do so in an *in vitro* situation, i.e. if it is exogenously added to a crude yeast extract. Consequently I first tested whether recombinant She2p pre-incubated with a *she2Δ* cell extract behaves like endogenous She2p in wild type extract (Section 2.4.1.) if separated on an 18% to 60% velocity sucrose gradient. Figure 20 A depicts a schematic overview of the *in vitro* gradient system. Similar to the situation with wild type cell extract, recombinant She2p can be detected in the high density fractions at the bottom of the gradients, in other words, it co-migrates with the ER marker just as well as endogenous She2p (Figure 20 B). This behaviour is not due to aggregation of recombinant protein since I did not observe She2p in high density fractions upon pre-incubation with buffer instead of cell extract (data not shown).

A



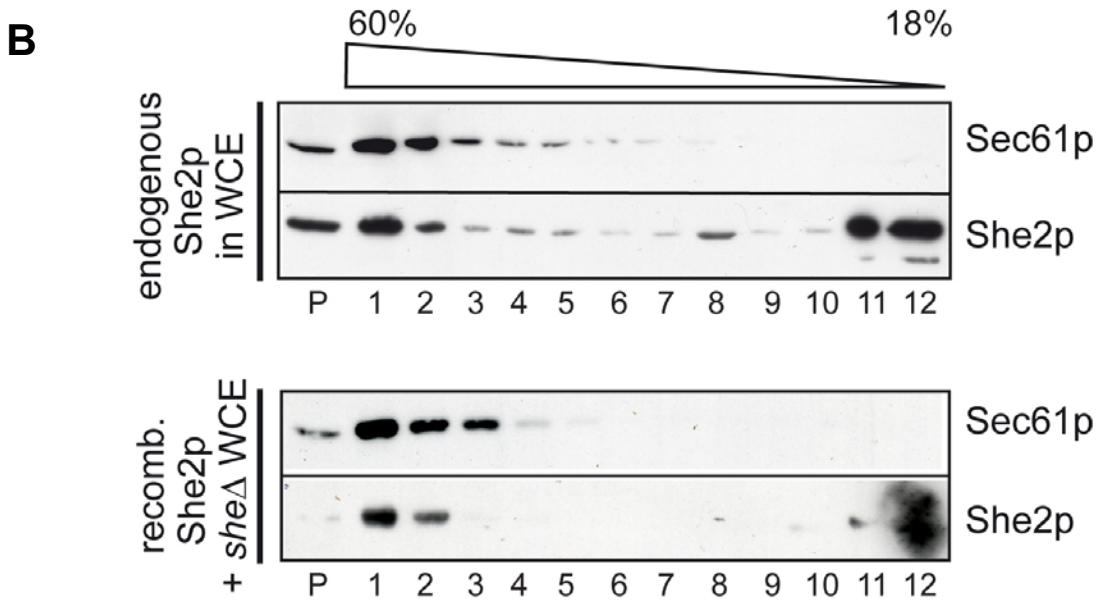


Figure 20: She2p co-migrates with ER *in vitro* just as well as *in vivo.*
(A) Schematic overview of the *in vitro* assay: Recombinant She2p is incubated for 25 min with WCE from a *she2Δ* (RJY2370) strain. Subsequent to pre-incubation the mix is spread on an 18-60% velocity sucrose gradient. After centrifugation the gradient is processed for Western Blotting as described earlier. (B) Comparison of endogenous versus recombinant She2p. Two velocity gradients were performed in parallel. One with wild type cell extract containing endogenous She2p, the other with a pre-incubation mix of recombinant She2p and *she2Δ* cell extract. She2p behaves the same in both situations.

Furthermore I performed the same *in vitro* assay with cell extract and recombinant GST instead of She2p. In strong contrast to She2p, GST remained exclusively in the light fractions on top of the gradient (Figure 21). GST represents an appropriate control for She2p since it not only has about the same molecular weight as She2p (GST: 26 kDa; She2p: 28 kDa) but also is known to form dimers (Ji *et al.*, 1992) as described for She2p (Niessing *et al.*, 2004).

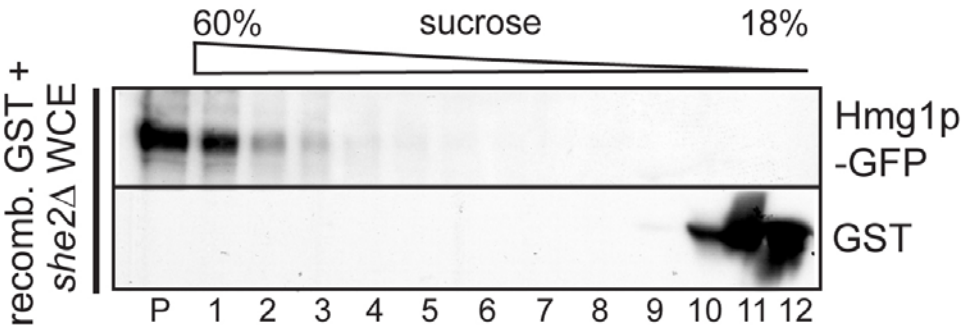


Figure 21: Recombinant GST does not co-migrate with ER in the *in vitro* assay
The *in vitro* assay on 18-60% linear sucrose gradients was performed with recombinant GST instead of recombinant She2p added to a *she2Δ* (RJY2370) cell extract.

Taken together, these observations demonstrate that the *in vitro* assay is an appropriate tool to study the effects of mutations in She2p. In addition, since even the recombinant protein behaves in the same way as endogenous She2p, these *in vitro* data reinforce the whole concept of an association between She2p and ER membranes.

2.8. The RNA binding mutant She2p-N36S,R63K is not impaired in ER association

With the *in vitro* system in hands I then intended to re-examine the issue of She2p's RNA binding activity in ER association. As mentioned earlier it is not possible to investigate this *in vivo* because the mutant She2p-N36S,R63K accumulates in the nucleus. Recombinant She2p-N36S,R63K was purified via GST affinity and TEV-cleavage steps as described above (Section 2.7.1.). Pure recombinant wild type- and N36S,R63K- She2 protein (Figure 22, left panel) was incubated in the same amounts with *she2Δ* cell extracts and their distribution compared to ER markers was analysed on 18 – 60 % velocity gradients.

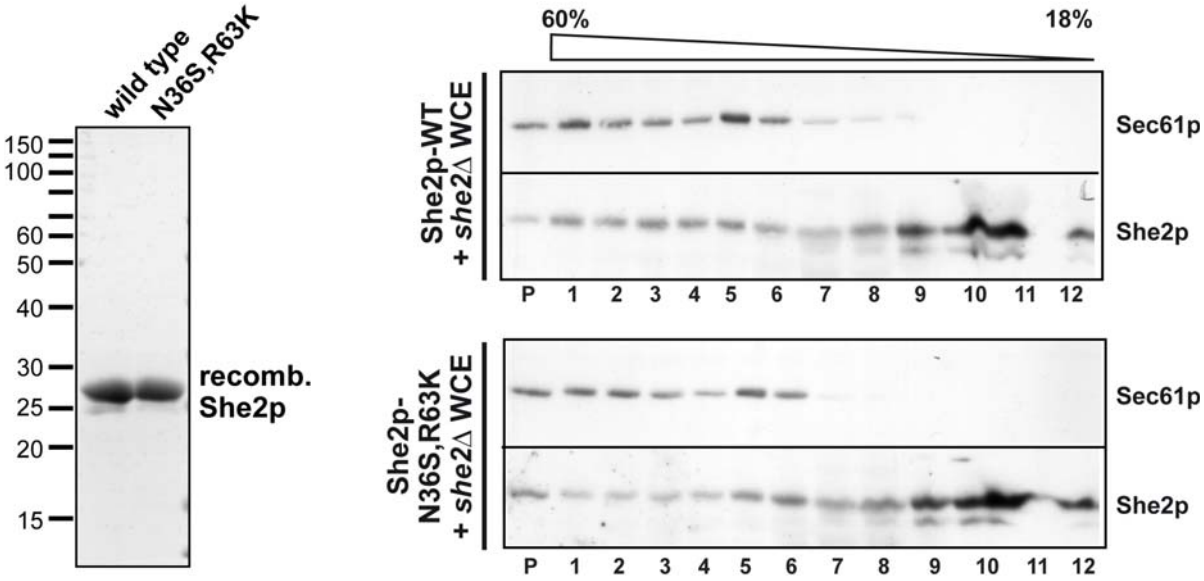


Figure 22: The RNA binding mutant She2p-N36S,R63K co-fractionates with ER like WT-She2p. Both She2p-WT (in RJB441) and She2p-N36S,R63K (in RJB448) were expressed and purified from *E. coli* as described earlier. Both proteins were added separately to *she2Δ* (RJY2370) cell extracts and *in vitro* assays were performed as described above.

In accordance with the results obtained from RNase treated samples (Section 2.6.1.) the RNA binding mutant co-migrates with the ER marker Sec61p to the same extent than wild type She2p (Figure 22, right panel). Altogether this strongly supports the notion that She2p – ER interaction is independent of RNA.

2.9. None of the best characterized mutations in She2p shows an effect on ER-association

As we have seen above, disruption of She2p's RNA-binding feature has no impact on its ER-binding capacity. As a consequence I wanted to explore whether this association is impaired in other She2p mutants already known to have a defect in mRNA localization.

The first amongst the group of She2p mutants tested was She2p-S120Y. This mutant was described to have a defect in its dimerization capability (Niessing *et al.*, 2004). Moreover it displays a loss of binding activity to the *ASH1* E3 element in filter binding assays and exhibits a failure to correctly localize *ASH1* mRNA *in vivo* (Niessing *et al.*, 2004) indicating that dimer formation is necessary for She2p's ability to bind RNA.

The second mutation investigated was She2p-L130S. This amino acid exchange was identified by a genetic screen as a mutant defective for *ASH1* mRNA localization and it does not bind to the *ASH1* E3 element as demonstrated by UV crosslinking experiments (Gonsalvez et al., 2003). In addition to this, it seems to be affected in its capability to bind the adaptor protein She3p (Gonsalvez et al., 2003).

Finally, I tested another mutant identified by Marisa Mueller (Niessing Lab). This She2p mutant bears a deletion of the small α-helix protruding at right angles from both sides at the middle of the She2 dimer (She2p-Δ amino acids 174-183). In the following, the latter will be called She2p-ΔHelix. Using filter binding assays M. Mueller had demonstrated that this mutant is significantly impaired in *ASH1* E3 element binding (M. Mueller, personal communication). In addition, upon investigating She2p-ΔHelix by fluorescent *in situ* hybridization I found, that apparently the deletion of the protruding helices also suppresses She2p's ability to correctly localize *ASH1* mRNA (data not shown).

Subsequently, in cooperation with a bachelor student, Milijana Mirkovich-Hoesle, all three She2p mutants were tested for their ability to co-migrate with ER markers, as described earlier, in an 18-60% velocity sucrose gradient. The assay was either performed *in vivo* with cell extracts obtained from a *she2Δ* strain transformed with a plasmid encoding the corresponding She2p mutant or it was carried out as an *in vitro* assay with recombinant She2p added exogenously to a *she2Δ* cell extract (Section 2.4.1. and 2.7.2.). After centrifugation, gradient fractions were processed and analyzed by Western Blotting as mentioned above.

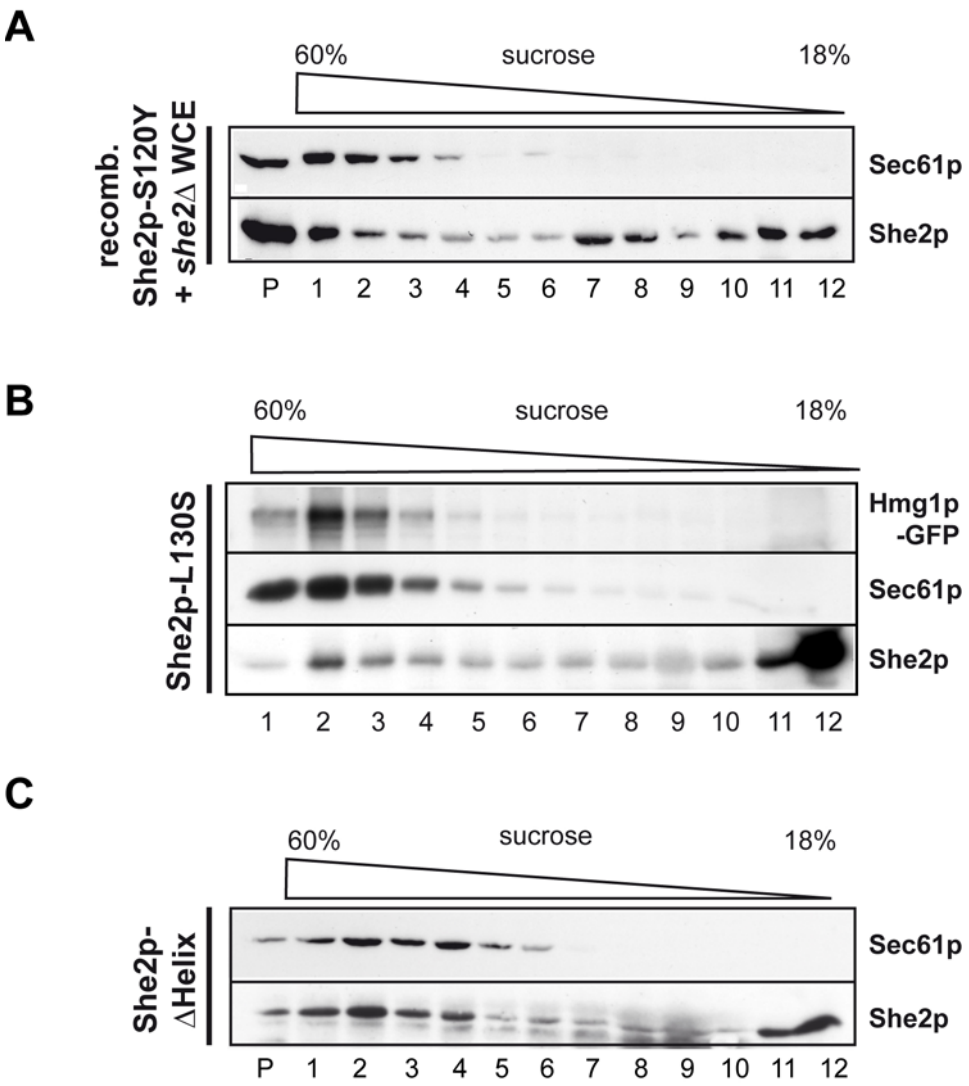


Figure 23: Already characterized surface residues of She2p are not responsible for its co-migration with ER membranes.
She2p mutants were either expressed and purified from *E. coli* and used in the *in vitro* gradient system as described earlier (A). For She2p-120Y plasmid pRJ1386 was transformed into BL21-pRIL. Alternatively the mutants were expressed *in vivo* from a plasmid transformed in a *she2Δ* yeast strain and *in vivo* gradients were performed as described in section 4.1. (B, C). For this, She2p-L130S (pRJ1605) and She2p-ΔHelix (pRJ1482) were both transformed into the *she2Δ* strain RJY2370.

Intriguingly, neither the She2p-L120S, nor She2p-L130Y nor She2p-ΔHelix mutant tested was affected to any extent in co-segregation with the ER marker (Figure 23 A, B, C respectively). This indicates that a yet unknown feature of She2p must be responsible for its interaction with ER membranes.

2.10. Search for a protein factor acting as mediator for the She2p – ER interaction

As we have seen in previous sections, She2p's RNA binding activity is not a prerequisite for its presence on ER membranes. As a consequence one of the most plausible explanations could be that She2p is tethered to ER membranes via a so far unidentified protein factor.
The most obvious and thus first candidates to test are the two other components of the core locasome: She3p and Myo4p. Both of them are known to be essential for inheritance of cortical ER and in addition they were found to co-localize with ER markers in subcellular fractionation assays (Estrada et al., 2003). I therefore tested whether co-segregation of She2p and ER is dependent on the motor protein Myo4p or its adaptor She3p.

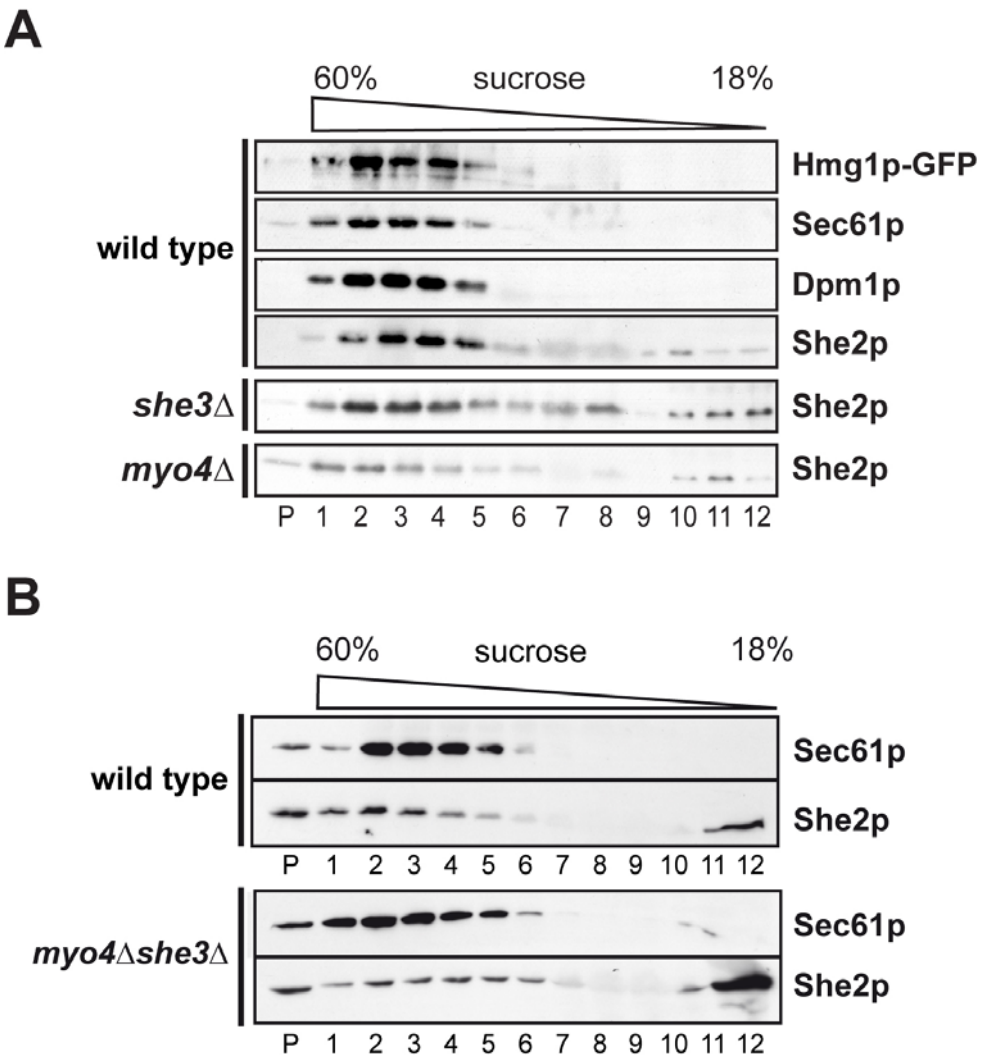


Figure 24: Myo4p and She3p are not necessary for She2p's binding to ER
(A) She2p co-segregates with ER to the dense fractions of the gradient not only in a *myo4Δ* (RJY2323) but also in a *she3Δ* (RJY2475) mutant. (B) Also in the double deletion strain *myo4Δ she3Δ* (RJY3307) She2p co-fractionates with the ER marker Sec61p to the bottom of the gradient.

Deletion of *MYO4* (RJY2323) or *SHE3* (RJY2475) however did not affect migration of She2p into the gradient (Figure 24 A). In *myo4Δ, she3Δ*, or wild-type extracts, She2p co-migrates with three ER marker proteins (Hmg1p-GFP, Sec61, and Dpm1p) into high-density fractions (lanes 2–5).

This indicates that She2p in its association to ER is not reliant on an intact Myo4/She3p motor complex.

However if only one of the two proteins – either Myo4p or She3p – is absent, one could still imagine a scenario in which She2p is tethered to the ER by the remaining protein. To rule out this possibility, I created a *MYO4*, *SHE3* double deletion strain (RJY3307) and investigated whether She2p distribution is altered under these circumstances. Interestingly, the RNA binding protein, along with the ER marker protein, could be still detected in the heavy fractions of the gradient (Figure 24 B).

These data strongly suggest that She2p-ER interaction is independent of Myo4p and She3p.

As deletion of the two core-locasome components mentioned above did not show any effect, additional putative linker candidates of the She2p-ER association were tested.

Firstly, I wanted to investigate whether one of the identified yeast two hybrid (Y2H) interaction candidates of She2p (Ito et al., 2001; Uetz et al., 2000) could be the "missing link". For this purpose I created deletion strains of *YBR027C, YOL073C, YJL048C* and *YML088W*. *YBR027C*, and *YOL073C* are still uncharacterized ORFs but the interesting feature about them is that both hypothetical proteins are predicted to contain transmembrane domains (TMDs): 2 TMDs for *YBR027C* and 4 TM-domains for *YOL073C* (SGD database http://www.yeastgenome.org). *YML088W* encodes the protein Ufo1p, a subunit of the SCF E3 ubiquitin ligase complex responsible for ubiquitylation and hence subsequent degradation of phosphorylated HO endonuclease (Kaplun *et al.*, 2003). At the time when I performed the database search, it was also predicted to contain a transmembrane domain and was therefore chosen as a candidate to test. *YJL048C* encodes Ubx6p, a UBX domain containing protein known to be localized at the nuclear periphery (GFP database: yeastgfp.ucsf.edu) (Huh et al., 2003). Since the outer nuclear envelope is continuous with the endoplasmic reticulum (Preuss et al., 1991), it seemed possible that this Y2H candidate could be responsible for the She2p-ER association.

Secondly, besides the She2p Y2H interactors, I wanted to investigate the effect of two other proteins, Scp160 and Asc1p. Scp160 is a 14 KH-domain containing RNA binding and polysome-associated protein. Interestingly, it was shown to be involved in *ASH1* mRNA localization (Darzacq *et al.*, 2003; Irie *et al.*, 2002; Trautwein *et al.*, 2004) and in addition to be localized to the ER (Frey *et al.*, 2001; Wintersberger *et al.*, 1995). Asc1p, a component of the ribosomal 40 S subunit (Gerbasi *et al.*, 2004), was recently published to be necessary for

Scp160-polysome association and thus could possibly act as a binding platform for Scp160 (Baum *et al.*, 2004). In the light of this, I considered Scp160 and Asc1p as promising candidates for mediators of the She2p-ER interaction.

Finally I assessed the possible linker role of another factor, namely Sec3p. This protein represents a component of the exocyst, an octameric protein complex that mediates targeting of post-Golgi vesicles to sites of active exocytosis (TerBush *et al.*, 1996). Furthermore it has not only been demonstrated that Sec3p is necessary for the correct inheritance of cortical ER into the bud (Wiederkehr *et al.*, 2003) but also that *ASH1* mRNA localization is disturbed in *sec3Δ* mutants (Aronov et al., 2007). Considering these data, Sec3p seemed to be a likely candidate acting as a linker between ER and She2p and I created a *sec3Δ* deletion strain to test this assumption.

I used all deletion strains mentioned above to prepare cell extracts and performed linear velocity sucrose gradients as described earlier to assess whether co-migration of She2p with ER markers was disrupted in one of those. Intriguingly however, She2p-ER co-segregation was not disturbed in any of the mutants tested (data not shown).

RJY	deleted protein	genotype	She2p-ER co-fractionation
2323	Myo4p	*myo4Δ*	+
2475	She3p	*she3Δ*	+
3307	Myo4p/She3p complex	*myo4Δ she3Δ*	+
3279	hypothetical protein	*YBR027C::natNT2*	+
3280	hypothetical protein	*YOL073C::kanMX6*	+
3281	Ubx6p	*YJL048C::natNT2*	+
3282	Ufo1p	*YML088w::natNT2*	+
2812	Scp160	*scp160Δ::kanMX6*	+
2814	Asc1p	*asc1Δ::kanMX6*	+
3031	Sec3p	*sec3Δ::HIS3*	+

Table 2: Summary of the deletion strains analyzed for She2p-ER co-fractionation via linear velocity sucrose gradients
In none of the knockout strains She2p co-migration with ER marker proteins was disrupted.

Table 2 depicts an overview of the different knockout mutants tested and the fractionation behaviour of She2p in velocity sucrose gradients of the respective deletion strains.

In summary this means that none of the proteins mentioned above (Table 2) serves to tether She2p to ER membranes. In contrast, the RNA binding protein is most likely linked via another, still unidentified protein or it can bind to ER membranes by itself.

2.11. She2 pellets with flotation purified ER membranes in an *in vitro* binding assay

From the aforementioned data obtained *in vivo* by different subcellular fractionation methods (Section 2.4.) and *in vitro* by gradients with recombinant She2p and *she2Δ* whole cell extracts (Section 2.7.) we inferred that She2p interacted with ER membranes. In order to further substantiate this notion, I next set up an *in vitro* binding assay to test whether recombinant She2p could bind to purified ER membranes. She2p-ER co-segregation had already been observed with a crude membrane fraction (Section 2.4.2.). However, an *in vitro* assay with purified ER microsomes is even more specific and could provide even more compelling evidence for She2p's association with ER.

2.11.1. *In vitro* binding assay with flotation purified ER membranes

An overview of the experimental setup for the *in vitro* binding assay applied is depicted in figure 25 A. For this experiment recombinant She2p was obtained as described earlier in section 2.7.1. (Figure 19). The second important step was to perform large scale purifications of ER microsomes from a yeast *she2Δ* strain (RJY2370) (Brodsky *et al.*, 1993; Rothblatt *et al.*, 1986). The latter will be entitled YRMs (yeast rough membranes) in the following text. Subsequent to the isolation of ER microsomes they were further purified via flotation through sucrose cushions (see coomassie gels and western blot depicted in figure 25 B). These successively purified membranes were then incubated with recombinant She2p to allow the binding reaction to occur. After pre-incubation, this mix was loaded on a 1.2 M sucrose cushion. Following ultracentrifugation, the resulting supernatant and the remaining pellet at the bottom of the cushion were analyzed by Western Blotting with regard to the distribution of ER marker and She2p.

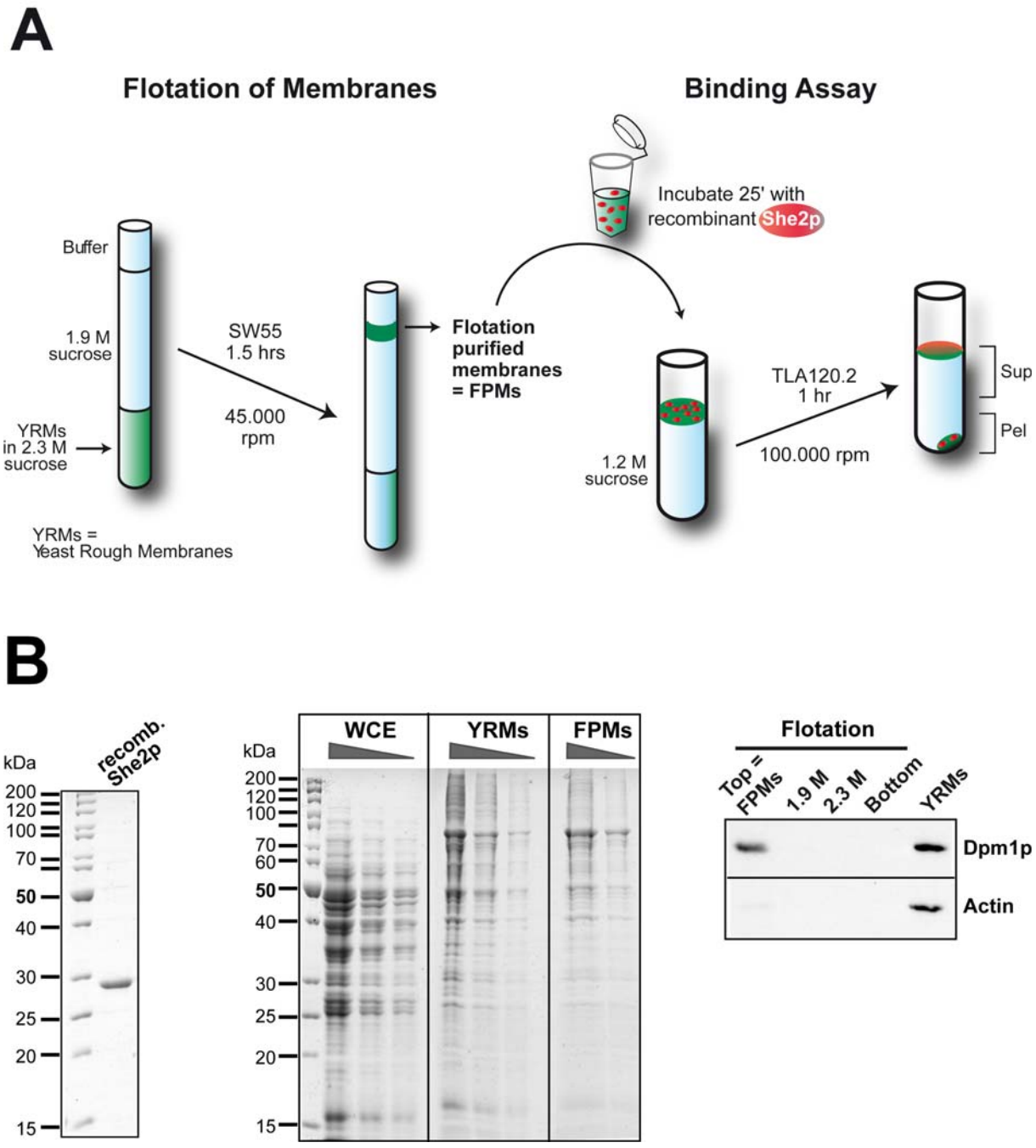


Figure 25: Schematic overview and components of the *in vitro* binding assay:
(A) Illustration of the experimental procedure constituting the *in vitro* binding assay of She2p with flotation purified ER microsomes.
(B) Components of the binding assay: Coomassie Gel of purified She2p (left panel) and comparative coomassie gel (middle panel) of yeast whole cell extract (WCE), yeast rough membranes (YRMs) and flotation purified membranes (FPMs). WCE, YRMs and FPMs originate from a *she2Δ* strain (RJY2370) Right panel: Analytical Western Blot of YRMs compared to different fractions of the flotation procedure (FPM = flotation purified membranes). The Western Blot was probed with antibodies against an ER-marker protein (Dpm1p) and cytosolic marker (Actin).

Most notably, it was observed that recombinant She2p indeed pellets through the sucrose cushion along with ER membranes. Conversely, She2p was not detectable in the pellet

fraction when it was incubated with buffer only instead of flotation purified membranes indicating that the pelleting is not due to aggregation of the recombinant RNA-binding protein (Figure 26 A).

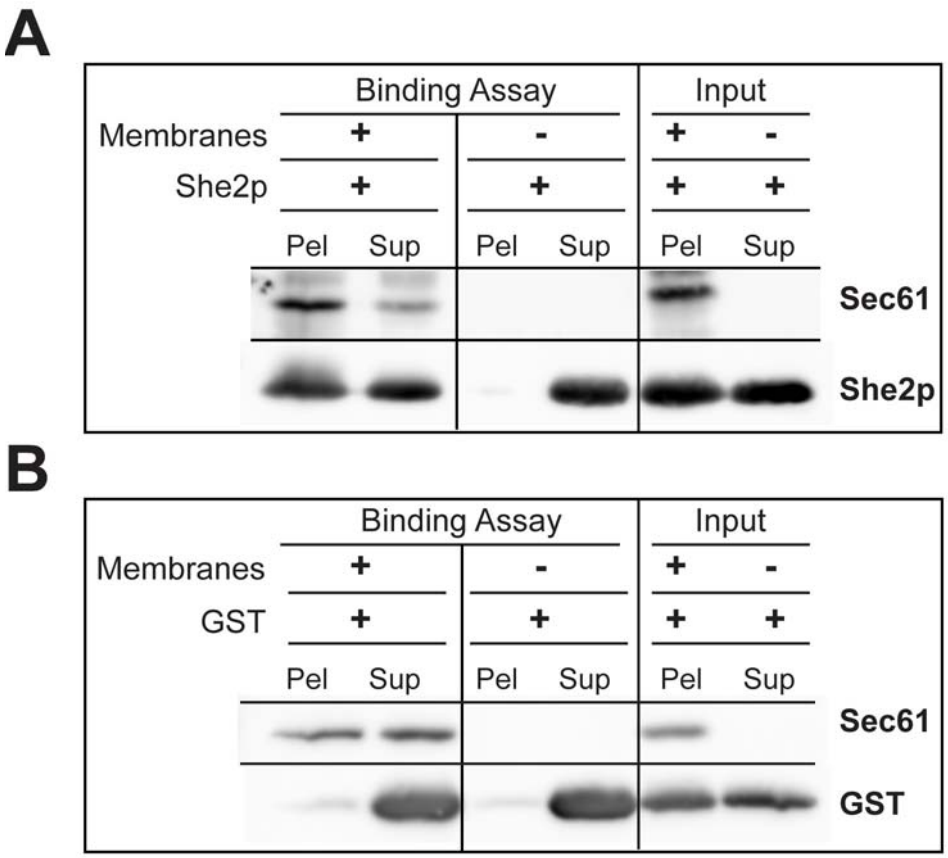


Figure 26: *In vitro* binding assay of recombinant She2p and GST with flotation purified ER membranes
(A) Recombinant She2p was either pre-incubated with flotation purified ER microsomes or buffer before loading on a 1.2 M sucrose cushion. After ultracentrifugation, She2p was detectable in the pellet only in presence of membranes but not with buffer. (B) The same assay was performed with GST instead of recombinant She2p but GST was neither pelleting with buffer nor with membranes.

For the same reasons as mentioned in section 2.7.2. (similar molecular weight, dimer formation like She2p) I again used GST as a control for the specific behaviour of She2p. In strong contrast to She2p, GST does not only stay in the supernatant when pre-incubated with buffer but it also does not pellet through the sucrose cushion in presence of flotation purified membranes (Figure 26 B) indicating that pelleting of She2p with these ER microsomes is specific.
The fact that She2p pellets with purified ER microsomes in an *in vitro* binding assay even stronger substantiates the idea that She2p can bind to ER membranes.

2.11.2. Protease treatment of ER membranes

With the *in vitro* binding assay in our hands, I wanted to assess whether She2p-ER association is mediated by a peripheral or integral membrane protein. To discriminate between these two possibilities, carbonate treatment of microsomes can be used. 0.1 M Na_2CO_3, pH 11.0 is known to strip off peripheral membrane proteins from ER membranes in contrast to integral membrane proteins which are retained (Fujiki *et al.*, 1982). Unfortunately I could not perform this binding assay with Na_2CO_3 treated, flotation purified membranes as the latter did not pellet properly through the sucrose cushion in the last step of the experiment. This was probably due to the fact that ER microsomes after Na_2CO_3 treatment are not only deprived of attached proteins but are also converted from their sealed vesicular shape to flat membrane sheets (Fujiki *et al.*, 1982).

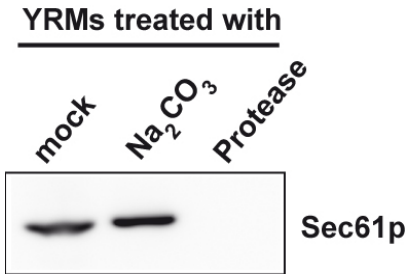


Figure 27: Carbonate and protease treatment of YRMs.
Isolated microsomes were either treated with 0.1 M Na_2CO_3 pH 11.5 to remove peripheral membrane proteins or with a protease mix consisting of Pronase E and Proteinase K to digest both peripheral and cytosolic domains of integral ER membrane proteins. Success of protease treatment was verified by probing for the integral ER marker protein Sec61p.

In the following, protease treatment of isolated ER microsomes was performed. By these means I sought to investigate whether She2p-ER interaction would be abolished upon removal off any ER resident protein component. To achieve this, I isolated YRMs and either mock treated them or digested them with a combination of Pronase E and Proteinase K. The effect of protease treatment was verified by probing for the integral membrane protein Sec61p (Figure 27). The anti-Sec61p antibody was originally directed against a peptide at the extreme C-terminus of the protein (Matthias Seedorf, personal communication) and the C-terminus itself is protruding to the cytosolic side of the ER membrane (Osborne *et al.*, 2005). Following several washing steps and inactivation of the protease cocktail, the *in vitro* binding assay with recombinant She2p was performed as described above. The resulting fractions were again analyzed by Western Blotting.

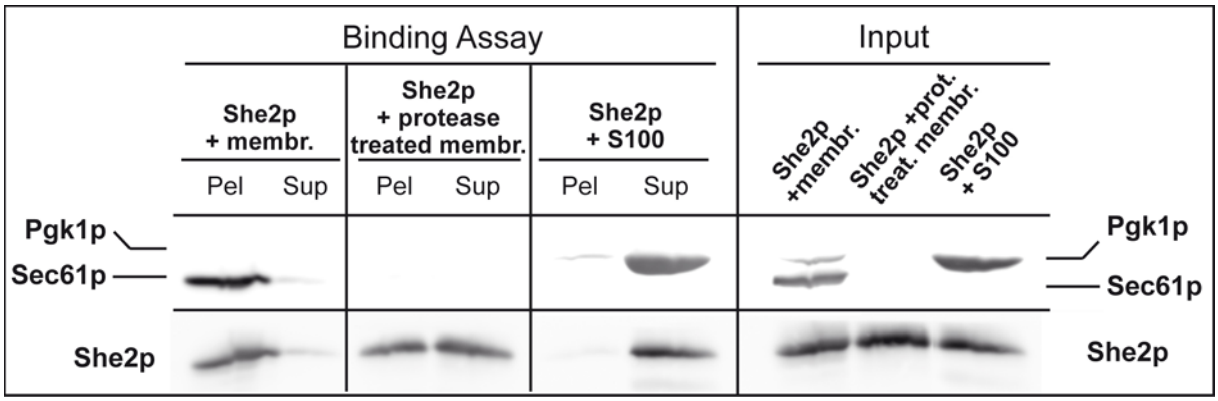


Figure 28: *In vitro* binding assay with protease treated membranes and a membrane-free cytosolic fraction (S100).
She2p was either incubated with mock treated membranes or membranes treated with a combination of Pronase E and Proteinase K. In addition She2p was mixed with a post 100,000 x g supernatant (S100) which is depleted of membranous components. After the binding reaction, all three samples were loaded on a 1.2 M sucrose cushion and the assay was performed as described above.

Compared to the situation with mock treated membranes (Figure 28, first lane) I observed a reduction of the pelleting of She2p with protease treated microsomes (Figure 28, second lane). Nevertheless, the amount of pelleted She2p was decreased but not completely abolished. Due to the lack of antibodies, the efficiency of protease digest could not be checked for numerous ER membrane proteins. The digest could have been partially incomplete as different resident ER proteins might display varying resistance against protease treatment. Therefore I could not judge at this point whether the data indeed indicate a decreased binding of She2p to ER membranes due to partial digest of a putative ER linker protein or whether the pelleting behaviour of She2p is disturbed by some other cause. One such reason could be the loss of integrity of protease treated membranes. A rather harsh experimental condition which constitutes the only difference to mock treated membranes is the protease heat inactivation by a 15 – 20 min 80°C incubation step. Membrane integrity is hard to assess since protease treated membranes cannot be detected any more by western blotting. But if integrity is indeed disturbed, less efficient pelleting of the membranes themselves as seen after carbonate treatment could be the consequence. This would finally also result in a reduced precipitation of She2p.
In summary, protease treatment of ER microsomes for the *in vitro* assay unfortunately did not lead to a clear-cut result.
Nevertheless I used the *in vitro* binding assay to assess another issue. Seeing that She2p pellets through the sucrose cushion together with ER membranes, the question arose whether components of a membrane-free fraction of yeast extracts could provoke the same effect for She2p via unspecific aggregation. To investigate this, I prepared a post 100,000 x g supernatant (S100) of a yeast whole cell extract which is devoid of any membranous

structures. This was then incubated with recombinant She2p and the binding assay was performed as described above. The distribution of S100 was assessed by a cytosolic marker protein, Pgk1p (phosphor-glycerol-kinase 1). As shown in figure 28 (third lane), She2p does not pellet through the sucrose cushion in presence of the post 100,000 x g supernatant which is in strong contrast to its behaviour in company of ER membranes.
This observation again strongly supports the notion that the pelleting of She2p with membranes is specific and that She2p indeed interacts with the ER compartment.

2.12.She2p directly binds to synthetic liposomes

We have seen earlier that the She2p-ER interaction is independent of ongoing translation or mRNA (Section 2.5. and 2.6.). The next rationale was that the association might be mediated via a protein linker. However, targeted deletion after “educated guesses” did not reveal such a factor (Section 2.10.). Since additionally the outcome of the protease treatment mentioned above was rather inconclusive, it remained elusive how the RNA binding protein might be linked to the membrane compartment.
Therefore I sought to move one step further and tried to investigate whether She2p itself can directly interact with lipid membranes.

2.12.1. She2p floats along with ER-like protein-free liposomes

For this approach I prepared synthetic, protein-free liposomes with a composition similar to ER membranes (ergosterol 16%, phosphatidylcholine PC 40%, phosphatidylethanolamine PE 24%, phosphatidylserine PS 10%, phosphatidylinositol PI 10%) (Schneiter *et al.*, 1999; Tuller *et al.*, 1999; Zinser *et al.*, 1991).
Shortly, phospholipids and ergosterol – each in organic solvent – were mixed, organic solvent was evaporated, the lipid film resuspended in membrane buffer and synthetic liposomes were created by passage through the membrane of an extruder.
The binding assay applied in the previous sections, however, did not work for such synthetic liposomes as they did not penetrate sucrose cushions of varying density. One possible explanation for this might be the complete absence of protein material in these synthetic lipid vesicles which reduces their density.
Therefore I proceeded to an alternative method suited to test association of proteins to lipid membranes, a flotation assay. As depicted in figure 29 A, it is without any trouble possible to identify the fraction with floated liposomes after ultracentrifugation even if one cannot probe for the synthetic vesicles by detection of a protein marker.

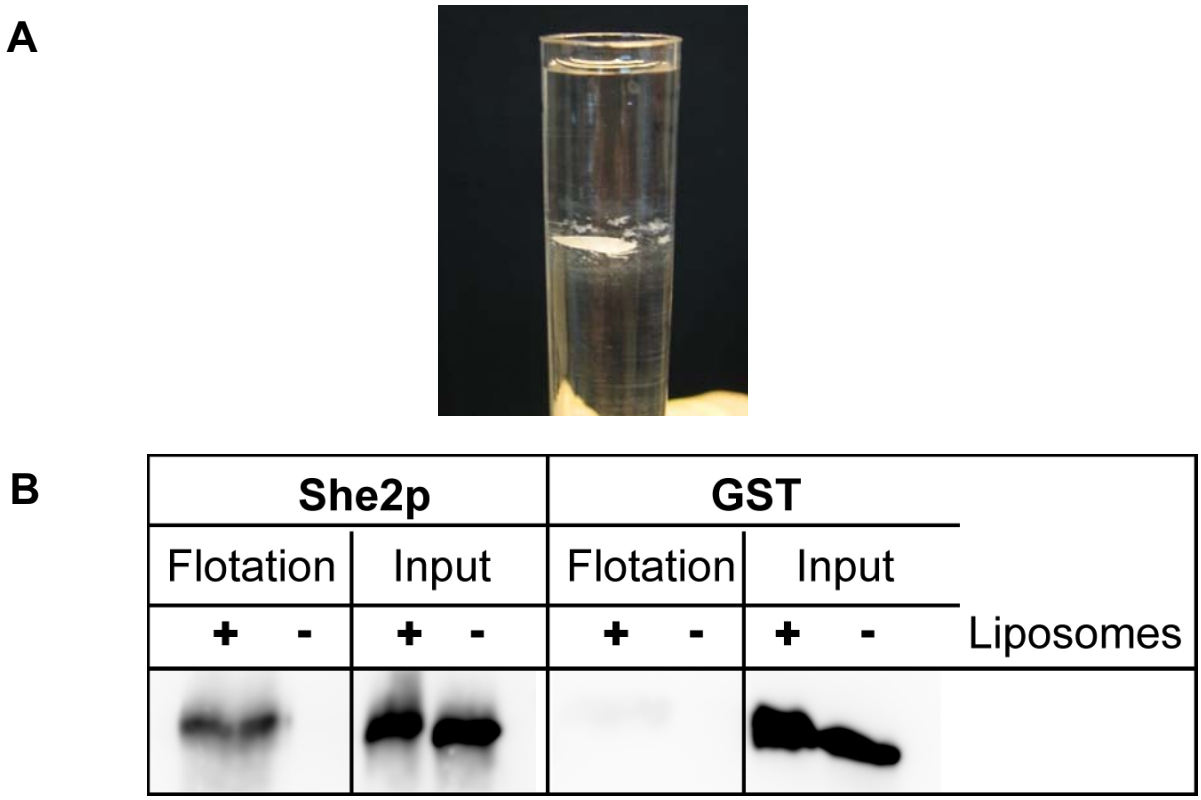


Figure 29: She2p interacts with ER-like, synthetic liposomes.
(A) Representative picture of a gradient with floated liposomes. Recombinant protein (She2p or GST) pre-incubated with liposomes or buffer was loaded into the bottom of a flotation gradient consisting of a 70% sucrose solution. This suspension was successively over-layered with three other cushions containing 50%, 40% and 0% sucrose. Liposomes visibly accumulate at the interphase between 40% and 0% sucrose.
(B) She2p but not GST floats to the top of the gradient together with the ER-like liposomes.

I incubated synthetic liposomes with either heterologously expressed She2p, GST or binding buffer alone before performing the flotation assay. Intriguingly, after harvesting the fractions with floated liposomes and analyzing them by western blotting, I found that She2p had floated along with the synthetic liposomes to the top of the gradient (Figure 29 B, left panel). This was in contrast to the GST control which was completely absent from the liposomal fraction (Figure 29 B right panel).
This result indicates that the RNA binding protein She2p can directly interact with lipid membranes.

2.12.2. She2p behaves like a bona fide peripheral membrane protein

After the aforementioned observation that She2p can directly interact with liposomes, I wanted to investigate how this association occurs. In general, the basis of protein – membrane interactions can be roughly categorized into hydrophobic or electrostatic

interaction forces (W. Cho *et al.*, 2005). Upon high salt treatment, electrostatic interactions should be disrupted whereas hydrophobic interactions should be even enhanced. Therefore I performed the pre-incubation of She2p and membranes under high salt conditions (1.0 M KCl) and also prepared flotation gradient solutions containing 1.0 M KCl. Under high salt conditions She2p was absent from the floated liposome fraction (Figure 30, flotation panel, lane 3) but it was also virtually gone in the input sample (Figure 30, input panel, lane 3). Thus, I unfortunately could not draw any conclusion regarding the mode of membrane interaction since She2p seems to be instable under this condition.

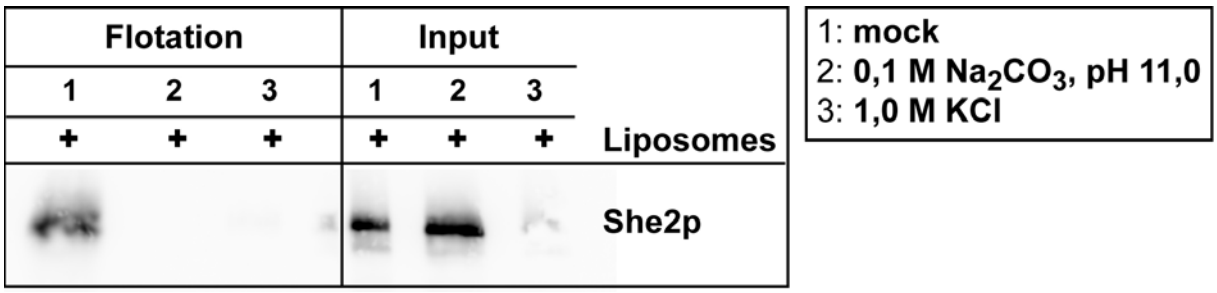


Figure 30: She2p-liposome interaction can be disrupted by carbonate treatment.
She2p is unstable under high salt conditions (lane 3, Input panel). However, She2p can be stripped off from liposomes by treatment with carbonate (lane 2, Flotation panel)

Simultaneously I wanted to investigate whether She2p behaves like a peripheral membrane protein. As already mentioned in section 2.11.2., peripheral membrane proteins can be removed from the membranes they bind to by treatment with 0.1 M Na_2CO_3, pH 11.0 (Fujiki *et al.*, 1982). When I performed the She2p-liposome flotation assay after pre-incubation in carbonate and with gradient solutions containing 0.1 M Na_2CO_3, pH 11.0, She2p was no longer detectable in the floated liposomal fraction though it was still present in the input samples (Figure 30, lane 2 flotation and input panels).
Hence, She2p indeed behaves like a genuine peripheral membrane protein.

2.12.3. Phosphatidylserine and phosphatidylinositol are not essential for She2p-liposome interaction

In case of electrostatic interactions between proteins and membranes, phospholipids like phosphatidylserine and phosphoinositides with acidic head groups are known to be the major binding targets (Lemmon, 2008). Our synthetic ER-like liposomes indeed contained phosphatidylserine (PS) but only contained phosphatidylinositol (PI), the unphosphorylated precursor of phosphoinositides. This phospholipid however still exhibits an overall negative

charge in contrast to phospholipids like phosphatidylcholine or –ethanolamine. Therefore I synthesized a second batch of liposomes lacking not only PS but also PI.

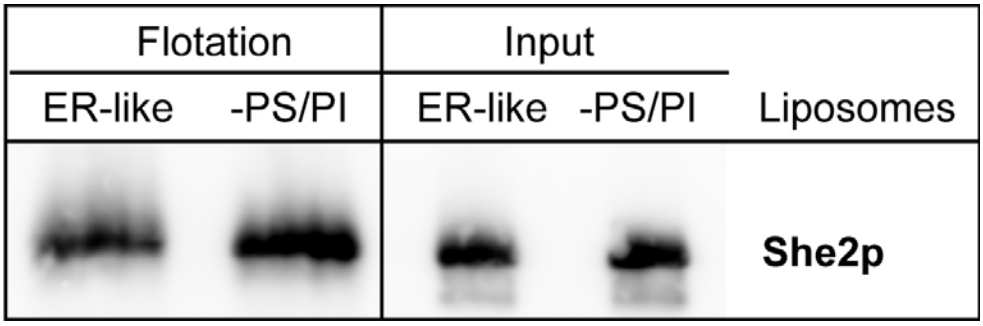


Figure 31: She2p interacts with liposomes deprived of phosphatidylserine and phosphatidylinositol.
She2p was floated with either ER-like liposomes or with similar vesicles lacking the two phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI).

With the newly prepared synthetic membranes I performed flotation assays in presence of She2p as described above. However I could not detect any difference in flotation behaviour of She2p between liposomes lacking PS- and PI and vesicles with an ER-like composition (Figure 31).
Thus, neither PS nor PI seems to be necessary for She2p's membrane interaction activity.

2.12.4. She2p interacts with liposomes in the presence of its RNA ligand

As already described in section 1.3.2.1.3., She2p harbours a helical hairpin that contains a cluster of basic amino acid residues (Figure 32). This hairpin is essential for the RNA-binding activity of the protein.
However, it seems possible that exactly the same positively charged basic residues could also be involved in the interaction with negatively charged head groups of membrane phospholipids. In light of this option I wanted to investigate whether She2p can still bind to phospholipid membranes in presence of its RNA ligand and whether the RNA is still bound to the She2p when the protein associates with synthetic membranes.

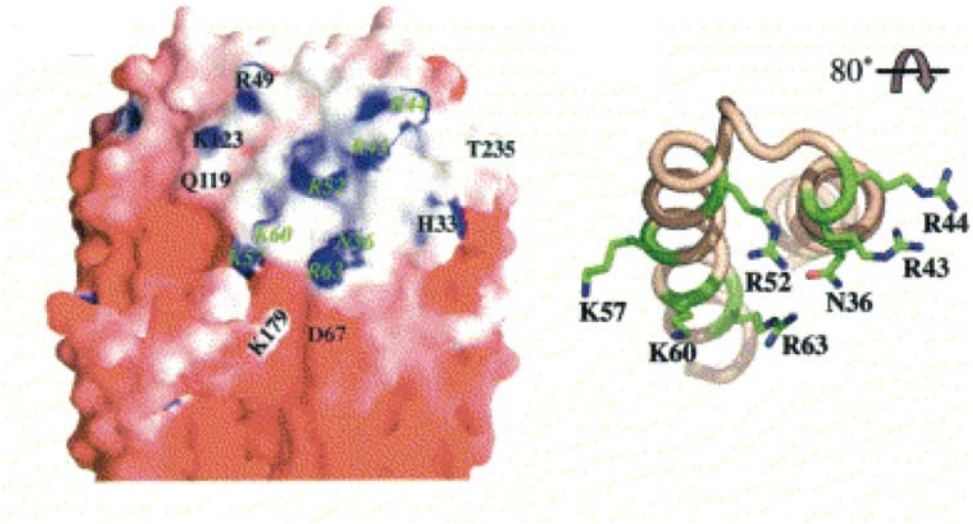


Figure 32: She2p contains a basic helical hairpin involved in *ASH1* mRNA binding.
Pictures taken from (Niessing *et al.*, 2004). Left picture: GRASP surface representation of the chemical properties of the solvent-accessible surface of She2p. The surface electrostatic potential is colour coded red and blue, representing electrostatic potentials between < −14 to > +14 k_BT, where k_B is the Boltzmann constant and T is the temperature. Right picture: Schematic of the basic helical hairpin of She2p. In both pictures residues involved in mRNA binding are depicted in green.

To test this I pre-incubated recombinant She2p with a 10 fold and 20 fold molar excess of the *in vitro* transcribed *ASH1* E3 element or with binding buffer only (mock). In the following, synthetic liposomes were added and flotation was performed as described earlier. The floated liposomal fractions were analyzed by SDS-PAGE and Western Blotting. From the remaining samples, RNA was isolated by Phenol/Chloroform extraction and analyzed on TBE / Ethidium Bromide gels. Interestingly, the RNA binding protein in presence of the RNA substrate floated along with liposomes as much as in the mock treated reactions without the *ASH1* E3 element (Figure 33 A, left panel).

Part of the *ASH1* E3 element had floated together with She2p and liposomes (Figure 33 B, left panel, fourth lane). The RNA fragment was a little bit smaller in size than the fragment in the input samples (Figure 33 B, right panel) which is most likely due to some degradation which occurred during the overnight flotation centrifugation.

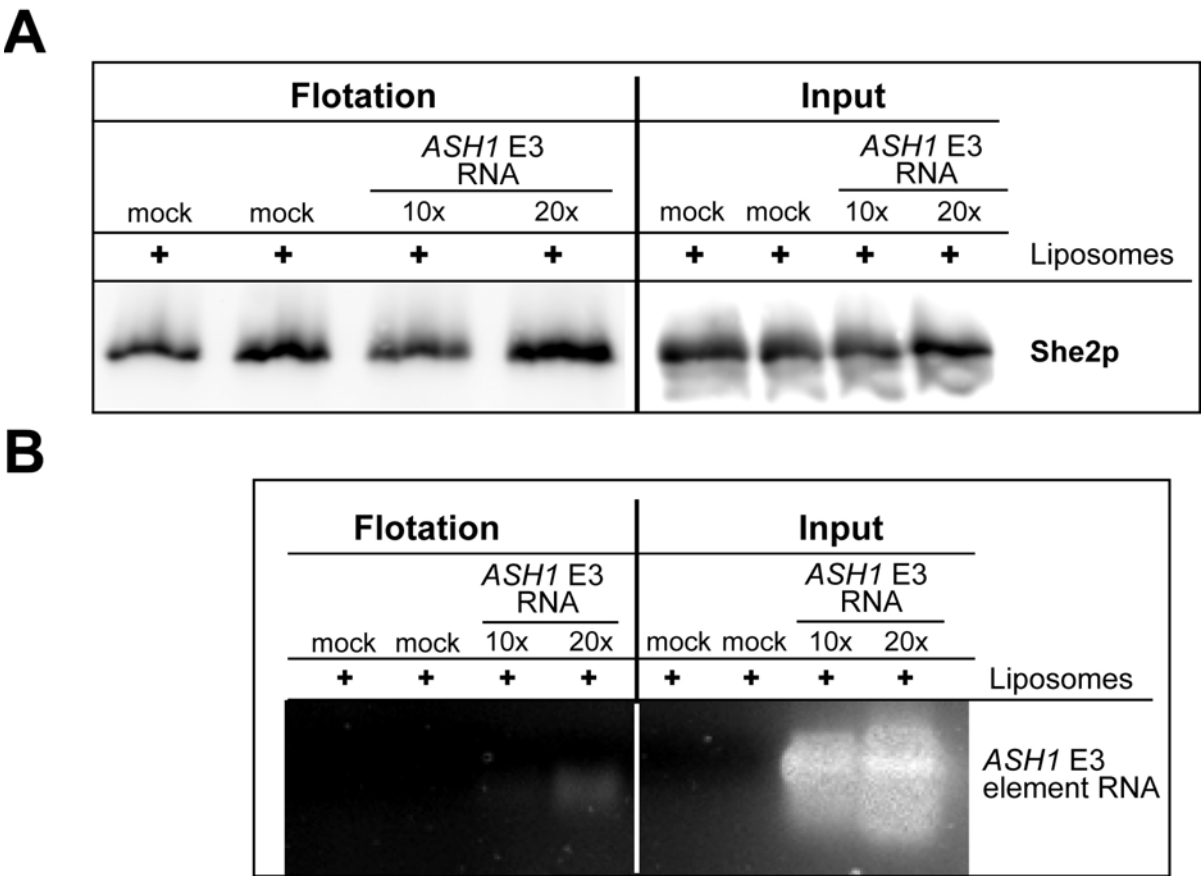


Figure 33: She2p binds to lipid membranes in presence of its RNA ligand and he *ASH1* E3 element floats along with She2p and liposomes.
(A) Western Blot of flotation assay of liposomes and She2p. Recombinant She2p was either pre-incubated with a 10x or 20x molar excess of *in vitro* transcribed *ASH1* E3 element or with buffer (mock). After pre-incubation, liposomes were added and flotation was performed as described earlier. (B) TBE-Ethidium bromide gel of RNA extracted from each floated liposomal fraction and from each input sample.

In summary, this data strongly support the notion that She2p can indeed not only bind to membranes but also that it can simultaneously associate with both its RNA substrate and lipid membranes.

She2p is already known to be an unconventional RNA binding protein not containing any so far characterized RNA interaction domain (Niessing *et al.*, 2004). From the data presented above it seems highly likely that it is also an unconventional lipid binding protein, though the nature of its lipid binding activity still remains to be elucidated.

3. Discussion

3.1. Functional linkage between mRNA localization and cortical ER inheritance

mRNA localization and cortical ER inheritance are both cell trafficking routes that occur during the process of bud-formation and cell division in *S. cerevisiae.* After the discovery that both transport courses employ the same locomotion machinery, the Myo4/She3p complex (Estrada et al., 2003), I sought to explore whether the two pathways were functionally linked. Indeed, I found that mutants impaired in ER inheritance like *myo4Δ*, *aux1Δ* and *srp101-47^{ts}* simultaneously displayed defects in localization of *ASH1* particles (Figure 10). The fact that both the defect in ER inheritance and in mRNA localization are not as prominent in the *srp101-47^{ts}* strain as in the other two mutants is most likely due to the fact that this mutant, in contrast to *myo4Δ* and *aux1Δ* is not a deletion mutant, but a temperature sensitive allele of the wt *SRP101* gene. A defect in the two transport routes was expected for the *myo4Δ* mutant as Myo4p is the motor driving both processes. However, the *aux1Δ* and the *srp101-47^{ts}* mutant so far were only known to have a deficiency in ER segregation (Y. Du *et al.*, 2001; Prinz *et al.*, 2000). Thus, the observation that in addition to ER inheritance defects those mutants are also impaired in mRNA localization provides the first evidence that mRNA localization and cortical ER inheritance are functionally linked and take place in a coordinated manner.

In contrast to our results, a previous study states that mRNA trafficking and ER tubule movement are independent (Estrada et al., 2003). This conclusion was mainly reached by demonstrating that ER segregation is independent of She2p and that in *aux1Δ* mutant cells the localized *IST2* mRNA can still be detected in the bud by *in situ* hybridization. At the moment I cannot thoroughly explain this discrepancy but one reason might be the use of different methodologies. Whereas Estrada *et al.* utilized *in situ* hybridization in fixed cells, I employed live cell imaging not only for ER structures but also for detection of *ASH1* mRNPs by using the MS2-System (figure 10).

The functional correlation between ER segregation and mRNA localization seen in ER inheritance mutants however is consistent with an earlier observation that by *in vivo* co-imaging, *ASH1* mRNPs co-localize and even stay associated with ER tubules (see section 1.5. and A. Jaedicke in (Schmid *et al.*, 2006)). These tubules move from the mother cell to the bud and are required for the segregation of cortical ER during early stages of the cell cycle (Y. Du *et al.*, 2004). Interestingly, another study using microarrays to distinguish pools of membrane associated and cytoplasmic gene products identified *ASH1* mRNA as an ER-

associated mRNA (Diehn *et al.*, 2000). This result is quite striking as *ASH1* mRNA does not encode a membrane or secreted protein but a nuclear transcription repressor and therefore cannot be recruited to the ER via the SRP-pathway (Figure 6). Therefore, this observation supports the aforementioned live cell co-imaging data and the hypothesis of an mRNA-ER co-transport.

In order to explain the discrepancy between Estrada *et al.* and my data, I employed a live cell imaging system using *IST2* as tagged mRNA to be imaged. However, when expressed from its own promoter and even when expressed from a *GAL1* promoter the bud localization efficiency for *IST2* mRNPs was not the same than for *ASH1* mRNPs. For *ASH1* in wt cells, localization to the bud occurs in about 92% of the cells. With the *IST2* mRNA only a value of 54% was reached in wt cells. As mRNA targeting was already inefficient in wt cells, it was not feasible to use this mRNA for statistical analysis in ER inheritance mutants. However, there is one possible explanation for the low bud-localization rate of *IST2* mRNA in live cell imaging. From recent publications it is known that even if targeting of its message is disrupted, there is still some Ist2 protein present in the daughter cell plasma membrane. To some extent Ist2p is transported to the bud independently of mRNA localization. A complex peptide-sorting signal is required for this transport pathway that also works independently of the classical secretory pathway (Franz *et al.*, 2007; Juschke *et al.*, 2004). The fact that for Ist2p, localization is not only mediated by its mRNA but that signals are also encoded on the protein level might be the reason why the transcript is targeted less efficiently to the bud than the *ASH1* message, where localization signals are exclusively harboured within the mRNA.

Recently, the finding that mRNA localization and cortical ER inheritance to the yeast bud seem to be coordinated processes was strongly supported by observations of Aronov *et al.*. Consistent with my data they found that mutations that affect cortical ER segregation to the bud also affect mRNA localization (Aronov et al., 2007). In this study, Aronov and co-workers identified an additional set of 9 bud localized mRNAs encoding polarity and secretion factors in yeast (POL mRNAs). These mRNAs localize asymmetrically to the tip of the emerging bud dependent on their 3'UTRs and the *SHE* genes and they are bound by the RNA binding protein She2p as demonstrated by IP and RT-PCR (Aronov et al., 2007). Thus, these newly identified mRNAs seem to utilize the same machinery like already described localized yeast messages. The conclusion that mRNA localization and cortical ER inheritance are connected was reached from the finding that POL mRNA localization to the tip of the emerging bud correlated directly with the presence of cortical ER. In analogy to my results Aronov *et al.* found defects in ER inheritance and mRNA localization not only in the *myo4Δ* strain but also in the *srp101-47*^{ts} mutant. Additionally, they tested Sec3p, a non-essential subunit of the exocyst complex which mediates targeting of post-Golgi vesicles to sites of active exocytosis (TerBush *et al.*, 1996). Sec3p is thought to act as a spatial

landmark for secretion but was also shown to be involved in ER segregation (Wiederkehr *et al.*, 2003). Similar to *aux1Δ, myo4Δ* and *srp101-47^{ts}* mutants, the *SEC3* deletion strain was not only impaired in ER inheritance but also displayed a defect in mRNA trafficking. Finally, not only *ASH1* but also the POL mRNAs were shown to associate with ER since they co-fractionated with ER microsomes (Aronov et al., 2007).

Our data in combination with the results from the aforementioned study strongly support the notion of an emerging interplay between the mechanism of mRNA transport and ER inheritance in the yeast bud.

3.2. A connection between mRNAs and membranes: lessons from other organisms

Our model of a coordination of mRNA and ER trafficking is not only supported by the findings of Aronov *et al.*. In addition there are also indications from multicellular eukaryotes for a connection between mRNAs and membranes suggesting that this is not only a yeast specific phenomenon.

In *Ascidians* (sea squirts) for instance, a variety of maternal mRNAs like *macho 1* and *HrPEM* associate with cortical ER in the developing embryo during the establishment of the animal-vegetal axis (Sardet *et al.*, 2003). Cortical ER and bound mRNAs build a structure termed the “cortical ER-mRNA domain” and in concert they are localized the cortex of the ascidien zygote (Prodon *et al.*, 2005; Sardet *et al.*, 2005; Sardet *et al.*, 2007).

In the plant species *Oryza*, two mRNAs encoding distinct seed storage proteins – prolamines and globulin-like glutelins – are directed to different and separated ER sub-domains. Prolamine mRNAs localize to ER-derived prolamine-enriched bodies known as type-I protein bodies, whereas glutelin mRNAs are targeted to the cisternal (or cortical) ER. Both storage proteins are translated from their respective mRNA on the ER surface, translocated into the ER lumen and then further sorted. Early mRNA-ER association in this case helps to separate the two different storage proteins and to avoid aggregation and non-productive interactions.

In neurons, mRNPs including RNA binding proteins involved in mRNA localization such as Staufen and FMRP were found to be associated with ER and it was proposed that a structure consisting of ER bound mRNPs might mediate dendritic transport and local translation of mRNAs (Ohashi et al., 2002). Furthermore, it has been suggested that vesiculated rER/Staufen complexes originating from the perinuclear ER might have a role in mRNA localization in neurons (Kiebler *et al.*, 2000).

In *Drosophila,* there are several examples for an interconnection between mRNA- and membrane-transport. For instance, *trailer hitch*, a factor necessary for proper secretion of the

dorsal-ventral patterning factor Gurken, is part of a large mRNA-protein complex that also includes the translation/mRNA localization factors Me31B and Cup. This ribonucleoprotein complex associates with specific ER subdomains (Wilhelm *et al.*, 2005) and thus creates a link between mRNA localization and membrane transport. Another study describes co-localization of RNP complex components with a specific subtype of ER membranes that are enriched in the reticulon-like 1 protein (Rtnl1). Since these ER membranes are actively translocated into the oocyte as it has been described for localized mRNAs, co-localization indicates a putative role for ER membranes in mRNP localization and transport (Roper, 2007). In the case of *bicoid* mRNA localization to the anterior pole of *Drosophila* eggs, it was recently found that the final steps of this pathway rely on the endosomal sorting complex required for transport (ESCRT-II) (Irion *et al.*, 2007) thus creating an additional link between mRNA and membrane trafficking. *gurken* mRNA in *Drosophila* is localized to the dorsal/anterior corner of the oocyte (see 1.1.2.1). In a recent study it was demonstrated that *gurken* transcript is exclusively deposited on ER structures in this restricted area in order to mediate efficient exocytosis of the encoded protein only at the dorsal/anterior part of the oocyte (Herpers *et al.*, 2004). ER association of a localized mRNA in this case facilitates asymmetric secretion of the respective protein. In contrast to *gurken*, *oskar* mRNA is targeted to the posterior pole of the *Drosophila* oocyte (see 1.1.2.1) and for this it depends in part on microtubule-base transport mechanisms. However, these cytoskeletal tracks do not reach beyond the middle of the oocyte (Cohen, 2005). Transport covering the remaining distance relies on Rab11, a protein involved in vesicle trafficking and organisation of posterior membrane compartments (Dollar *et al.*, 2002). Finally, results from a different study demonstrate that the localization of *oskar* mRNA during *Drosophila* development is also disturbed in several mutants defective in the assembly of ER structures (Ruden et al., 2000) and the authors suggest an involvement of the ER in *oskar* mRNA localization.

In the amphibian *Xenopus laevis*, an interplay between ER transport and mRNA trafficking has been observed during embryonic development. As mentioned in section 1.1.2.2., Vg1 mRNA is localized to the vegetal pole of the embryo via the late pathway by transport through the ER. Interestingly, the VgLE binding protein Vera (VgLE binding and endoplasmic reticulum association) associates with these ER membranes and is thought to mediate this mRNA-ER co-transport (Deshler *et al.*, 1998; Deshler *et al.*, 1997).

Altogether there is a growing number of examples from higher eukaryotes for an interconnection between the transport of mRNAs and membranes supporting our observations made in yeast.

3.3. The RNA binding protein She2p associates with ER membranes

In *S. cerevisiae*, it had been observed earlier by double live imaging that *ASH1* mRNPs co-localize with ER tubules (Section 1.5.). Here I sought to investigate whether She2p, a protein binding to all bud localized yeast mRNAs (Aronov *et al.*, 2007; Bohl *et al.*, 2000; Shepard *et al.*, 2003), is also associated with ER. She2p is not required for ER segregation like She3p and Myo4p, two locasome components that also co-fractionate with ER markers (Estrada et al., 2003). Nevertheless it could tether localizing transcripts to the ER and thereby effect the observed connection between mRNA and ER transport (Section 2.1.).

In order to test if She2p is associated with ER membranes, I tried to perform *in vivo* co-imaging of ER tubules and She2p. As She2p could not be expressed as an RFP-tagged version but only as a GFP fusion protein, an RFP-labelled ER membrane marker had to replace the Hmg1p-GFP ER marker. Unfortunately, all RFP-ER markers that were tested exhibited extensive photo bleaching. Due to these bleaching problems, She2p-ER co-imaging over time was not possible during this study.

I also used a biochemical approach to investigate the issue of whether She2p associates with ER. Indeed, She2p was found to co-fractionate with ER marker proteins in a set of different subcellular fractionation assays such as sucrose velocity gradients, ER isolation procedures and membrane flotation assays (Section 2.4.). These data strongly indicate that the RNA binding protein She2p is associated with ER and might serve as the linker which tethers localizing mRNPs to this membrane compartment.

This idea is consistent with the observation that, in contrast to wt cells where a dynamic co-migration of cytoplasmic ER tubules and *ASH1-MS2* RNPs can be detected (see 1.5.), mutants lacking She2p contain *ASH1-MS2* particles that stay in close proximity to the nuclear envelope and do not associate with ER tubules emanating from the perinuclear ER (A. Jaedicke in (Schmid *et al.*, 2006)). Similar to this, *in situ* hybridization of untagged *ASH1* mRNA expressed under similar conditions in *she2Δ* cells shows perinuclear staining (Figure S1 in (Schmid *et al.*, 2006)). Moreover, another study demonstrated by subcellular co-fractionation that *ASH1* mRNA and POL mRNAs bind to the ER in a She2p dependent manner. In cells lacking She2p, bud-localizing mRNAs are released from ER membranes (Aronov et al., 2007).

In light of these data it seems highly likely that She2p is the candidate that mediates ER membrane anchoring of localized mRNAs.

Interestingly, She2p is not the only RNA localization factor that can associate with ER. For instance, such observations have also been made for Staufen 1, a conserved double-stranded RNA binding protein involved in mRNA localization in various cell types (Allison *et*

al., 2004; Roegiers *et al.*, 2000). In *Xenopus* oocytes (Allison et al., 2004) as well as in certain mammalian cell types, a population of Staufen 1 co-fractionates and co-localizes with rough ER (Duchaine *et al.*, 2000; Gautrey *et al.*, 2005; Kiebler *et al.*, 1999; Marion *et al.*, 1999; Wickham *et al.*, 1999). However, in case of Staufen 1 it is still unclear if this ER association is needed for mRNA localization. In *Xenopus laevis* oocytes, VgRBP (Vg1 RNA binding protein) binds to several localized mRNAs including Vg1 (King *et al.*, 2005). As mentioned earlier, the protein has also been named Vera (VgLE-binding and endoplasmic reticulum association) since it co-fractionates and co-localizes with ER membranes (Deshler *et al.*, 1998; Deshler *et al.*, 1997) and in case of Vg1 mRNA it is thought that co-transport with ER is essential for its localization (Cohen, 2005).

It is therefore interesting to reveal the molecular basis of the She2p-ER interaction in yeast. As mentioned before, the other two core locasome components, She3p and Myo4p, were shown to co-fractionate with ER (Estrada et al., 2003). Association of She2p with ER could therefore in principle occur via adaptor proteins like She3p (Bohl *et al.*, 2000; Long *et al.*, 2000). Although previous results suggest a direct interaction between She2p and She3p (Bohl *et al.*, 2000), She3p is not required for She2p co-fractionation with ER (Section 2.10., figure 24 A) in sucrose gradients and is therefore unlikely to be the linker between She2p and ER. The same is true for the motor protein Myo4p: She2p still co-fractionates with ER markers in a *myo4Δ* mutant. This result is consistent with data from live cell microscopy, as in cells lacking Myo4p, *ASH1-MS2* particles are still associated with ER tubules even though these membrane structures do no longer migrate into the growing bud (A. Jaedicke in (Schmid *et al.*, 2006)). In velocity sucrose gradients She2p – ER interaction was even not disturbed in a *myo4Δshe3Δ* double knockout strain (Figure 24 B). This rules out a possible scenario in the respective single knockouts in which Myo4p could compensate for the loss of She3p and *vice versa*. She2p appears to attach to ER without the assistance of She3p or Myo4p. Consistent with these results an independent study observed that She3p does not co-fractionate with ER markers in the concurrent absence of She2p and Myo4p yet does so if only one of them is missing (Long, 2007). This indicates that either She2p or Myo4p are necessary for the She3p-ER interaction to occur. Simultaneously it implies that both Myo4p and, most relevant for this study, She2p are associated with ER independently from each other.

She2p's ER binding activity does also not depend on polysomes, i.e. ongoing translation, and SRP-mediated recruitment of mRNAs to the ER membranes (Section 2.5.). In theory, this would be one possible explanation as a large number of localized mRNAs and thus She2p substrates encode membrane or secreted proteins. From recent data it is known that membrane and non-membrane encoding messages are co-assembled into and co-transported in common mRNPs (Lange et al., 2008). Such a particle harbouring She2p and

different localizing mRNA ligands could be tethered to the ER via mRNA/ribosome/nascent polypeptide chain complexes and the SRP pathway. By these means She2p would be anchored only indirectly to the ER. In neurons this is for instance the case for an ER bound mRNP comprising Staufen and FMRP: Upon EDTA treatment, which is known to disrupt polysomes, these RBPs detach from the ER (Ohashi et al., 2002). In strong contrast to this, She2p still co-fractionates with ER membranes in presence of EDTA. Even more notably it shifts along with ER membranes to slightly lighter fractions. This is presumably due to the removal of polysomes by EDTA (Figure 65) indicating that She2p stays associated with ER membranes independently of polysomes and translation.
She2p is neither bound to ER via the mRNA translation machinery as mentioned before, nor via the mRNA itself. The latter was demonstrated by RNase treatment (Section 2.6.1.) and by use of She2p-N36S,R63K, a She2p mutant impaired in mRNA binding (Section 2.8.).

To summarize, the association of an *ASH1-MS2* mRNP with ER tubules and the co-fractionation of She2p with ER occur independently of other She proteins like the motor protein Myo4p or the adaptor She3p. Since She2p itself is required for co-localization of *ASH1-MS2* RNPs with ER tubules and co-fractionates with ER membranes independently of polysomes (thus ongoing translation) and of mRNA, it is suggestive that localizing mRNPs associate with ER tubules via She2p.

If polysomes, mRNA and other She proteins do not mediate She2p's interaction with ER, an unknown protein might act as bridging factor.
Different potential candidates were tested that might tether She2p to ER (Section 2.10.) but none of them turned out to be essential for this association. Aronov *et al.* observed the detachment of *ASH1* mRNA from ER upon deletion of Sec3p, an exocyst component also involved in ER segregation (Aronov et al., 2007), indicating a role for Sec3p in *ASH1*-ER anchoring. However, in contrast to this, velocity sucrose gradient experiments in this study demonstrated that She2p is still co-fractionating with ER markers in a *sec3Δ* mutant (see 2.10.). Even if I cannot explain this discrepancy at the moment, in light of my data it appears that She2p is stably associated with ER even in absence of Sec3p. Hence it seems unlikely that Sec3p serves to tether She2p to ER membranes.
In summary, every attempt to determine the She2p-ER bridging factor by such "educated guesses" failed (see 2.10.). However, the nature of linking factors can be too exotic to be identified via mere speculation. For example it was found recently that the G1 cyclin Cln3 is retained at the ER via an unusual chaperone-regulatory J domain which in turn is bound by the ER attached J-chaperone Ydj1 (Verges *et al.*, 2007).

In order to identify the putative protein factor via a more general approach, I also carried out chemical cross-linking experiments using formaldehyde or *bis*-maleimide cross-linkers for conjugation between sulfhydryl-groups (data not shown). These were performed in whole cell extracts lacking She3p in order to avoid background signal due to cross-linking between core locasome components (She2p, She3p and Myo4p). In parallel, cross-linking was executed with a fraction containing recombinant She2p and purified ER membranes prepared via the She2p-ER pelleting assay (described in section 2.11.1.). In this purified ER membrane fraction, only integral and peripheral membrane proteins, no cytosolic components, should be present and therefore the range of possible candidates should be more restricted. However, none of these cross-linking approaches resulted in a specific band that would indicate a potential binding partner of She2p on ER membranes (data not shown). In addition, She2p-ER pelleting assays with protease treated ER microsomes (Section 2.11.2.) did not show a sole protein dependency of the She2p-ER interaction.
Taken together, these data infer that it is highly likely that She2p binds to ER membranes directly.

3.4. She2p has the ability to directly interact with lipid membranes

The notion of a direct interaction of She2p with membranes was further substantiated by binding assays with synthetic, protein free membranes: in membrane flotation assays She2p – in contrast to the control protein – floated along with artificial, ER-like liposomes (Section 2.12.1.) suggesting that it can directly bind to membranes.
From these observations the question arises how She2p might attach to membranes, i.e. which domain might serve as a lipid binding domain.

In general, according to the nature of their association, membrane proteins can be divided into intrinsic (integral) or extrinsic (peripheral) membrane proteins (Goni, 2002).

She2p is not an integral membrane protein since it does neither contain an N-terminal signal peptide or any predicted transmembrane domains nor does it possess a carboxy-terminal hydrophobic segment like tail-anchored ER membrane proteins do (Borgese *et al.*, 2003).

Within the class of peripheral membrane proteins, some contain covalently attached fatty acid- or prenyl-moieties such as myristate, palmitate, farnesyl and geranylgeranyl, which they embed in the lipid bilayer for membrane anchorage (Resh, 2006). However, since even recombinantly expressed She2p binds to liposomes, such a lipid binding mode can be

excluded for She2p. The same rationale applies to another posttranslational lipid modification, the glycosylphosphatidylinositol (GPI) anchor (Orlean *et al.*, 2007).

Recently, a targeting signal responsible for directing a number of proteins to the cytosolic surface of the ER was identified (Loewen *et al.*, 2003). This so called FFAT motif (with [FF] standing for diphenylalanine) has the consensus amino acid sequence EFFDAxE and mediates binding to a highly conserved class of ER transmembrane proteins, the VAP protein familiy (Kaiser et al., 2005). However, no such motif is present in the She2p primary sequence and therefore this anchoring mode does not apply for She2p.

Nevertheless, it was observed in this study that She2p behaves like a genuine peripheral membrane proteins since it could be removed from liposomes by Na_2CO_3 treatment (Section 2.12.2.). Na_2CO_3, pH 11 is known to shear off extrinsic membrane proteins (Fujiki *et al.*, 1982).

Another class of peripheral membrane proteins contains globular domains specialized for lipid binding, which are also termed membrane-targeting domains (W. Cho *et al.*, 2005). This group includes various members and amongst them are protein kinase C conserved 1 (C1), conserved 2 (C2), and annexin domains. C1 binds to phorbolesters and diacylglyerol (DAG) whereas C2 and annexin domains are known to bind phosphatidylserine (PS) in a Ca^{2+}-ion dependent manner (Lemmon, 2008). Other membrane-targeting domains like PH (pleckstrin homology), FYVE (Fab1, YOTB, Vac1, and EEA1), PX (Phox-homology), ANTH (AP180 N-terminal homology), BAR (Bin amphiphysin Rvs), FERM (band 4.1, ezrin, radixin, moesin), PDZ (postsynaptic density, disk large, zonula occludens), and tubby domains all have a specificity for different phosphoinositides (PIPs), derivatives of phosphatidylinositol (PI) with one ore more phosphates attached by specific kinases to the 3, 4 or 5 positions of the inositol ring (Balla, 2005). However, attempts to detect similarities between She2p and those domains failed, suggesting that She2p does not contain homology to any known globular lipid binding domains (analysis performed by Johannes Söding, Genecenter, LMU Munich). Yet it is interesting to note at this point that, concerning its mRNA association activity, She2p turned out to be an unconventional RNA-binding protein not belonging to any previously identified classes of RNA binding proteins (Niessing *et al.*, 2004). In analogy to this it is tempting to speculate that She2p might also constitute a so far uncharacterized type of lipid binding protein.

Finally there are also peripheral membrane proteins that do not have special globular lipid-binding domains but rather interact with the membrane via their molecular surface or an amphiphatic secondary structure (W. Cho *et al.*, 2005). In this case, membrane anchoring can occur either via hydrophobic forces with non-polar parts of the membrane matrix or via electrostatic or polar forces with charged head groups of the phospholipids (Goni, 2002).

Concerning membrane polarity, zwitterionic phospholipids like PC (phosphatidylcholine) or PE (phosphatidylethanolamine) have a zero net charge and in total behave electrically neutral like the yeast steroid ergosterol. In contrast, PS (phosphatidylserine) and PI (phosphatidylinositol) are phospholipids with a single net negative charge whereas phosphoinositides (PIPs) can be highly negatively charged. Often, clusters of basic amino acids in extrinsic membrane proteins interact with negatively charged lipids like PI, PS or PIPs and such interaction can be even stronger than the interaction between lipids and the aforementioned specific membrane targeting domains (McLaughlin *et al.*, 2005). In case of the yeast exosome component Sec3p such a polybasic region lies within its N-terminal domain and mediates interaction with PS and a specific type of PIP, thereby facilitating membrane recruitment (Zhang et al., 2008).
Analysis with the web-based tool ConSurf (http://consurf.tau.ac.il) (Glaser et al., 2003; Landau et al., 2005) to identify highly conserved and basic residues in She2p yielded one larger region that simultaneously displays amino acids matching both criteria (Figure 34).

However, this surface exposed region, also called the “basic helical hairpin”, has been identified as a She2p domain involved in mRNA binding (Niessing *et al.*, 2004). After this analysis, one major flaw of the *in vitro* lipid binding assay using liposomes and recombinant She2p had to be addressed: In absence of its mRNA ligand, She2p might non-specifically associate with negatively charged phospholipids in synthetic vesicles via its basic helical hairpin. To investigate this, the She2p-lipid binding assay was performed in presence of *in vitro* transcribed *ASH1* E3 localization element, one of the three RNA elements bound by She2p. The RNA ligand floats together with the She2p/vesicle fraction (see 2.12.4.) suggesting that She2p can simultaneously bind RNA and liposomes and does not merely attach randomly to lipid membranes if the RNA ligand is absent. This notion is further supported by the observation that She2p-N36S,R63K, a protein which carries mutations in the basic helical hairpin and is incapable of binding mRNA, can still bind as efficient to liposomes as wild type She2p (data not shown). Moreover, *in vivo*, She2p is known to associate with ER membranes even upon over-expression of its mRNA ligand from a 2µ plasmid (Section 2.4.1., figure 13) and it has been shown that both mRNA and She2p co-fractionate simultaneously with ER membranes (Aronov et al., 2007). Together, these results strongly suggest that the mRNA and lipid binding activities in She2p are not mutually exclusive and that the basic helical hairpin is not necessarily involved or at least is not a major player in the process of membrane attachment. Consistent with these results She2p’s ability to interact with synthetic membranes remains even if liposomes are lacking PS and PI and thus do not contain any phospholipids with a negative net charge (Section 2.12.3.). In summary these observations indicate that She2p does not interact unspecifically with lipid membranes via its polybasic stretch.

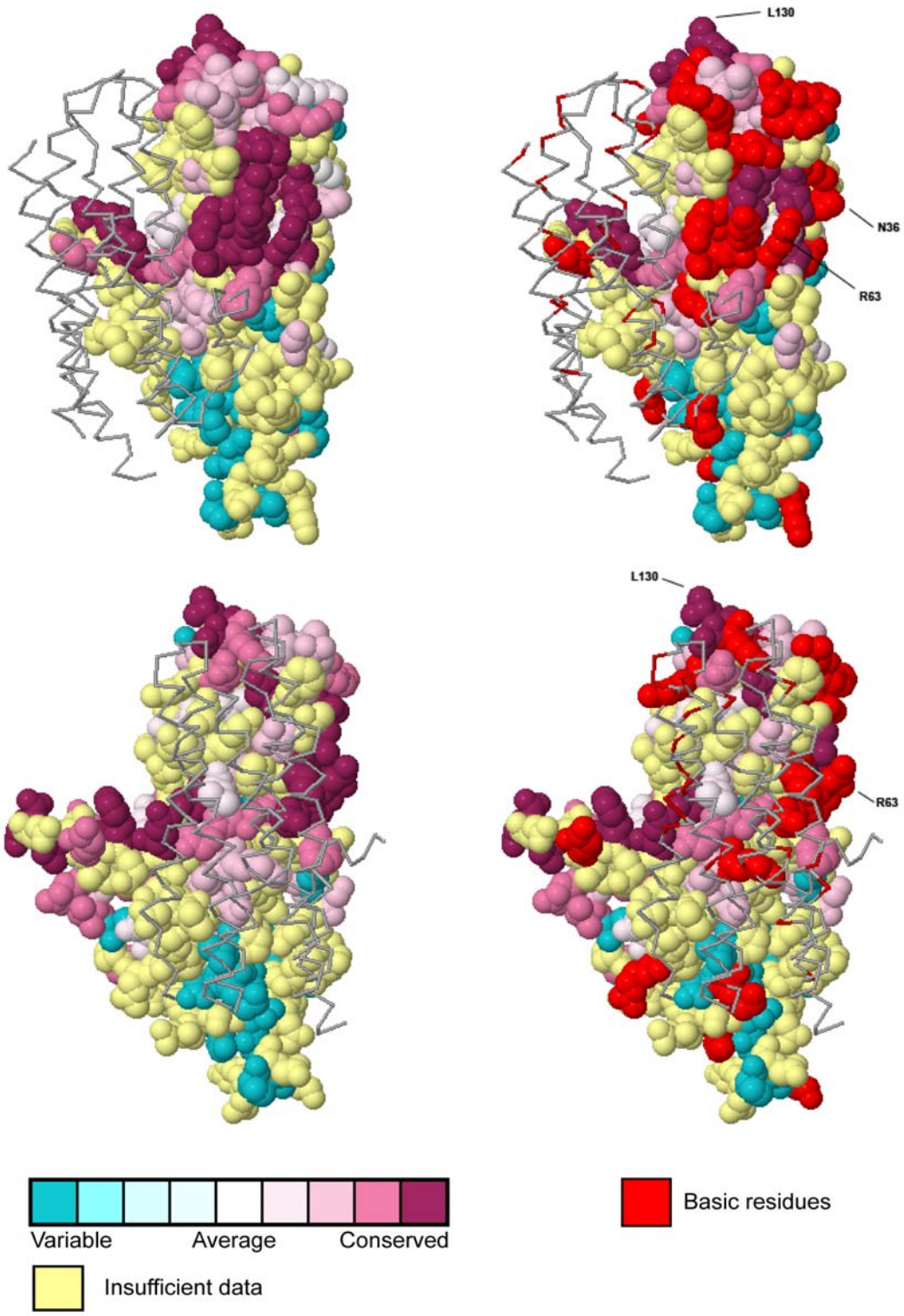


Figure 34: The surface exposed basic helical hairpin of She2p is highly conserved and harbours a stretch of basic amino acids: Surface residue conservation and distribution of negatively charged amino acids of She2p was analysed using CONSURF (http://consurf.tau.ac.il). Left panel: the degree of conservation is depicted in a colour range where green indicates high variability and deep purple represents a high degree of conservation. Right panel: structures are duplicates from the ones in the left panel with the additional information that basic amino acid residues are depicted in red. Please note that only the fully elaborated A chain of the She2p PDB structure is illustrated with its atoms in colour whereas the incomplete B chain is only shown in ribbon form.

Another option for She2p would be membrane attachment via hydrophobic interaction forces. One possible candidate for such a binding mode could be Leu130 which is positioned on the top of the She2p dimer (Figure 35). However, the mutant She2p-L130S is not impaired in its association with ER in fractionation assays (see 2.9.). Moreover this hydrophobic region is implied in the oligomerization of She2p (Marisa Müller, personal communication). Another possible hydrophobic surface patch is the region above the aliphatic amino acid Val202 (Figure 35). This amino acid was identified in the same screen for mutants defective in mRNA localization as residue Leu130 (Gonsalvez et al., 2003). Yet, due to limitations in time, V202 was not analyzed within this study. Currently one can only speculate but this amino acid and the adjacent hydrophobic region should be investigated for involvement in She2p-membrane interaction in future.

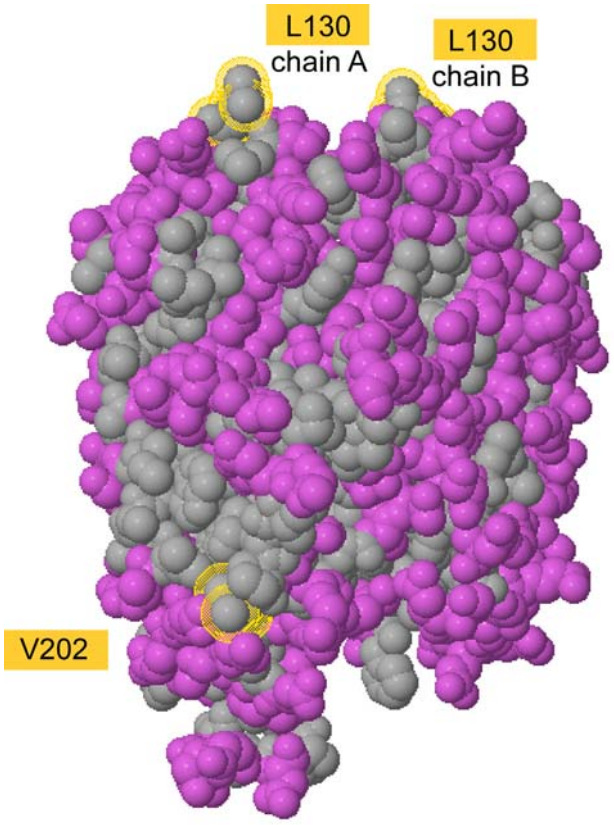


Figure 35: Hydrophobic residues on the She2p surface: Hydrophobic patches were displayed using CONSURF (http://consurf.tau.ac.il); polar (charged & uncharged) amino acids are depicted in purple, hydrophobic residues in grey.

In order to analyze She2p for possible candidate regions involved in membrane binding, I used MAPAS, a web-based tool to predict membrane-contacting protein surfaces (http://cancer-tools.sdsc.edu/MAPAS/pro2.html) (Sharikov et al., 2008). The results of this analysis for She2p are depicted in figure 36.

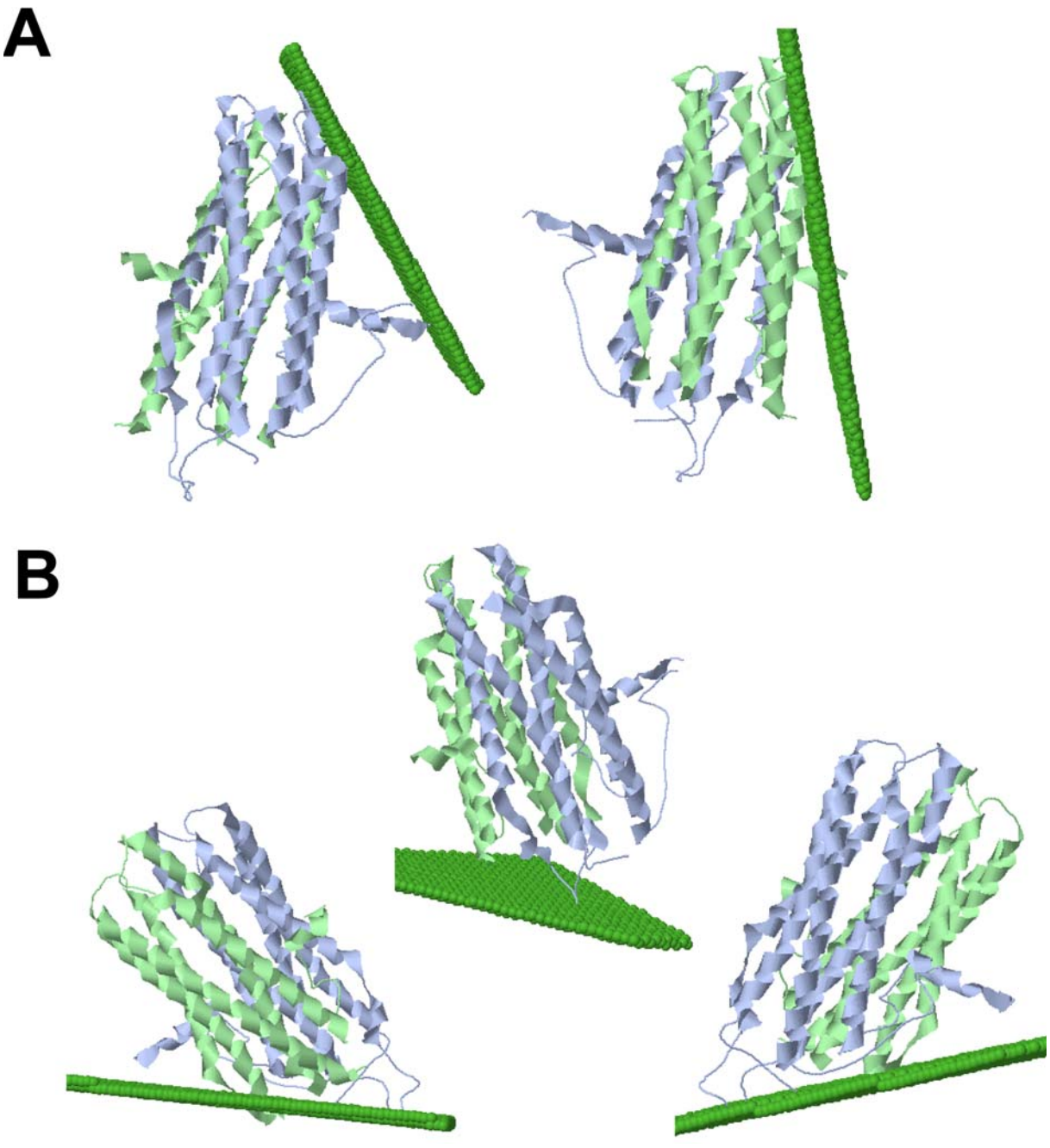


Figure 36: Prediction of possible She2p-membrane interactions by MAPAS analysis (http://cancer-tools.sdsc.edu/MAPAS/pro2.html): The two She2p monomers of the PDB structure are depicted in different colours, chain A in blue, chain B in light green. Note that due to the PDB data chain B is incomplete and lacks e.g. the unstructured loops at the bottom of the dimer.

Figure 36 A shows one possible interaction mode in which parts of the protruding helix of the She2p dimer and hydrophobic residues including Leu130 in the head domain interact with the membrane. However, as mentioned above, mutation of Leu130 did not affect membrane association. Concerning the α-helix which protrudes at right angles from both sides at the middle of the She2 dimer, its effect was tested via She2p-ΔHelix (She2p-Δ amino acids 174-183), a protein bearing a deletion in this secondary structure. When analyzed by velocity sucrose centrifugation, She2p-ΔHelix was not impaired in its association with ER membranes indicating that it is not involved in lipid binding (see 2.9.).
In figure 36 B, a second potential lipid contact is illustrated which consists of the interaction between membranes and the unstructured loops at the bottom end of the She2p dimer (ranging approximately from Asn79 to Ser91). It is important to note at this point that chain B of the She2p dimer (depicted in light green in figure 36) is incomplete in the PDB structure and lacks the region of the unstructured loop which was therefore also not considered in the MAPAS analysis. So far, no mutational analyses have been performed with residues in this unstructured loop which might contact membranes according to MAPAS. Even if the probability of being a lipid interacting domain according to MAPAS is not too high, it will be interesting in the future to investigate its role in She2p membrane anchoring.
Unfortunately amino acid Val202 did not come up in the MAPAS analysis, although the residues Asn203, Ser204, Glu205, Glu206 in its near surrounding were amongst the amino acid group which was involved in the potential lipid interactions depicted in figure 36 B.

Besides the question about the molecular basis of the She2p-membrane interaction another key issue that emerges is specificity: how does She2p recognize ER structures and discern them from any other intracellular membranes?
In general it was believed for a long time that specific subcellular localization of peripheral membrane proteins can be only based on protein-protein interactions with integral membrane proteins, because lipid-protein interactions could not confer such precise specificity. Today it is known that the majority of extrinsic membrane proteins binds two families of compounds: The first class consists of numerous activated GTPases that are displayed on the surface of the respective organelles. The second class of molecules that contributes to the unique identity of membrane compartments are specific lipids (Behnia *et al.*, 2005). Recent studies have indicated that high specificity and affinity can be also attained through lipid-protein interactions (W. Cho *et al.*, 2005). Cellular membrane compartments have different lipid compositions and recognition of these specific lipid species is another mode for extrinsic membrane proteins to distinguish one intracellular organelle from another (Lemmon, 2008).

Within the class of lipids, polyvalent phosphoinositides (PIPs) are major players in defining organelle identity and in recruiting proteins to specific membranes even though they

are only low abundant (Behnia *et al.*, 2005; Lemmon, 2008; McLaughlin *et al.*, 2005; van Meer *et al.*, 2008). For instance PI(3)P is exclusively found on early endosomes, $PI(3,5)P_2$ on late endosomes and lysosomes, PI(4)P on trans-Golgi stacks, and PI(4)P, $PI(4,5)P_2$, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ on plasma membranes (Behnia *et al.*, 2005; van Meer *et al.*, 2008). However, from recent studies it is known that not only PIPs but also the more abundant, anionic lipid phosphatidylserine (PS) is involved in specific recruitment events (Behnia *et al.*, 2005). By use of a biosensor it was shown to be accumulated in the cytosolic leaflets of plasma membrane, endosomes and lysosomes and to redirect cationic proteins to the endocytic pathway (Yeung et al., 2008).

But in contrast to other intracellular compartments, the ER itself is exceptional since there are no specific lipids found on this organelle (Behnia *et al.*, 2005). PS is only present at low levels and PIPs are completely absent. The ER-like liposomes for my binding assay were prepared according the yeast ER lipid composition (see methods section 5.7.1.) (Schneiter *et al.*, 1999; Tuller *et al.*, 1999; Zinser *et al.*, 1991). In order to assess whether the two anionic phospholipids PS and PI act as landmarks for She2p-ER recruitment, I prepared synthetic vesicles lacking both PS and PI and tested them for She2p-lipid interaction. She2p was floating with these PS-/PI-minus liposomes just as well as with the ER-like vesicles (Section 2.12.3.) indicating that neither PS nor PI are the components which mediate specificity in the She2p-ER interaction. This result was anticipated due to absolutely “basic” phospholipid equipment of the ER compartment from which one can conclude that in case of the ER, proteins should still act as organelle markers. Consistent with this idea I had observed a slight but not complete reduction in the pelleting of She2p with protease treated ER microsomes (Section 2.11.2.). One could speculate that this is due to the loss of a factor which – although not acting as the ER-linker for She2p – might in contrast act as a landmark for efficient ER-recognition and the adjacent recruitment of She2p.

In summary, the data of this study strongly suggest that She2p has indeed the ability to directly interact with lipid membranes even though the nature or molecular basis of this association still has to be elucidated. However, extensive mutational analysis based on the surface analyses as discussed above should finally help to elucidate the domains involved in lipid binding. Thus, it seems likely that the specificity for She2p-membrane interaction is not only mediated by lipids but might also be mediated by accessory protein factors. In order to further analyse lipid specificity in the She2p-ER interaction, additional studies could be performed. Phospholipid strips could be used to quickly identify a potential preference of She2p for a certain species of phospholipids (Membrane Lipid Array™; Echelon Biosciences). In addition, surface plasmon resonance (SPR) technology could be used to investigate in detail affinities between She2p and different membranes. The lipid specificity of protein-membrane interactions can be easily studied with this method by manipulating the

lipid composition of immobilised synthetic membranes. But such Biocore^{TM} sensor chips do not only allow the capture of liposomes but even of subcellular membrane preparations (Besenicar *et al.*, 2006). This would permit investigating the strength of She2p interaction with natural ER membranes in contrast to other intracellular membrane compartments and in comparison to e.g. protease treated ER microsomes or microsomes from various yeast mutants in search of the aforementioned hypothetical accessory factor. Finally, different She2p mutants could be analyzed for their binding affinities to lipid membranes.

3.5. Benefits of coordinated mRNA and ER transport and implications for a possible model

In summary, I observed a functional correlation between mRNA localization and ER transport in yeast. The RNA binding protein She2p associates with ER membranes and thus most likely serves as the linker that attaches localizing mRNPs to this subcellular compartment. The She2p-ER interaction occurs independently of polysomes (thus ongoing translation), of mRNA and other She proteins like Myo4p or She3p. In the contrary, She2p was shown to directly interact with lipid bilayers even though the molecular details of this membrane binding activity still have to be unravelled.
Taken together, these results support the notion that She2p and the She3p/Myo4p motor complex attach to ER independently and that the trimeric She2p-She3p-Myo4p locasome that has been considered as the device for mRNA localization in yeast (Bohl *et al.*, 2000; Gonsalvez *et al.*, 2005; Long *et al.*, 2000; Takizawa & Vale, 2000) is only a component of a still more complex transport machinery that includes ER tubules.

For both mRNAs encoding cytosolic ($mRNA_{cyt}$) or mRNAs encoding membrane or secreted proteins ($mRNAs_{memb/sec}$), a co-transport together with ER membranes would have several benefits compared to separate transfer processes.
In case of $mRNAs_{memb/sec}$, common transport with ER would allow the pre-assembly with structures responsible for synthesis and processing of the encoded proteins. The localized mRNAs would be already present firstly near ER-resident ribosomes on which the proteins are synthesized in the following; secondly they would be already close to the translocon, the machinery that mediates transfer of membrane proteins or secreted proteins to the ER lumen. Finally, mRNAs for secreted proteins would be in close proximity of ER structures like tER from which their encoded proteins are packed into transport vesicles and then released into the secretory pathway. This would therefore enable a much more efficient performance of processes like ER-located synthesis, membrane insertion and ER import and secretion of

the respective proteins. Another example of mRNA-organelle targeting and a resultant more efficient transfer process of the encoded protein, is the localization of transcripts like metallothionein-1 (MT-1) and transcription factors c-FOS and c-MYC to the nuclear periphery (Chabanon *et al.*, 2005; ChabanonNury *et al.*, 2004; Dalgleish *et al.*, 2001). Upon disruption of perinuclear localization of its message, MT-1 protein is not efficiently imported into the nucleus any more indicating that mRNA targeting to the nuclear periphery enhances nuclear transport (Levadoux *et al.*, 1999).

As mentioned earlier (see 1.3.3.), the majority of localized mRNAs in yeast encodes membrane or secreted proteins and therefore this possible advantage is of special importance. In view of the following facts, the co-transport model seems even more plausible: Firstly, it is known that localized mRNAs are transported in a translationally silenced state (Deng *et al.*, 2008; Paquin *et al.*, 2007). Therefore, it is questionable whether targeted $mRNAs_{memb/sec}$ can in fact be recruited to the ER via the SRP pathway, a process completely dependent on translation (Halic *et al.*, 2005; Keenan *et al.*, 2001). Even though this will have to be investigated, the probability is high that SRP recruitment might not work for localizing mRNAs and that recruitment via She2p might help to circumvent this problem. Secondly this idea is consistent with the recent finding that the mRNAs for membrane and secreted proteins can be also directed to ER membranes in a signal peptide and translation independent manner, putatively via specific RNA binding proteins (Pyhtila et al., 2008), like She2p.

In the case of localized mRNAs for cytoplasmic proteins, it has been found recently that different mRNA species, encoding either soluble factors or membrane proteins, are co-assembled and simultaneously transported in the same mRNP (Lange et al., 2008). A common recruitment of $mRNA_{cyt}$ and $mRNAs_{memb/sec}$ to ER membranes is therefore highly likely to occur. This notion is supported by the striking observation of a cDNA-microarray based screen for novel secretory and integral membrane proteins: in addition to identifying novel genes for this group, a large number of mRNAs encoding soluble proteins, amongst them also *ASH1* mRNA, was found to be ER associated (Diehn *et al.*, 2000). Independently, other results show that $mRNAs_{cyt}$ are present and sometimes even enriched on the ER and are also translated by ER bound polysomes (Lerner *et al.*, 2003; Nicchitta *et al.*, 2005).

In summary, the model of a co-trafficking of both $mRNAs_{memb/sec}$ and $mRNA_{cyt}$ with ER membranes is substantiated by these observations.

Concerning local protein synthesis at peripheral cell regions, the two types of localizing mRNAs, the ones for soluble and the ones for membrane or secreted proteins would both equally benefit from a co-transport with ER. In yeast the final destination of mRNA and ER trafficking is the newly forming bud and in higher eukaryotes like in neurons this would be distant cellular areas in axonal and dendritic processes. The co-assembly and co-transport of

localizing mRNAs and ER would ensure that the transcripts and their necessary translational infrastructure such as cortical ER associated polysomes (Baba, 1987), ER resident translocon and tER areas reach the peripheral regions together. This in turn could greatly enhance the efficiency of local translation and, in case of $mRNAs_{memb/sec}$, accelerate membrane insertion and secretion of the respective proteins.

The concept of coordination between mRNA and ER transport is also supported by the fact that several recent reviews also describe the emerging interplay between the two processes (Cohen, 2005; Gerst, 2008; Paquin *et al.*, 2008).

However, there is one point which seems to be controversial in the concept of a common transport of mRNAs and ER. In live cell microscopy studies using MS2 tagged full-length *ASH1* mRNA, the *ASH1*-MS2 particles reach a mean transport velocity of 546 nm/sec (Lange et al., 2008). Values in the same range (200-440 nm/sec) were reported in another study though in that case fusion constructs of bacterial lacZ mRNA with one *ASH1* localization element was used (Bertrand et al., 1998). This speed of mRNP transport is also compatible with that expected for a myosin V motor (Cheney et al., 1993). In contrast to this, the rate of ER tubule movement was estimated to an average of 13 nm/sec (Estrada et al., 2003) and thus much slower than the observed speed of *ASH1* mRNA trafficking. I cannot explain this discrepancy at the moment. However, recent *in vivo* imaging experiments using the *ASH1*-MS2 detection system and a GFP-tagged ER tubule marker clearly demonstrated co-localization and co-migration of the ER tubule and an attached *ASH1*-MS2 mRNP particle (A. Jaedicke in (Schmid *et al.*, 2006), see also section 1.5.).

Another issue arises from the observation that ER tubules migrate only into the newly forming bud during S-phase, when the yeast bud is small to medium sized (Y. Du *et al.*, 2001; Estrada *et al.*, 2003; Preuss *et al.*, 1991). Is this compatible with the observed co-transport of ER tubules and *ASH1*-MS2 mRNPs, an mRNA which is expressed during late anaphase at the end of mitosis? At this point one has to consider that in our experiments, *ASH1* mRNA was expressed from a constitutive promoter (Section 1.5.) and therefore was present throughout the whole cell cycle.

As mentioned earlier, amongst the group of cell cycle regulated localized mRNAs, *ASH1* is expressed very late in cell cycle. However, others like *WSC2* or *SRL1* are expressed early in S and G1 respectively. Therefore it is tempting to speculate that in *S. cerevisiae* there might be chronologically different mechanisms for mRNA localization, in analogy to the transport routes that exist in *Xenopus laevis*. As mentioned earlier, dependent on the time-scale there are two different mechanisms for mRNA targeting in *Xenopus*: the early and the late pathway. The early one uses a structure called METRO and the late one involves the ER in order to achieve translocation of the respective messages (see 1.1.2.2.). Analogously one could imagine for *S. cerevisiae* that there is on the one hand an early pathway which

depends on ER and occurs by co-trafficking of early expressed mRNAs and ER tubules; localized mRNAs like the POL mRNAs would also be likely candidates for such a transport mechanism because they start to localize very early when buds are just about to grow (Aronov et al., 2007). On the other hand, the late pathway would proceed without ER membrane involvement since ER tubules no longer move to the bud and peripheral ER already extends through the complete cortex of the bud at this stage of cell cycle (Figure 37).

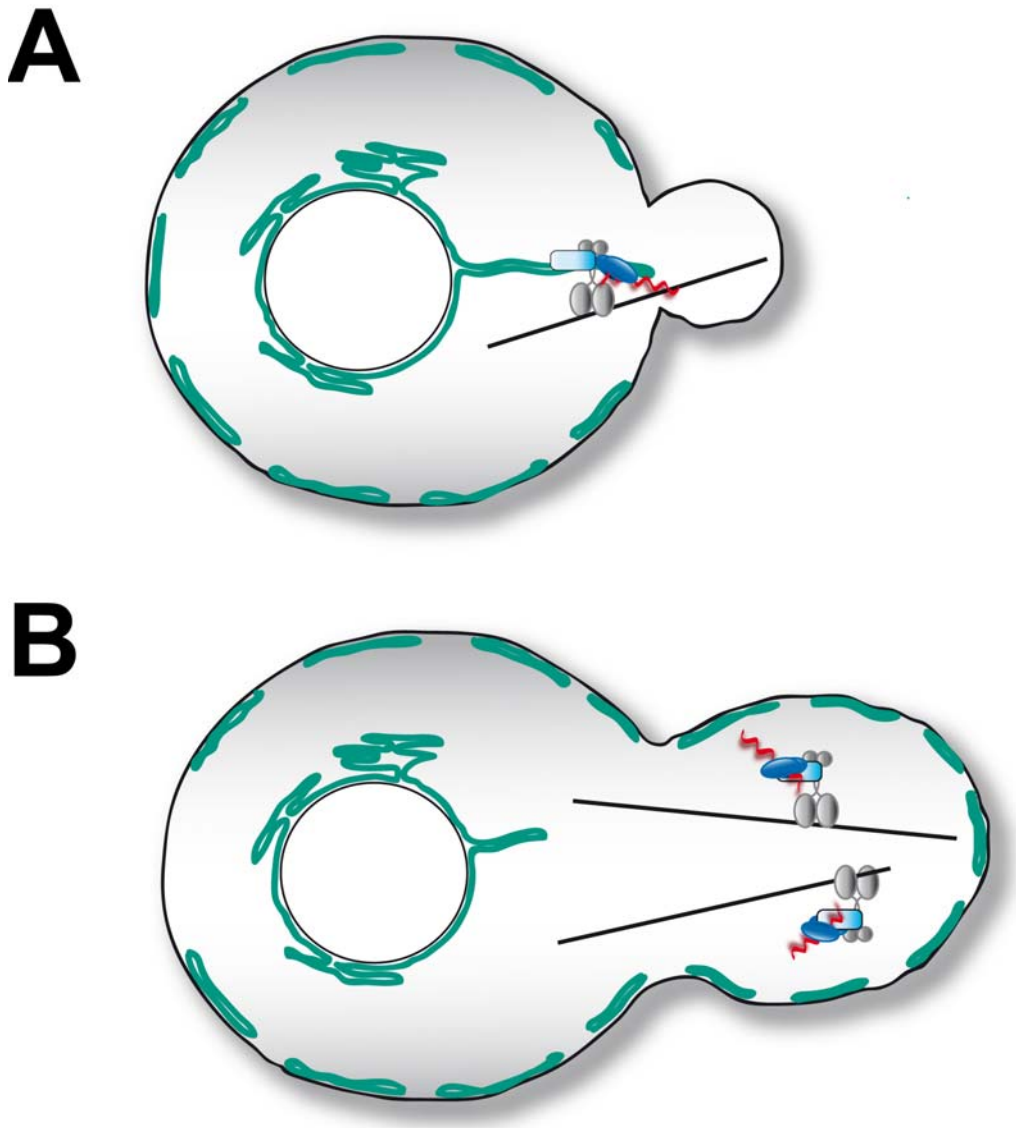


Figure 37: Hypothetical model for early and late mRNA localization mechanisms in *S. cerevisiae*. (A) Early mechanism involves co-transport of ER tubules and mRNAs to the small to medium sized buds. (B) Late mechanism: No ER tubules move to the bud since cortical ER in the bud is already established. mRNA targeting proceeds independently of ER tubule movement.

However, to date this model is only based on speculation and additional experiments have to be performed to test this hypothesis. For instance, localized mRNAs expressed early in cell cycle such as *WSC2* could be used to investigate the impact of ER inheritance on the trafficking of such early localizing transcripts.

4. Materials

4.1. Consumables and Chemicals

Consumables and chemicals were purchased from the following companies:
Acros Organics (Geel, Belgium), Applichem (Darmstadt), Applied Biosciences (Darmstadt), Apollo Scientific Limited (Bredbury, UK), Axon (Kaiserslautern), Becton Dickinson, (Heidelberg), Beckman Coulter (Krefeld), Biaffin (Kassel), Biomol (Hamburg), Biorad (Munich), Biozym (Hess. Oldendorf), Chemicon (Temecula, Canada), Fermentas (St. Leon-Rot), Formedium (Norwich, UK), GE Healthcare (München), Gilson (Bad Camberg), Invitrogen (Karlsruhe), Macherey & Nagel (Düren), Medac (Hamburg), Medigenomix (München), Membra Pure (Bodenheim), Merck Biosciences (Darmstadt), Millipore (Molsheim, France), Mobitec (Göttingen), MP Biomedical (Illkirch, France), NEB (Frankfurt), Neolab (Heidelberg), Nunc (Wiesbaden), Peske (Aindling-Arnhofen), Promega (Mannheim), Qiagen (Hilden), Roche (Mannheim), Roth (Karlsruhe), Santa Cruz (Santa Cruz, USA), Sarstedt (Nümbrecht), Semadeni (Düsseldorf), Serva (Heidelberg), Sigma (Taufkirchen), Stratagene (Amsterdam, The Netherlands), VWR (Ismaning).

4.2. Equipment

Abimed	Pipetman Gilson P10, P20, P200, P1000
Avestin	Extruder LiposoFast-Basic
Beckman Coulter	Ultracentrifuge L-80; rotors SW40, SW55 and SW60 Ultracentrifuge Optima MAX; rotor TLA120.2
Biorad	*E. coli* Pulser
Biometra	T1 Thermal Cycler
Branson	Sonifier 200
Eppendorf	Thermomixer Compact, Cooling centrifuge 5415R, BioPhotometer
Fuji Europe	Fuji LAS-3000 mini imaging system
Haereus	Tabletop centrifuge Biofuge *pico*, Multifuge 3 L-R
Ika	Vibrax VXR basic
Julabo	Waterbath Shaker SW 2
Liebherr	Freezer -20°C, Fridge
Mitsubishi	Gel documentations system

MJ Research	PCR cycler PTC-200
Neolab	Akku-Jet
New Brunswick	Freezer -80°C, Shaking incubator
Olympus	Fluorescence Microscope BX60
Peqlab	Semi-dry blotting device
Sartorius Universal	Analytical balance
Scientific Industries	Vortex Genie 2
Thermo Fisher Scientific	Sorvall Evolution RC; rotors SLC6000, SS-34, GS-3, GSA; RC M120 Ex Micro-Ultracentrifuge; rotor RP120-AT
Zeiss	Light microscope ICS/KF 2
ZMBH (Heidelberg)	Gel electrophoresis chamber

4.3. Commercially available kits

Ambion	MEGAshortscript T7 Kit
Invitrogen	Topo TA Cloning®
Machery&Nagel	Nucleospin Miniprep Kit
New England Biolabs (NEB)	Quick Ligation Kit
Qiagen	QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit
Stratagene	QuickChange® Site-directed Mutagenesis

4.4. Enzymes

Axon	Taq Polymerase
Biomol	Lysozyme
Fermentas	CIP (Calf Intestine Alkaline Phosphatase); Restriction endonucleases
New England Biolabs (NEB)	Quick-T4-DNA-Ligase, Vent® DNA Polymerase
Roche	Micrococcal Nuclease, RNase A
Seikagaku America, ICN	Zymolyase 20T,100T
Stratagene	Herculase® II Fusion DNA Polymerase

4.5. Oligonucleotides

RJO	name	sequence	purpose
73	ASH1-RTPCR2A	TACATGGATAACTGAATCTC	RT-PCR
74	ASH1-RTPCR2B	CAGGATGACCAATCTATTGC	RT-PCR
214	ASH1_1383_r	GGGGGAGAGTCGAGAGC	sequencing
282	ASH1_639_f	CGCGAAGAAGTGGCTCATTTC	sequencing
166	Yxplac-5'	cccgactggaaagcgggcag	gap repair cloning
167	Yxplac-3'	ggagaaaataccgcatcaggc	gap repair cloning
184	Myo4-ko-forw.	CCAGTTTGGCGAGACAATTTATTTTCAATACGATACCCCAGCTGAAGCTTCGTACGC	knockout
185	Myo4-ko-rev	CAGCTTCGCCCTTGTTAGCAGGCTTGTATTTCAACGCATAGGCCACTAGTGGATCTG	knockout/tagging
434	s MYO4ko	GTTACCAGTTTGGCGAGACA	yeast colony PCR
541	She3-Tag-forw.	GGGAAATAGTATGGTTGTTCACGGGGCCCAATCCcgtacgctgcaggtcgac	tagging
542	She3-tag-rev	TCCTATATATATACTCCCTTGTGTCGGCATATTatcgatgaattcgagctcg	knockout/tagging
850	IST2-BamH1-F(2544)	ttttggatcCTGAACAAACAAAAAAG	sequencing
1110	URA3_+365_R	CAATACCTGGGCCCACCACACCG	yeast colony PCR
1082	WSC2-1F	ATGCACCTAGATCTCATAC	RT for *in situ*
1083	WSC2-1R-T7	TAATACGACTCACTATAGGGACTGTAACTGAGCTACGG	RT for *in situ*
1084	WSC2-2F	GTGGTGGTTCTTCTGCCATG	RT for *in situ*
1085	WSC2-2R-T7	TAATACGACTCACTATAGGGGAGGAAGTAGTTGTCTC	RT for *in situ*
1086	WSC2-3F	GCCTCTTCCAGTTCAGAAACG	RT for *in situ*
1087	WSC2-3R-T7	TAATACGACTCACTATAGGGCTAGTAGAGGAGGACGTAGACG	RT for *in situ*
1088	WSC2-4F	GAGTGGTGGCGCCATCGCAGG	RT for *in situ*
1089	WSC2-4R-T7	TAATACGACTCACTATAGGATCTCTGCCGCCCGGCG	RT for *in situ*
1992	MYO4_epi_S3_f	CAGCAATACAGAGGGCTTAGCTACTGTCAGTAAAATTATAAAATTAGACAGAAAAcgtacgctgcaggtcgac	tagging
1993	MYO4_epi_S2_r	GTAGGATATATGTATATATACATATATACATATATGGGCGTATATTTACTTTGTTCatcgatgaattcgagctcg	knockout/tagging
1999	She2_N36S_for	CTCATCTTATATTCACGTGCTGAgCAAGTTCATCAGTCATTTGCG	site directed mutagenesis

2000	She2_N36S_rev	CGCAAATGACTGATGAACTTGCTCA GcACGTGAATATAAGATGAGAGATA CC	site directed mutagenesis
2001	She2_R63K_for	GATTAAATTTGTTAAGAAATTGAaAT TTTACAACGATTGTGTGTTAAGC	site directed mutagenesis
2002	She2_R63K_rev	GCTTAACACACAATCGTTGTAAAATt TCAATTTCTTAACAAATTTAATC	site directed mutagenesis
2020	TRP1_sequ_f	GAGGTTCCAGTTCCCACAGG	yeast colony PCR
2021	TRP1_sequ_r	CCTGTGGGAACTGGAACCTC	yeast colony PCR
2027	kann_C_primer_f	tgattttgatgacgagcgtaat	yeast colony PCR
2028	SHE3_ko_S3_f	CGTTAGCTCGTCTATCAAGCACGC CAAGGTTCAACGACACTACTTTTGT GTAAGcgtacgctgcaggtcgac	knockout
2029	SHE3_ko_S2_r	CTTTTGTTCTATTATCTAAATGAATC CTATATATATACTCCCTTGTGTCGG CATATTatcgatgaattcgagctcg	knockout/tagging
2033	She2_L130S_f	CAAAAGGAAATTTTATCTAAAACTTc GAACGAGGACCTAACGCTAAC	site directed mutagenesis
2034	She2_L130S_r	GTTAGCGTTAGGTCCTCGTTCgAAG TTTTAGATAAAATTTCCTTTTG	site directed mutagenesis
2035	She2_L130Y_f	CAAAAGGAAATTTTATCTAAAACTTa cAACGAGGACCTAACGCTAAC	site directed mutagenesis
2036	She2_L130Y_r	GTTAGCGTTAGGTCCTCGTTgtAAGT TTTAGATAAAATTTCCTTTTG	site directed mutagenesis
2039	She3_ko_check_f	CGTTGGTAGATCTTGATGG	yeast colony PCR
2068	SHE2_ORF486_r	CAAAGACTCAATCATCCATTGAG	sequencing
2069	SHE2_ORF262_f	GAGGCGGATTCGTTTGACAAG	sequencing
2070	SHE2_epiK_f	GAATTTGATGTTGTCGCTACTAAAT GGCATGACAAATTTGGTAAATTGAA AAACcgtacgctgcaggtcgac	tagging
2071	SHE2_epiK_r	CTATTAACTAGTGGTACTTATTTGCT CTTTTTGAGCTAAAAACTGAAGGCC atcgatgaattcgagctcg	tagging
2090	PDA1_RT_f	CCATCTTGTTTGAAGACGTCTAC	RT-PCR
2091	PDA1_RT_r	gggaagaatatcatgcgatcac	RT-PCR
2092	SEC61_RT_f	GTACTTTAGGTTCTGGGGCATC	RT-PCR
2093	SEC61_RT_r	ggaggggtgtggctaaatgcg	RT-PCR
2150	SHE2_ORF558_r	CGTTCCGTCCTCATCTGCG	sequencing
2147	AUX1_ko_f	AACCTATTCCTGTGCTTCTGGAAAG GACGCAGCCTGCAAGAAACAGTCA ACATCAcgtacgctgcaggtcgac	knockout
2149	AUX1_ko_r	ATTTGTATAAAGTACATATCAAAAAC AACTGAGCGAAGCAGGCACACAAG GGAAAatcgatgaattcgagctcg	knockout
2165	Aux1_5UTR_check_f	ggctcaatgagagcgtggc	yeast colony PCR

2166	Aux1_3UTR_check_r	GATACGCCTTCCTTGACC	yeast colony PCR
2235	GFPamp_pYM12_f	cgcGGATCCggagcaggtgctggtgctgg	GFP Amplification
2236	GFPamp_pYM12_r	GAagatctTTTGTACAATTCATCCATACC	GFP Amplification
2330	clonNAT_for	AATCGGACGACGAATCGGACG	yeast colony PCR
2347	f_BamHI_IST2UTR	GAAGCTTTAAAAAAAAGCTAGGAtccTAACAAATTTTATTTTTATAATATGG	site directed mutagenesis
2348	r_BamHI_IST2UTR	CCATATTATAAAAATAAAATTTGTTAggaTCCTAGCTTTTTTTTAAAGCTTC	site directed mutagenesis
2356	scp160_check	GAACGTCTAAGTACACAACAGC	yeast colony PCR
2357	asc1_check	CATTGGGCTATTCCTTTAATTG	yeast colony PCR
2360	clonNAT_rev (maria)	CCGTGTCGTCAAGAGTGGTAC	yeast colony PCR
2395	MYO4_del_f	CTAATTCTAAAACACAAAAAAACAAAAAAAATCCTATAACCAGTTCTCCCGCCgtacgctgcaggtcgac	knockout
2420	SHE2_ko_f	GTAAACCCTCCTTAATTTTCCTTTTGCATAATACCAGACACTTAAAAcgtacgctgcaggtcgac	knockout
2425	MYO4_5'UTR_f	cattgttaccagtttggcgagac	yeast colony PCR
2426	HMG1_bp64_r	TATGAATTGGTCGTTTCGCCG	sequencing
2427	HMG1_bp2037_f	GACTACGACCGCGTATTTGGCG	sequencing
2428	IST2_RT_f	CTACAGATGCTACTCAGCC	RT-PCR
2429	IST2_RT_r	GCTTCTTTTTCAGCTTATGC	RT-PCR
2495	kanMX_outfor	GCAGTTTCATTTGATGCTCGATGAG	yeast colony PCR
2496	natNT2_outfor	CGCTCTACATGAGCATGCCCTGCCC	yeast colony PCR
2497	IST2_ORF2718-39_f	GCTGGAGTGAAGAATGTCACG	sequencing
2550	pGEX_Tev_sequ_f	GCATGGCCTTTGCAGGGCTG	sequencing
2551	pGEX_Tev_sequ_r	CATGTGTCAGAGGTTTTCACCGTC	sequencing
2568	YBR027C_ko_f	GAGAGAAGGCTCGGATCTGCACTGACTTACTTTTTTTTGTTTTTTTGcgtacgctgcaggtcgac	knockout
2569	YBR027C_epi_r	CTGATGAGCTGAGGACGACATGTAACGTTTGAATGTGGGAAGCATCTTGatcgatgaattcgagctcg	knockout
2570	YOL073C_ko_f	GGAGAGAAAGTCAACGACATAAAAAGCAAACACAATAGTCTACAAATAcgtacgctgcaggtcgac	knockout
2571	YOL073C_epi_r	CTAAACCGTTGCTATGTTTATTTGTTTATGTAGGTATATGCTGATATAAAatcgatgaattcgagctcg	knockout
2572	YJL048C_ko_f	GACATTTTTGACCCTCAAAGGAAGTGAATTACAGGTATTGAATAACAGAAcgtacgctgcaggtcgac	knockout

2573	YJL048C_epi_r	GAAAGAAAATATGTGTGAATAACCAAATAGGAAATAAACAAAAGCACATatcgatgaattcgagctcg	knockout
2574	YML088w_ko_f	GAGTTAAAAACCTTTATCAGGTGGCCGACACTAGGGAATAAGACAGCcgtacgctgcaggtcgac	knockout
2575	YML088w_epi_r	CTATAAATAAAATATTTAACATATGCTCTTCCAAATGTACATACTTatcgatgaattcgagctcg	knockout
2611	YJL048C_5UTR_f	GATTCGTTAGTAGTTGTATAGGGAC	yeast colony PCR
2612	YJL048C_3UTR_r	CACGTTGACGAACTGAGTGAC	yeast colony PCR
2613	YML088w_5UTR_f	CATTTTCTCGCATGTGGCGG	yeast colony PCR
2614	YML088w_3UTR_r	GAGGAATAAAGTCCGACATTTTTTTC	yeast colony PCR
2615	YBR027C_5UTR_f	GCAAGTCCCCGGATATGTTC	yeast colony PCR
2616	YBR027C_3UTR_r	CTGACAAAACCTTGGTACAATCC	yeast colony PCR
2617	YOL073C_5UTR_f	GAATGCCATTGATGTGAAGATGG	yeast colony PCR
2618	YOL073C_3UTR_r	CAACCCAACAATACTAAAGCCAG	yeast colony PCR
2598	SEC3_ko_f	GCCAGATATCTCCAGCTAGGTAACAAGGCTACGCAATTTATTCTATATTcgtacgctgcaggtcgac	knockout
2599	SEC3_epi_r	CTTAATTAGTCTAAATATGTAATATGAAGCGACAATGCAGAGGTTACatcgatgaattcgagctcg	knockout/tagging
2585	IST2_ORF_1113_r	CCATGCTAAATTGAATCAGTTGG	sequencing
2586	IST2_ORF_619_r	CAACATGATAACCGAACTAGC	sequencing
2587	IST2_ORF_2408_r	GTTATTTTCGCCACCAGTAACAG	sequencing
2588	IST2_ORF_1923_r	CCGAGTTCTTGGTGCAACTTC	sequencing
2589	IST2_ORF_313_r	GATAGGTTGTACAACTCATACTG	sequencing
2619	SEC3_kocheck_f	GGCAAATACTAACTTGGTGAACAC	yeast colony PCR
2620	SEC3_kocheck_r	CAATGAAGCTAACTAATATTCTGTTCC	yeast colony PCR
2621	SHE3_kocheck_r	ccgttgtgagtgaccgaaagtg	yeast colony PCR
2631	SEC3_kocheck_f	CTGAGTCGGTGCCAGATATC	yeast colony PCR
2632	HIS3 MX6 rev	CGACTCTTCAGGTAAGGGAGC	yeast colony PCR
2633	HIS3 MX6 out for	GTAATGACCATCATCGTGCTG	yeast colony PCR
2650	Ash1_-110bpStart_f	cctatcgctcctgtcctatcc	sequencing
2656	Ash1_XhoI_3'UTR230 bp_r	CCGCTCGAGgagaagttattagaatgatttcac	sequencing
2734	ASH1_ORF586_f	CTGATCTTACCCATTGGTGTAAGG	sequencing
2735	ASH1_ORF486_f	CTACCATCACTAAGGCATCTGC	sequencing
2736	ASH1_ORF1272_r	CGTCGGTGTGGAGGGAGATGG	sequencing

2737	ASH1_ORF1029_r	GGCATGGGAAATGAATTTCCACG	sequencing
2774	SHE3tag_f	CCACTCATAAGAAAAAGGGAAATAG TATGGTTGTTCACGGGGCCCAATC Ccgtacgctgcaggtcgac	tagging
3024	Kann_ORF_rev	GAAACGTGAGTCTTTTCCTTACCC	yeast colony PCR
3141	ASH1_E3_r	GTGTCGAATGAAAATGAAAGAAAAT G	RT
3140	ASH1_E3_f_T7	ttaatacgactcactatagggCCGTTGCTTAT TTTGTAATTACATAAC	RT
3158	5'RDN18-1_RT2	GGGATCGGGTGGTGTTTTTT	RT-PCR
3159	3'RDN18-1_RT2	CCAGAACCCAAAGACTTTGATTTC	RT-PCR

4.6. Plasmids

pRJ	name	origin
88	YEplac181-*ASH1*	pC3319 in Long *et al.*, 1997
132	YEplac195-*ASH1*	Jansen lab plasmid collection
135	pFA6a-*HIS3MX6*	Wach *et al.*, Yeast , 1994
138/144	YCplac22	Gietz and Sugino, Gene, 1988
139	YCplac33	Gietz and Sugino, Gene, 1988
140	YIplac128	Gietz and Sugino, Gene, 1988
141	YIplac204	Gietz and Sugino, Gene, 1988
142	YIplac211	Gietz and Sugino, Gene, 1988
143	YEplac181	Gietz and Sugino, Gene, 1988
138/144	YCplac22	Gietz and Sugino, Gene, 1988
145	YCplac111	Gietz and Sugino, Gene, 1988
146	pRS303	Sikorski & Hieter, Genetics, 1989
276	pYM2	Knop *et al.*, Yeast, 1999
286	pYM12	Knop *et al.*, Yeast, 1999
308	pRS426	Christianson *et al.* , Gene, 1992
413	YEplac195	Gietz and Sugino, Gene, 1988
577	pRS426-*IST2*-full length	Matthias Seedorf, ZMBH Heidelberg
630	pGEX-GST-Tev-She2p	Jansen lab plasmid collection
673	pRS426-*WSC2-full length incl. UTRs*	Matthias Seedorf, ZMBH Heidelberg
700	pRS424	Christianson *et al.* , Gene, 1992

721	pRS313	Sikorski & Hieter, Genetics, 1989
741	pG14-pGPD-NLS-HA-MS2-DSRed	A. Jaedicke in Schmid *et al.*, 2006
915	YIp211-pGAPDH-HMG1-GFP	Wihovsky *et al.*, MBC, 2000
916	YCplac111-*SHE2*-ATG-KpnI	Thesis, T.G. Du
920	YCplac111-GFP::She2	Thesis, T.G. Du
921/2	YCplac111-GFP::She2	Thesis, T.G. Du
1062	pRS423	Christianson *et al.* , Gene, 1992
1063	pRS313-pGAL1-ASH1-MS2(6)	A. Jaedicke in Schmid *et al.*, 2006
1101	YCplac22-She2 A1/2-16	Thesis, T.G. Du
1146	YCplac111-She2p N36S	this study
1147	YCplac111-She2p R63K	this study
1148	YCplac111-She2p N36S R63K	this study
1149	YCplac111-She2p N36S,R63K	this study
1150	pRS313-She2p N36S,R63K	this study
1213	pFA6a-*natNT2*	Janke *et al.,* Yeast, 2004
1313	2xGFP-tag-cassette	this study
1314	pRS426-IST2-minusXho/BamHI sites	this study
1337	pRS426-*IST2-BamHI3'UTR*	this study
1338	pRS426-IST2-6xMS2	this study
1341	YCp22-*pGAPDH-HMG1-GFP*	this study
1342	YCp33-*pGAPDH-HMG1-GFP*	this study
1343	YCplac111-*pGAPDH-HMG1-GFP*	this study
1347	pFA6a-*kanMX6*	Wach *et al*., Yeast, 1994
1605	YCplac111-She2p L130S	this study
1606	pRS313-She2p L130S	this study
1384	pGEX-GST-Tev-She2p N36S	this study
1385	pGEX-GST-Tev-She2p R63K	this study
1386	pGEX-GST-Tev-She2p S120Y	this study
1387	pGEX-GST-Tev-She2p L130S	this study
1398	Sec63p-RFP (2 µ, *URA3*)	S. Michaelis, John Hopkins, Baltimore, USA (pSM1960)
1399	p431-*GAL1-IST2-6xMS2*	this study
1403	pGEX-GST-Tev-TIS11	this study
1404	pGEX-GST-Tev-SEC53	this study
1412	pGEX-GST-Tev-She2p N36S,R63K	this study
1482	YCplac111-She2p-ΔHelix	Marisa Mueller; Gene Center, Munich
1517	YCplac22-She2p-ΔHelix	Marisa Mueller; Gene Center, Munich

4.7. *E. coli* strains

Strain	essential genotype
TOP10 cells (molecular biology)	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen)
XL1-Blue (molecular biology)	hsd R17 rec A1 end A1 gyrA46 thi-1 sup E44 relA1 lac [F' pro AB lacI^{q}ZΔ M15 Tn10 (Tet^r)] (Stratagene)
BL21 (DE3)/RIL (protein expression)	B F$^-$ ompT hsdS($r_B^-m_B^-$) dcm+ Tet^r galλ (DE3) EndA Hte [argU ileY leuW Cam^r] (Stratagene)

RJB	transformed bacterial strain	+ pRJ	Plasmid name
441	BL21(DE3)pRIL	630	pGEX-GST-Tev-She2p
442	BL21(DE3)pRIL	1384	pGEX-GST-Tev-She2p N36S
443	BL21(DE3)pRIL	1385	pGEX-GST-Tev-She2p R63K
444	BL21(DE3)pRIL	1386	pGEX-GST-Tev-She2p S120Y
445	BL21(DE3)pRIL	1387	pGEX-GST-Tev-She2p L130S
448	BL21(DE3)pRIL	1412	pGEX-GST-Tev-She2p N36S,R63K

4.8. *S. cerevisiae* strains

RJY	essential genotype
358	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+*
359	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+*
361	*MAT a, his1*
362	*MAT alpha, his1*
2049	*MATa ; his3Δ1 ; leu2Δ0 ; met15Δ0 ; ura3Δ0*
2050	*MAT alpha; his3Δ1; leu2Δ0; ura3Δ0*
2053	*MAT alpha; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; she2::kanMX4*
2292	*MAT a his3 leu2 HMG1-GFP::URA3, ash1::TRP1*, pRJ88 (YEplac181-*ASH1*)
2299	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 myo4::kanMX6*
2321	*MAT a URA3::HMG1-GFP*
2323	*MAT a URA3::HMG1-GFP ash1::TRP1 myo4::kanMX6*, pRJ88 (YEplac181-*ASH1*)
2339	*MAT a URA3::HMG1-GFP*, pRJ1063 (pRS313-*prGAL1-ASH1-MS2(6)*) pRJ741 (pG14-*prGPD1-MS2CP-RedStar*)
2369	*MAT alpha his3 leu2 TRP1 HMG1-GFP::URA3*, *myo4::kanMX6*,

2370	*MAT a his3 leu2 TRP1 she2::kanMX6 HMG1-GFP::URA3,* pRJ88 (YEplac181-*ASH1*)
2372	*MAT alpha URA3::HMG1-GFP myo4::kanMX6,* pRJ1063 (pRS313-*prGAL1-ASH1-MS2(6)*) pRJ741 (pG14-*prGPD1-MS2CP-RedStar*)
2474	*MAT a his3, leu2,TRP1, HMG1-GFP::URA she3::kanMX6*
2475	*MAT a his3 leu2 TRP1 HMG1-GFP::URA she3::kanMX6,* pRJ88 (YEplac181-*ASH1*)
2479	*MAT a URA3::HMG1-GFP ash1::TRP1 MYO4-HA3::HIS3MX6*, pRJ88 (YEplac181-*ASH1*)
2656	*MAT a his3, TRP1, she2::kanMX4, HMG1-GFP::URA3,* pRJ88 (YEplac181-*ASH1*), pRJ1150 (pRS313-She2pN36SR63K)
2657	*MAT a his3, TRP1, she2::kanMX4, HMG1-GFP::URA3,* pRJ88 (YEplac181-*ASH1*), pRJ721 (pRS313)
2731	*MAT a URA3 ::HMG1-GFP aux1 ::kanMX6*
2794	*MAT a URA3::HMG1-GFP aux1::kanMX6*, pRJ1063 (pRS313-*prGAL1-ASH1-MS2(6)*) pRJ741 (pG14-*prGPD1-MS2CP-RedStar*)
2807	*MAT alpha ura3-52 his3Δ200 leu2Δ1 srp101-47-ts*
2812	*MAT a his3 leu2 met15 ura3 scp160 ::kanMX4*
2813	*MAT alpha his3 leu2 lys2 met15 ura3 scp160::kanMX4*
2814	*MAT a, his3, leu2, met15, ura3, asc1::kanMX4*
2815	*MAT alpha, his3, leu2, lys2, met15, ura3, asc1::kanMX4*
2830	*MAT alpha ura3-52 his3Δ200 leu2Δ1* (FY68 WT; for 2807, 2858, 2859, 2866, 2867)
2847	*MAT a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; trp1Δ*
2848	*MAT alpha; his3Δ1; leu2Δ0; ura3Δ0; trp1Δ*
2858	*MAT alpha ura3-52 his3Δ200 leu2Δ1 srp101-47-ts*, pRJ1063 (pRS313-*prGAL1-ASH1-MS2(6)*) pRJ741 (pG14-*prGPD1-MS2CP-RedStar*)
2859	*MAT alpha ura3-52 his3Δ200 leu2Δ1 srp101-47-ts*, pRJ1343 (YCplac111-*HMG1-GFP*)
2862	*MAT a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0, aux1::kanMx6*
2866	*MAT alpha ura3-52 his3Δ200 leu2Δ1, pG14-MS2-DSRed (p741), pRS313-pGAL1-ASH1-MS2(6) (p1063)*
2867	*MAT alpha ura3-52 his3Δ200 leu2Δ1, YCplac111-pGAPDH-HMG1-GFP (p1343)*
3279	*MAT a, his3, leu2, TRP1, HMG1-GFP::URA3, YBR027C::natNT2*
3280	*MAT a, his3, leu2, TRP1, HMG1-GFP::URA3, YOL073C::kanMX6*
3281	*MAT a, his3, leu2, TRP1, HMG1-GFP::URA3, YJL048C::natNT2*
3282	*MAT a, his3, leu2, TRP1, HMG1-GFP::URA3, YML088w::natNT2*
3031	*MAT a sec3::HIS3MX6* (ts, grows at 25°C on –His, does not grow on YPD!)
3283	*MAT a; his3D1; leu2D0; met15D0; ura3D0; trp1D, p1179 (MS2CP), p1398 (SEC63-RFP), p1063 (ASH1-6MS2), p922 (GFP-She2p)*
3307	*MAT a his3 leu2 TRP1 HMG1-GFP::URA she3::kanMX6 myo4::natNT2*

4.9. Antibodies

Name	Source	Dilution	Company
Primary Antibodies			
anti-Dpm1 (5C5)	mouse	1:5 000 (Western)	Molecular Probes
anti-GFP (B-34)	mouse	1:25 000 (Western)	Covance
anti-GST (B-14)	mouse	1:10 000 (Western)	Santa Cruz
anti-HA (3F10)	rat	1:1 000 (Western)	Roche Applied Sciences
anti-HA (16B12)	mouse	1:1 000 (Western)	HISS Diagnostics GmbH
anti-myc (9E10)	mouse	1:1 000 (IF,Western)	Evan *et al.*, 1985
anti-Pgk1 (22C5)	mouse	1:10 000 (Western)	Molecular Probes
anti-Rpa49p	rabbit	1:50 000 (Western)	Gift from H. Tschochner, Ratisbon
anti-Rpl13p	rabbit	1:10 000 (Western)	Gift from M. Seedorf, Heidelberg
anti-Sec61	rabbit	1:10 000 (Western)	Gift from M. Seedorf, Heidelberg
anti-She2p (323/4)	rabbit	1:2000 (IF)	Thesis T.-G. Du, 2007
anti-She2p (134/3)	rabbit	1:2 000 (Western)	Thesis T.-G. Du, 2007
Secondary Antibodies			
Alexa®488 anti-mouse-IgG	goat	1:250 (IF)	MoBiTec
Alexa®488 anti-mouse-IgG	rabbit	1:250 (IF)	MoBiTec
Alexa®488 anti-rabbit-IgG	goat	1:250 (IF)	MoBiTec
Alexa®488 anti-rat-IgG	donkey	1:100 (IF)	MoBiTec
Alexa®594 anti-mouse-IgG	goat	1:250 (IF)	MoBiTec
Alexa®594 anti-mouse-IgG	rabbit	1:250 (IF)	MoBiTec
Alexa®594 anti-rabbit-IgG	goat	1:250 (IF)	MoBiTec
anti-mouse-IgG-HRPO	goat	1:5 000 (Western)	Dianova
anti-rabbit-IgG-HRPO	goat	1:5 000 (Western)	Dianova
anti-rat-IgG-HRPO	goat	1:5 000 (Western)	Dianova

5. Methods

Many of the following microbiological, biochemical methods and in particular molecular biological methods such as restriction digest, dephosphorylation of fragments, ligations and separation of DNA in agarose gels are based on standard techniques (Ausubel, 2000; Sambrook *et al.*, 2001). Commercially available kits were used according to the manufacturer's instructions. Point mutations were inserted by quick change site directed mutagenesis using the QuickChange® Site-directed Mutagenesis Kit (Stratagene). Plasmids were sequenced by the in-house sequencing service (AG Blum, Gene Center Munich). For all methods described, deionised sterile water, sterile solutions and sterile flasks were used.

5.1. *E. coli*-specific techniques

5.1.1. Preparation of competent *E. coli* cells

E. coli cells were grown at 37°C in 1l of LB medium (16 g bacto tryptone, 10 g yeast extract, 5 g NaCl pH 7.4). At an OD_{600} ~0.7–0.8 cells were chilled on ice for 30 min and then harvested by centrifugation (15 min, 5000 rpm, 4°C). In the following, all steps were performed at 4°C, with prechilled sterile materials and solutions. For the preparation of electrocompetent bacteria, sedimented cells were washed once with 1l water centrifuged and washed a second time with 0,5 l water containing 10% (v/v) glycerol. After another centrifugation step, cells were resuspended in 3 ml 10% (v/v) glycerol and after shock freezing stored as aliquots at -80°C in 100 µl. For the preparation of chemically competent cells, sedimented cells were carefully resuspended in half of the culture volume of 0.1 M $CaCl_2$ and cooled on ice for 30 min. Finally competent cells were pelleted and resuspended in 1/40 volume of 0.1 M $CaCl_2$/10% glycerol, divided into aliquots of 100 µl, shock frozen in LN_2 and stored at -80°C.

5.1.2. Transformation of competent *E. coli* cells

Both types of competent *E. coli* cells were thawed on ice shortly before transformation. For electroporation, 25 µl competent cells were mixed with 10 ng plasmid DNA or 10 µl plasmid isolate from yeast which before had been micro-dialyzed on a nitrocellulose filter (Millipore, ∅13 mm, 0.025 µm pores) against water. This mix was then electroporated in a pre-chilled cuvette (0.1cm electrode gap) with a pulse of 1.8 kV and 25 µF at a resistance of 200 Ω. For transformation of chemically competent cells, 1-10 ng of plasmid DNA or 10 µl of a ligation

mix was pre-cooled and incubated with 50 µl thawed cells for 15 min on ice. Cells were then heat shocked for 1min at 42°C and incubated on ice for 2 min. Subsequent to both transformation methods, cells were recovered in 1ml pre-warmed LB medium at 37°C for 1h and plated on antibiotic containing LB agar plates overnight at 37°C. Candidate colonies were picked to inoculate a 3ml LB Medium containing the appropriate antibiotics (e.g. 100 µg/ml ampicillin) for plasmid preparation (Miniprep), and incubated in a 37°C-shaker over night.

5.1.3. Preparation of Plasmid-DNA

Isolation of pure plasmid DNA for restriction analysis and sequencing was performed with the Nucleospin Miniprep Kit (Machery&Nagel).

5.2. *S. cerevisiae*-specific techniques

5.2.1. Cell density of yeast cultures

The cell density of a yeast culture was determined in a spectrophotometer at a wavelength of 600 nm. One OD at 600 nm (1 OD_{600}) corresponds to 2.5 x 10^7 cells.

5.2.2. Culture of *S. cerevisiae*

Yeast strains were cultured in either full-medium or synthetic complete (SC) medium at 30°C or at 24°C in case of temperature sensitive strains. Full-medium contained 1% yeast extract (Becton Dickinson, Heidelberg), 2% Bacto-Peptone (Becton Dickinson, Heidelberg) and either 2% glucose (YPD) or 2% galactose (YPG). Synthetic complete media contained 0.67% yeast nitrogen base (Formedium, Norwich, UK), 0.06% CSM dropout mix (including all essential amino acids except the amino acids used as auxotrophy markers, i.e. leucine, tryptophane, histidine, uracil and adenine) and either 2% glucose (SDC) or 2% galactose (SGC). 5-FOA, G418 and clonNAT were added to a final concentration of 1g/l, 300 mg/l or 100 mg/l respectively.

5.2.3. Transformation of yeast cells

'One-step' transformation with plasmid DNA was performed according to Chen *et al.* (Chen *et al.*, 1992). The protocol for high-efficiency yeast transformations with linear DNA fragments and PCR products was adapted from Gietz and Schiestl (Gietz *et al.*, 1992; Schiestl *et al.*, 1989). Cells from a mid-log phase growing culture were harvested by

centrifugation (500g, 5 min, room temperature), washed first with 1/5 volume sterile water, then with 1/10 volume SORB solution (0.1 M LiOAc, 10 mM Tris-HCl pH 7.5, 1mM EDTA, 1.0 M Sorbitol) and resuspended in 200µl SORB solution. 50 µl of this cell suspension were gently mixed with 10µl of carrier DNA (salmon sperm DNA, 2 mg/ml), up to 4 µg of linear PCR fragment, 6 volumes of PEG (40 % PEG 4000 in 0.1 M LiOAc, 10 mM Tris-HCl, pH 7.5, 1mM EDTA, 1.0 M Sorbitol) and incubated at 30°C for 30 min. To enhance the transformation rate DMSO in 10% final concentration was added (Hill *et al.*, 1991) before heat shocking the cells for 15 min at 42°C. Cells were then centrifuged at 400g for 3min at room temperature, resuspended in 100µl sterile water and plated on the selective SC medium plates. For transformation of temperature sensitive strains the 30°C step was performed at 25°C for 40 min and the duration of the 42°C heat shock was reduced to 5 min. If G418 or clonNAT was used for selection, transformed cells were first shaken for 3h or over night respectively in liquid YPD medium before plating. Selection of transformants was carried out for 2-3 days at 30°C (or 24°C for temperature sensitive strains). If necessary, transformants were replica-plated on selection plates to remove the background.

5.2.4. Preparation of yeast genomic DNA

Genomic DNA from yeast was extracted in order to use it as a template for PCR amplification of a desired gene. Cells from a (24 h) stationary yeast culture (15ml) were pelleted by centrifugation (3000 xg, 5 min, 4°C), washed once with 0.5 ml water and resuspended in 200 µl breaking buffer (2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). Subsequently, 200µl phenol/chloroform/isoamyl alcohol (24:24:1 v/v/v; Roth) and 300 mg acid washed glass beads (∅ 425-600µm; Sigma) were added, and the mixture was vibraxed for 5 min (highest speed, 4°C). The lysate was mixed with 200µl TE buffer, centrifuged for 5 min at 16000 xg, 23 °C and the upper, aqueous layer transferred to a new tube. The genomic DNA was precipitated by addition of 1ml 100 % ethanol followed by a 3 min centrifugation at 16000 xg, 23°C. The pellet was resuspended in 0.4 ml TE buffer and RNA contaminants were digested by treatment with 30 µl of DNase-free RNase A (1 mg/ml) for 5min at 37°C. Next, the genomic DNA was re-precipitated by addition of 10µl ammonium acetate (4M) and 1ml ethanol (100%). After a brief centrifugation, the pellet was shortly dried at 37°C to remove residual traces of ethanol and finally resuspended in 50 µl TE buffer. The quality of isolated genomic DNA was assessed by agarose gel electrophoresis.

5.2.5. Isolation of plasmid-DNA from yeast

After cloning in yeast via gap repair for example, the plasmid DNA was re-isolated for its direct propagation in *E. coli*. 15 ml overnight yeast culture were harvested (5 min, 3000 xg,

4°C), resuspended in 300 µl lysis buffer (1% (v/v) SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0), and after addition of an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1 v/v/v; Roth), the liquid volume was filled with acid-washed glass-beads (∅ 425-600µm; Sigma). Cells were then lysed by shaking on a vibrax (5 min, highest speed). The DNA was recovered by a 5 min centrifugation at 16000 xg, 4°C. The aqueous upper phase containing DNA was transferred to a new tube. For ethanol precipitation, 0.1 volume sodium acetate (3 M, pH 4.8) and 2.5 volumes pre-chilled ethanol were added to the DNA solution and incubated at –20°C for 30 min. The mixture was then centrifuged for 20min at 16000 xg, 4°C. The DNA pellet was washed once with 0.5 ml 70% ethanol. After centrifugation, the DNA was air-dried and resuspended in an appropriated volume of TE buffer. The recovered plasmid DNA was subsequently transformed into *E. coli* by electroporation.

5.2.6. Gap repair cloning via homologous recombination

In *S. cerevisiae* one molecular feature namely its capability for homologous recombination can be used for plasmid construction via gap repair. A PCR fragment (including a gene of choice accompanied by a promoter and terminator) can be amplified with two primers each containing short stretches homologous to the backbone of the target vector. The amplification product is then co-transformed with the linearized target vector in yeast. Recombination of the two linear DNA fragments subsequently results in an intact plasmid which must be recovered from yeast for further propagation in *E. coli*.

5.2.7. Genomic integration via homologous recombination

Chromosomal gene deletions or insertions of epitope tags were performed by a PCR strategy (Janke et al., 2004; Knop et al., 1999; Longtine et al., 1998). Basis of this genomic manipulation are special cassette modules consisting of a selection marker and in case of C-terminal tagging an additional sequence encoding for a tag. The PCR products generated from these template cassettes contain flanking homologous sequences which allow their targeted integration into the correct genomic loci. This is achieved by using PCR primers that have a 5' end (45-55 bp) corresponding to the respective target genes and 3' ends (22 bp) that anneal on and permit amplification of the chosen cassette. For gene deletions, the forward oligonucleotide contains 55bp immediately upstream of the start ATG whereas the reverse primer constist of a stretch of up to 55 bp downstream of the STOP codon. For integration of C-terminal epitope tags the forward primer must instead include 55 bp of the ORF just 5' of the STOP codon (excluding the STOP). After amplification of the cassette, the PCR fragment was concentrated by ethanol precipitation and transformed into the desired yeast strains. In case of gene disruption, homologous recombination leads to replacement of

the complete ORF by the marker gene contained within the PCR cassette. Upon tag integration however the STOP codon of the target gene is substituted by the tag's sequence followed by a marker gene. To assess whether the correct integration event had occurred, candidate yeast clones were subjected to yeast colony PCR (for gene deletion) or WCEs were prepared and used for Western Blot analysis (in case of epitope tagging).

5.2.8. Yeast colony PCR

After transformation single yeast colonies growing on selective plates were subjected to colony PCR in order to test whether they have integrated the gene disruption cassette at the correct locus. Simultaneously each colony was streaked out on new selective plates. For the colony PCR, one inoculation loop of cells from each colony was resuspended in 100 µl 0.02 M NaOH. For cell lysis an equal volume of glass beads was added and the suspension was incubated on a thermo-mixer for 5 min at 99°C, 1400 rpm. Finally the lysate was shortly incubated on ice, pelleted for 30 s at 16000xg and 5 µl of the supernatant was used as a template in 50 µl PCR reactions.

5.2.9. Analytical whole cell extracts (WCEs)

Analytical WCEs were used to check yeast clones for correct integration of the tagging cassettes. Preparation was performed as described previously (Knop et al., 1999). About 2 OD_{600} of a logarithmically grown yeast culture or one inoculation loop of cells from a freshly grown yeast plate were resuspended in 1 ml of water. This cell suspension was vigorously mixed with 150 µl of a freshly prepared alkaline solution (1.85 M NaOH, 7.5% ß-MeEtOH) and cells were lysed by incubation on ice for 15 min. After addition of 150 µl of 55% TCA, precipitation of the cell lysate was performed for 15 min on ice. Following a 30 min centrifugation at 16 000xg and 4°C the supernatant was discarded and the tubes were centrifuged shortly for a second time to remove residual traces of TCA. The pellet was then resuspended in 100 µl of HU-buffer (8 M urea, 5% SDS, 200 mM Tris pH 6.8, 1 mM EDTA, with a trace of bromophenol blue as colouring and pH indicator, 1.5% DTT; the buffer is stored without DTT at -20°C). In case the samples turned yellow they were neutralized with 1-3 µl 2 M Tris base. Proteins were then denatured at 65°C for 10 min in a thermomixer and insoluble aggregates were pelleted by centrifugation for 2 min at 16 000xg. Aliquots of the samples were analyzed by SDS Page and Immunoblotting.

5.3. SDS-PAGE and Western Blotting

SDS-PAGE was performed according to Laemmli (Laemmli, 1970) on a Hoefer SE 260 Mighty Small II system (Amersham Pharmacia). Proteins were transferred onto nitrocellulose membrane using a semi-dry blotting machine (Peqlab) for 1 h at 7 V. After transfer, the membrane was blocked with blocking buffer (2% milk-powder in PBS) and incubated overnight at 4°C with the first antibody dissolved in blocking buffer. Excess of first antibody was removed by washing the membrane 6 times for 5 min with blocking buffer at RT. The membrane was incubated with secondary antibodies diluted in blocking buffer for 1h at RT. Visualization of immuno-decorated proteins was performed using an ECL-Kit (Applichem), either followed by exposure of the membrane to light-sensitive films (GE Healthcare) and subsequent developing using a Kodak Xomat M35 developing machine or by direct analysis with the Fuji LAS-3000 mini chemiluminescence imaging system.

5.4. Purification of recombinant She2p and She2p mutants from *E. coli*

5.4.1. Recombinant expression in *E. coli*

GST-She2p and GST-She2p-Mutants were recombinantly expressed in the *E. coli* strain BL21(DE3)/RIL (RJB343) using pGEX-GST-TEV-*SHE2*/*she2* plasmids. 800 ml of LB medium containing ampicillin and chloramphenicol were inoculated with a stationary overnight culture to a starting OD_{600} of 0.2 and grown at 37°C until an OD_{600} of 0.8. 200 ml cold LB (4°C) medium was added, and expression was induced by the addition of 1 mM IPTG and incubation for 5-6 hours at 25°C. Cells were harvested in a SLC6000 rotor at 7800x g for 10 min. After washing with 200 ml water, cells were pelleted in a GSA rotor for 10 min at 7800x g, and pellet was frozen in LN_2 and stored at -20°C.

5.4.2. Lysis of cells

For the lysis, cells were resuspended in 30 ml lysis-buffer (25 mM Hepes-KOH pH 7.5, 0.1 mM EDTA pH 8, 1 M NaCl, 2 mM DTT, 1x protease inhibitors) containing 100 mg/l Lysozyme and rotated in a 50 ml Falcon tube for 30 min at 30°C. After 3 rounds of freezing (LN_2) and thawing (37°C water bath), cells were subjected to sonification with a flat tip in a 50 ml steal beaker. Cells were broken 4 times for 5 min with a pause of 5 min on ice in the Sonifier® (70% output, 40% duty cycle). NP40 was added to the lysate in a final concentration of 0.1% and rotated for 30 min. Cell debris was pelleted in a SS34 rotor at 4°C at 15.000 rpm for 30 min. Samples of 100µl of pellet and supernatant were taken and analyzed via SDS-PAGE.

5.4.3. Affinity purification

The GST-She2 fusion protein was purified using 500 µl slurry of Glutathione Fast Flow Sepharose (Amersham Pharmacia). Beads were pre-washed with 10 ml lysis buffer and added to the lysate for binding in a 50 ml Falcon tube while rotating at 4°C for one hour. After binding, beads were washed with 10 ml wash buffer I (25 mM Hepes-KOH pH 7.5, 0.1 mM EDTA pH 8.0, 12.5 mM $MgCl_2$, 1 M NaCl, 0.1% NP40, 2 mM DTT, 1x protease inhibitors) and washbuffer II (25 mM Hepes-KOH pH 7.5, 0.1 mM EDTA pH 8.0, 12.5 mM $MgCl_2$, 0.7 M NaCl, 0.1% NP40, 1 mM DTT), and finally equilibrated in 10 ml TEV-buffer (50 mM HEPES-KOH pH 7.5, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.15% NP40, 1 mM PMSF – add DTT and PMSF freshly before use) using a Poly-Prep Chromatography Column (BioRad). The remaining slurry was then transferred to a Mobicol column (MobiTec). After addition of 500 µl TEV-buffer and 10 µl of TEV-protease, the column was rotated for 1 ½ hour at 16°C for TEV-cleavage. She2p was eluted by centrifugation of the Mobicol at 2000 rpm for 2 min. An aliquot (10 µl) of the eluate (~500 µl) was removed for analysis in SDS-PAGE. The His_6-tagged TEV-protease was removed using NiNTA sepharose (Quiagen). NiNTA-beads were washed 3 times with 10 ml TEV–buffer in a Mobicol. The TEV-eluate was added to the beads and rotated for 2 hours at 4°C. Recombinant She2p was eluted into a fresh eppendorf tube by a short spin for 1 min at 2000 rpm in a cooling centrifuge. If necessary, concentration of the recombinant eluate was further increased using vivaspin® concentrators. The protein concentration was determined using the Bradford assay (Biorad) according to the manufacturer's manual. As standard a BSA solution (NEB) with a concentration of 10 mg/ml was employed. The She2p eluate was adjusted to a glycerol content of 20%, divided into small aliquots, shock frozen in liquid N_2 and stored at -80°C.

5.5. Subcellular Fractionation Experiments

5.5.1. Spheroplasting of yeast and cell lysis

Cell disruption by shearing the cell wall with glass beads is very fast and effective method. Nevertheless it is not applicable for most subcellular fractionation experiments as the strong mechanical forces arising damage intracellular organells and might possibly disrupt protein-membrane interactions. In order preserve subcellular integrity, it is therefore more recommendable to enzymatically convert yeast cells to spheroplasts and to disrupt them by application of gentle mechanical forces. For the subcellular fractionation experiments described here, yeast cells were treated as follows: In short, the appropriate amount of cells (up to 530 OD_{600}) was harvested and spheroplasted by Zymolyase treatment for 1h at 30°C under gentle shaking in 5 ml isotonic buffer SB (1.4 M sorbitol, 50 mM KP_i pH 7.5, 10 mM

NaN_3, 0.4% ß-MeEtOH, 2 mg/ml Zymolyase 20T). After this treatment intact spheroplasts were harvested through 8 ml of a sorbitol cushion (1.7 M Sorbitol, 50 mM KP_i pH 7.5) by centrifugation for 10 min, 600 xg, 4°C. The spheropellet was then resuspended in 6 ml Hepes lysis buffer (20 mM Hepes/KOH, 140 mM KOAc, 1 mM $MgOAc_2$, 1 mM EDTA, 100U/ml Superasin RNAse inhibitor (Ambion, Huntingdon, UK) and a protease inhibitor cocktail) and lysis was performed by either 12 passages through a 25 gauge needle or by dounce homogenisation using a loose pestle. After pelleting cell debris (5x 5 min at 400xg) the homogenate was further processed as described in each fractionation assay.

5.5.2. Velocity gradient centrifugation on discontinuous sucrose gradients

Velocity gradient centrifugation on 18-60% sucrose gradients was essentially performed according to Barrowman *et al.*, 2000 and Estrada *et al.*, 2003 (Barrowman *et al.*, 2000; Estrada *et al.*, 2003). In short, cells corresponding to 400 OD_{600} units were harvested, spheroplasted, lysed with a needle and cleared from cell debris as described above. 1 ml of the homogenate (corresponding to 66 OD_{600} units) was then loaded onto a linear 18%-60% gradient of sucrose in 20 mM Hepes/KOH, 140 mM KOAc, 1 mM $MgOAc_2$. Gradients were spun in a SW40 rotor for 2.3 h at 38000xg. 12x 1 ml fractions were collected starting close to the bottom of the gradient and the remaining pellet was resuspended in 1 ml lysis buffer. Fractions were TCA precipitated and resuspended in 100 µl SDS sample buffer. 20 µl of these were used for western blot analysis except for the top three fractions where only 7 µl were used in order to avoid overloading of the gel.

5.5.2.1. RNase Treatment

To entirely deplete extracts from mRNA, RNAse inhibitors were omitted from the lysis buffer and the lysate was treated with 0.2 mg/ml RNaseA (Roth) and 25 U/ml Micrococcal Nuclease (SIGMA) in presence of 1 mM $CaCl_2$ for 15 min at RT. The reaction was stopped by addition of 2 mM EDTA and by on cooling on ice. The RNAse/Micrococcal Nuclease treated as well as the mock treated lysate were carefully loaded on discontinuous gradients and further processed as above. After collecting the centrifuged gradient in 1 ml fractions, 800 µl of each sample were TCA precipitated for SDS-PAGE and immunoblotting. The remaining 200 µl were processed for RT-PCR analysis. Shortly, RNA was isolated by Phenol/Chloroform extraction, treated with RNase-free DNase (Promega) and reverse transcription was performed using oligo d(T)18 primers (NEB) and the BD PowerSript Reverse Transcriptase (Clontech). Different primer pairs was used on the cDNA for RT-PCR to asses the complete digest of RNA (*ASH1*: RJO 73+74; *IST2*: RJO 2428+2429; ribosomal rRNA *RDN18*: RJO 3158+3159).

5.5.2.2. EDTA Treatment

By addition of EDTA, ribosomes and polysomes can be disrupted into 40S and 60S subunits. To achieve this, lysis and gradients were performed in lysis buffer and sucrose solutions containing 10 mM EDTA. After SDS-PAGE and immunoblotting, polysome disruption was verified by using an antibody directed against a ribosomal protein (anti-Rpl13p). For quantification, ECL western blots were exposed for the shortest time possible that still gave a detectable signal on film, films were scanned at 600 dpi resolution and pixel values of the corresponding bands were determined using ImageJ 1.36b (http://rsb.info.nih.gov/ij/). After background subtraction, pixel values (= 'amount of antigenic material' in supplementary figure 3) were calculated using the integrated 'Analyze Gels' function of ImageJ. In order to compensate for unequal loading of the top 3 fractions (see above), these values were multiplied by three.

5.5.3. *In vitro* binding assay: Velocity gradient with WCE and recombinant protein

For *in vitro* binding experiments, a crude yeast lysate was prepared by spheroblasting and preclearing as described above except that instead of a *SHE2* wt strain, a *she2Δ* strain (RJY2370) was used. 1 ml of the lysate (corresponding to 66 OD_{600}) was pre-incubated with recombinant She2p and She2p-mutants (about 1.5 µg protein per reaction) for 30 min on ice. The suspension was then carefully loaded on an 18%-60% gradient. Centrifugation with a SW40 rotor, collection of the fractions and processing of the fractions was done exactly as described earlier.

5.5.4. Purification of ER membranes on a 2-step sucrose gradient

For the two-step microsome purification we used a scaled-down variant of a published protocol (Rieder *et al.*, 2000; Wuestehube *et al.*, 1992) originally designed to separate ER membranes from vacuolar and Golgi membranes. Logarithmically growing cells corresponding to 40 OD_{600} units were spheroplasted, lysed by dounce homogenisation and the cell homogenate pre-cleared as described above. Subsequently the lysate was spun for 10 min at 16000xg, the supernatant was removed and the membrane pellet spun again for 1 min at 13000xg to get rid of remaining supernatant. The resulting pellet was resuspended in 200 µl HEPES lysis buffer and loaded on top of a two-step sucrose gradient (1.5 ml cushions of 1.2 M and 1.5 M sucrose in HEPES lysis buffer). After centrifugation in a SW60Ti rotor (1.6 h at 80000xg), two bands at the interphases of the sucrose cushions were visible. The upper band was collected as fraction 2 and the lower band, representing microsomes, as fraction 4. Five additional fractions were harvested (F1 = layer on top of upper band, F3 =

layer between upper and lower bands, F5 and F6 = layer below lower band, F7 = pellet). Protein from these fractions was precipitated with TCA and processed as above.

5.5.5. Flotation Assay

For membrane flotation of ER membranes via equilibrium density centrifugation we adapted a protocol from Kanai *et al.* (Kanai *et al.*, 2004). 530 OD_{600} units of logarithmically growing cells were harvested, spheroplasted and lysed by dounce homogenisation and cleared from cell debris as described above. The crude lysate was mixed with gradient buffer (50 mM Hepes/KOH, 150 mM KOAc, 5 mM $MgOAc_2$) containing 85 % sucrose to give a final sucrose percentage of 50%. 3 ml of this suspension was layered on top of 3 ml 70% sucrose in gradient buffer. Two additional 3 ml cushions (40% sucrose and 0% sucrose) were layered on top. The 4-step gradient was spun in a SW40 rotor for 8.6 h at 38000xg. After centrifugation, fractions were collected from each cushion and the interphases (in total 7 fractions). Protein was precipitated with TCA and processed as above.

5.6. *In vitro* binding assay with flotation purified ER membranes

5.6.1. Preparation of yeast microsomal membranes

Preparation of yeast microsomes was in principle performed as described previously (Brodsky *et al.*, 1993; Rothblatt *et al.*, 1986). 4.5 l of yeast cell culture from a *she2Δ* strain (RJY2370) were harvested at an OD_{600} of 1.0-2.0 (SLC 6000 rotor, 5min, 5000 rpm, RT) and washed once with water. After weighing the cell pellet it was resuspended in 100 mM Tris-SO_4 pH 9.4, 10 mM DTT to a final concentration of 50 OD_{600}/ml and incubated for 15 min at RT. Following this preincubation, cells were washed once with 1.2 M sorbitol and then resuspended in 80 ml of YPD medium containing 0.7 M sorbitol, 20mM KP_i pH 7.4 and Zymolyase 20T (in a concentration of 2.5 mg Zymolyase/g cells). Spheroplast formation was allowed to proceed for 90 min at 30°C with very gentle swirling. The spheroplast suspension was then layered on “cushion 1” (0.8 M sucrose, 1.5% ficoll 400, 20 mM Hepes/KOH pH 7.4) in 50 ml conical tubes and was centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was carefully removed, and the spheropellet was resuspended in 0.7 M sorbitol, 20mM KP_i pH 7.4 at 1000 OD_{600}/ml and frozen in LN_2 for storage at -80°C. Alternatively, spheroplasts were directly further processed. In the latter case, the spheropellet was resuspended to 100-200 OD_{600}/ml in ice cold lysis buffer (100 mM sorbitol, 50 mM KOAc pH 7.4, 2 mM EDTA, pH 8.0, 20 mM Hepes/KOH, pH 7.4,1 mM DTT, 1 mM PMSF) and homogenized extensively on ice in a glass potter with tight fitting pestle. To collect microsomes, 15 ml “cushion 2” (1 M

sucrose, 50 mM KOAc pH 7.4, 20 mM Hepes/KOH pH 7.4, 1 mM DTT) were over layered with 15 ml lysate in several 50 ml conical tubes and centrifuged (10 min, 4000 rpm, 4°C). Material remaining in the upper 15 ml layer was recovered avoiding the interface and centrifuged (21000 xg, 10 min, 4°C). The membrane pellet was washed once with buffer 88 (20 mM Hepes/KOH pH 6.8, 150 mM KOAc pH 7.4, 250 mM sorbitol, 5 mM $MgOAc_2$) and pelleted again at 21000 xg for 10 min at 4°C. The microsome pellet was then resolved in about 2 ml of buffer 88 to a final concentration of OD_{260}= 40 in 2% SDS corresponding to a protein concentration of 10-12 mg/ml. Finally these yeast rough membranes (YRMs) were aliquoted, shock frozen in LN_2 and stored at -80°C.

For protease treatment of YRMs, purified microsomes before aliquoting and storage were adjusted to 10 mM $CaCl_2$ and either mock treated or treated with a combination of 2mg/ml Pronase E (20mg/ml stock; SIGMA) and 2mg/ml Proteinase K (20mg/ml stock; Roche) for 30 min at 37°C. Subsequent to digest, both mock and protease containing samples were treated with excess EDTA and EGTA and 1 mM PMSF to inhibit proteases. Only the protease-containing membranes were additionally incubated for 15 min at 80°C to inactivate protease activity by heat denaturation. This step was not performed for mock treated membranes to maintain the integrity of ER marker proteins. Both membrane preparations were then washed 2 times with buffer 88, resuspended in the original volume of buffer 88 and treated for storage at -80°C as described above.

5.6.2. Flotation purification of ER membranes

For flotation purification of YRMs, 5 µl of the above prepared microsomes were mixed with 200 µl cushion III (2.3 M sucrose in 50 mM Hepes/KOH pH 7.5, 5 mM $MgOAc_2$, 150 mM KOAc, 1.5 mM DTT). This cushion was layered at the bottom of an ultra clear SW55 tube (5x41 mm) and carefully covered with 360 µl of cushion II (1.9 M sucrose in 50 mM Hepes/KOH pH 7.5, 5 mM $MgOAc_2$, 150 mM KOAc, 1.5 mM DTT) followed by 120 µl of cushion I (50 mM Hepes/KOH pH 7.5, 5 mM $MgOAc_2$, 150 mM KOAc, 1.5 mM DTT). After centrifugation with adaptors in a SW55 Ti rotor for 90 min at 45 000 rpm and 4°C the membrane fraction was collected at the interface between cushion I and II. Harvested membranes were either TCA precipitated to check for composition via SDS-PAGE and Western blotting or they were used directly for the *in vitro* binding assay (see below).

5.6.3. *In vitro* pelleting assay

For the binding-assay, four fractions of flotation-purified membranes were combined and diluted with 2 volumes of binding-assay buffer (50 mM HEPES/KOH pH 7.5, 5 mM $MgOAc_2$, 150 mM KOAc, 1,5 mM DTT) to reduce the sucrose concentration. After re-solubilisation of

the membranes they were mixed and incubated with recombinant protein (850 ng She2p or GST) for 10 min at RT and 15 min on ice. This *in vitro* binding mix was then layered over a 500 µl sucrose cushion (1.2 M sucrose, 50 mM HEPES pH 7.5, 5 mM $MgOAc_2$, 150 mM KOAc, 1.5 mM DTT) and centrifuged in a TLA 120.2 rotor for 1h at 100 000 rpm, 4°C. Subsequent to centrifugation, the supernatant was collected, the lowest level of the cushion (225 µl) including the pellet was resuspended in 1 ml of binding-assay buffer and both were TCA precipitated. Samples were then analyzed by SDS-PAGE and immunoblotting.

5.7. Flotation assay with ER-like protein-free liposomes

5.7.1. Preparation of ER-like, protein free Liposomes:

Liposomes with ER-like lipid content (ergosterol 16%, phosphatidylcholine PC 40%, phosphatidylethanolamine PE 24%, phosphatidylserine PS 10%, phosphatidylinositol PI 10%) (Schneiter *et al.*, 1999; Tuller *et al.*, 1999; Zinser *et al.*, 1991) were prepared as follows (Qbadou, JCS 2003). All lipids used were solved in chloroform/methanol (Avanti Polar Lipids, Roth, Sigma). They were mixed to a total amount of 100 mg lipid in a round bottom flask darkened with aluminium foil and filled with N_2 gas. The lipid mixture was then dried with a rotary evaporator at 95 mbar under N_2 atmosphere and slow rotational speed for 2-3 h until all residual organic solvent was completely removed. The created lipid film was completely dissolved to a final total lipid concentration of 10mg/ml in degassed liposome buffer (20 mM HEPES pH 7.4, 100 mM NaCl) by gentle swirling at RT for about 1 h. The lipid emulsion was then passed 21 times through a 400 nm pore polycarbonate filter membrane mounted in an extruder (“LiposoFast-Basic”, Avestin) in order to create unilamellar liposomes. The liposomes were then aliquoted, shock frozen in LN_2 and stored at – 80°C.

For preparation of liposomes lacking PS and PI, the lipid composition was changed to 18% ergosterol, 41% PC and 41% PE. Apart from this change liposomes were prepared as described above.

5.7.2. *In vitro* binding and flotation of liposomes

In order to assess protein-liposome interaction, 1.36 µg (48 pmol) She2p or 1.45 µg GST were combined with 100 µl liposomes and 140 µl binding buffer (50 mM Hepes/KOH, 150 mM KOAc, 1 mM $MgOAc_2$, 1mM EDTA, 1mM DTT) and incubated for 15 min at RT followed by 10 min on ice to allow binding. 40 µl of sample were kept as “input” for SDS-PAGE and Western blotting. 200 µl were mixed with 3 ml binding buffer containing 70% sucrose to form the bottom of the gradient in a SW40 ultraclear polycarbonate tube. The sample containing

cushion was then over layered with 3 ml binding buffer containing 50%, 40% and 0% sucrose respectively. After centrifugation to equilibrium (22000 rpm, 16.5 h, 4°C, SW40 rotor) the lipid containing fraction of about 1ml was collected at the 40%-0% sucrose interface, TCA precipitated and resolved in 45 µl HU buffer. Flotation samples together with "input" samples were analysed by SDS-PAGE and immunoblotting.

For carbonate treatment, which is known to shear off peripheral membrane proteins (Fujiki *et al.*, 1982), 1 M Na_2CO_3, pH 11.5 solution was added to the She2p/liposome resuspension to a final concentration of 0.1 M Na_2CO_3, pH 11.5. Flotation was performed as described above with the exception that all sucrose cushions contained 0.1 M Na_2CO_3, pH 11.5.
For high salt treatment to test for ionic interaction forces, 2.5 M KCl was added to the She2p/liposome mixture to a final concentration of 1 M KCl. The flotation assay was carried out in a gradient consisting of sucrose cushions in binding buffer containing 1M KCl.

For the RNA competition assay, 48 pmol She2p was pre-incubated with either mock (binding buffer) or 480 pmol (10x) or 960 pmol (20x) of *in vitro* transcribed *ASH1* E3 element for 15 min at RT and 10 on ice in 140 µl RNAse free binding buffer containing RNasin® (Promega). Liposomes were added only afterwards and were incubated with the protein – mRNA mix under the same conditions. Centrifugation and sample processing was performed as mentioned above. The *ASH1* E3 element mRNA was synthesized with the Ambion MEGAshortscript T7 Kit according to the manufacturer's instructions. As template for reverse transcription, a PCR fragment amplified from pRJ88 with the oligonucleotide pair RJO 3140, 3141 was used.

5.8. Indirect immunofluorescence

Cellular distribution of a protein was detected in the microscope by indirect immunofluorescence (IF) using specific antibodies.

5.8.1. Preparation of cells

Cells of a logarithmically growing culture (10 ml) were fixed with formaldehyde in a final concentration of 3.7%. Fixation was performed in a shaking incubator for one hour either at 30°C or at 37°C in order to maintain non-permissive conditions. Cells were centrifuged and washed three times with spheroplasting buffer (1.2 M Sorbitol, 0.1 M Potassium phosphate (pH 7.4), and 0.5 M $MgCl_2$). They were subsequently spheroplasted in 500µl spheroplasting buffer containing 100 µg/ml of Zymolyase 100T and 0.2% 2-Mercaptoethanol for 45 minutes at 30°C. Spheroplasts were pelleted at low speed (3000 rpm/1000x g) in a tabletop

centrifuge for one minute. They were washed and finally resuspended in 200 µl spheroplasting buffer. The cell suspension was stored in aliquots at -80°C or directly used for immunofluorescence.

5.8.2. Immunofluorescence

Multi-well slides (Neolab) used for immunofluorescence microscopy were coated with drops of 0.02% Poly-L-Lysine for 5 min and washed with distilled water. A drop of the cell suspension (~10 µl) was applied onto each well for 5 min. Cells were blocked for 5 min with blocking solution (1x PBS, 1% BSA). A dilute solution of the primary antibody was put onto each well and incubated for 2 hours in a wet chamber. After three rounds of washing (1x PBS, 1% BSA, 0.1% Triton X-100), cells were incubated with diluted Alexa®-coupled secondary antibodies (Molecular Probes) in a darkened wet chamber for one hour. After another three rounds of washing, nuclei were stained with Hoechst Stain Solution (SIGMA) and cells were mounted in mounting solution (1x PBS, 80% glycerol). Cells were inspected with an Olympus BX60 fluorescence microscope (Olympus) and a 100x NA 1.3 DIC oil objective. Images were acquired using an ORCA ER CCD camera (Hamamatsu Photonics) controlled by Openlab 4.01 software (Improvision).

5.9. Fluorescent *in situ* hybridisation using oligonucleotides (FISH)

Cellular localization of mRNAs was determined in the microscope using fluorescently labelled antisense oligonucleotides for *ASH1* and DIG labelled anti-sense probes for *WSC2* mRNA.

5.9.1. Preparation of FISH probes

5.9.1.1. Fluorescently labelled (Cy3-conjugated) antisense DNA oligonucleotides (*ASH1*)

A stock solution containing 100 ng/µl of each oligonucleotide was diluted with DEPC treated water to yield aliquots sufficient for 6 wells. Aliquots of 10µl were dried in a speed-vac and stored at -80°C. (1 ng/µl oligonucleotides, 1 mg/ml *E. coli* tRNA and 1 mg/ml salmon sperm DNA in DEPC water)

5.9.1.2. DIG-labelled antisense probes (*WSC2*)

The anti *WSC2* probe was prepared by generating four different digoxigenin (DIG)-labelled, antisense *WSC2* RNA fragments with the MEGAshortscript T7 Kit (Ambion) according to the manufacturer's protocol. Shortly, four DNA fragments (250-310 bp long) were amplified from a *WSC2* template (pRJ675) with a set of primer pairs (RJO 1082 – 1089) with each reverse primer harbouring the T7 promoter for *in vitro* transcription. After Phenol-Chloroform

extraction of the fragments, reverse transcription with simultaneous DIG-labelling was performed with the MEGAshortscript T7 Kit to create antisense RNA probes. The four different anti-*WSC2* RNA-fragments were then mixed in equal amounts to yield an anti-*WSC2* probe which allows annealing over a long stretch of the *WSC2* ORF. The mix of the 4 probes (3.3 µg/µl) was diluted to 660 ng/µl, split into 10 µl aliquots and frozen at -80°C.

5.9.2. Preparation of cells

Cells of a logarithmically growing culture (10 ml) were fixed with formaldehyde (3.7 % final concentration). Fixation of cells was performed in a shaking incubator for one hour either at 30°C. Cells were pelleted and washed three times with Buffer B (1.2 M sorbitol, 100 mM potassium phosphate pH 7.4). Cells were spheroplasted for 10 min at 30°C in 200µl spheroplasting buffer (1ml contains: 100 µg Oxalyticase (Enzogenetics); 720 µl 1.4 x Buffer B (1.7 M sorbitol, 140 mM KP_i pH 7.4); 3.5 µl AEBSF (4-(2-Aminoethyl)-benzensulfofluorid; Applichem); 100 µl RVC (Ribonucleoside-Vanadyl-Complex, Sigma); 3 µl RNasin® (Promega); 2 µl ß-MeEtOH; 171.5 µl DEPC-water). After spheroplasting, cells were pelleted carefully for one minute at low speed (3000 rpm/1000x g) and washed with buffer B. Spheroplasts were finally resuspended in 100 µl buffer B. Multi-well slides were coated with 0.02% Poly-L-Lysine for 5 min and washed with DEPC water. 5µl of the cell suspension was applied onto each well for 30 min at 4°C. After washing with Buffer B, the slide was fixed and stored in 70% ethanol at -20°C.

5.9.3. Hybridisation procedure

5.9.3.1. Hybridisation of fluorescently-labelled oligonucleotides (*ASH1*)

Multi-well slides were re-hydrated in a jar with 2x SSC (20x SSC: 3 M NaCl; 0.3 M Na_3-citrate) and 2x SSC, 40% formamide for 5 min. In the meantime a frozen aliquot of the *ASH1* probe was resuspended in 15 µl of solution 1 (49.3 µl formamide, 0.63 µl 1M Na-phosphate pH 7.0, 11.7 ul DEPC-water) and incubated at 80°C for 3 min. Probes were then mixed with 15 µl of ice-cold solution 2 (12.3 µl BSA (20 mg/ml); Roche), 12.3 µl 20 x SSC, 0.75 µl RNasin® (Promega), 36.2 µl DEPC-water) and centrifuged at full speed for 5 min. Each well was wetted with 5µl of the probe solution, covered with a large cover slip and hybridised over night at 37°C in a darkened wet chamber.

After hybridisation, the slide was washed in a jar with pre-warmed (37°C) solution of 2x SSC, 40% formamide at 37°C for 15 min, two times with 2x SSC, 0.1% Triton for 15 min at RT and finally with 1x SSC for 15 min at RT. Nuclei were stained with Hoechst Stain Solution (SIGMA) for 15 min and cells were mounted in mounting solution (1x PBS, 80% glycerol).

5.9.3.2. Hybridisation of DIG-labelled probes (*WSC2*)

Slides with adherent cells were re-hydrated in a 50 ml jar for 5 min at RT with 5xSCC buffer. After this, they were pre-hybridised with 40 ml of hybridisation mix (for 40 ml: 20 ml 100% formamide; 10 ml 20xSSC; 400 µl 500 mM EDTA pH 8.0; 400 µl 10% Tween; 800 µl 50x Denhardt's; 400µl 10% CHAPS; 400µl 10mg/ml tRNA; 400µl 10mg/ml herring sperm DNA; 400µl 10mg/ml Heparin; 6,8ml DEPC-water) for 1h at RT. In the meantime, 10 µl of *WSC2*-probe solution was mixed with 90 µl of hybridisation mix, applied to the multi-well slides after pre-hybridisation and incubated over night at 37°C in a darkened wet chamber.

Subsequent to hybridisation, slides were washed in a jar with pre-warmed (37°C) solution of 2x SSC, 40% formamide at 37°C for 15 min and two times with antibody solution (1x PBS, 10% FCS, 0.1% TritonX-100) for 15 min at RT. After this, the primary mouse-anti-DIG antibody was applied to the wells (diluted 1:250 in antibody solution) and incubated at RT for 2 hrs. After three washing steps with 1x PBS, 0.1% BSA, the secondary rabbit-anti-mouse-Alexa®488 antibody (1:1000 in antibody solution) was applied at RT for 1 h. Again after 3 washing steps with 1x PBS, 0.1% BSA, the tertiary goat-anti-rabbit-Alexa®488 antibody was used in a 1:1000 dilution for 1 hr at RT. Slides were washed again twice and nuclei were stained with Hoechst Stain Solution (SIGMA) for 15 min at RT before the stain was washed away and cells were mounted with 1x PBS, 80% glycerol.

6. Summary

mRNA localization is a widespread mechanism in most eukaryotic cells to spatially restrict protein synthesis. During cell propagation of the budding yeast *S. cerevisiae*, mRNA localization is the basis for an asymmetric, stem-cell like division process. At least 24 transcripts are known to be localized to the yeast bud tip and the common core machinery mediating this mRNA translocation pathway consists of three components: the type V motor protein Myo4p, its adaptor She3p and the mRNA binding protein She2p. Recently, Myo4p and She3p were identified as essential factors of another bud-directed transport process, the inheritance of cortical endoplasmic reticulum (ER). In addition, live cell imaging revealed a co-localization and co-migration of localizing mRNPs with ER tubules that move to the yeast bud, implying for the first time that there might be a common transport of mRNAs and ER membranes.

Within the scope of this study it was demonstrated that mutants defective in ER segregation are simultaneously impaired in mRNA localization indicating that there is indeed a connection between the two processes. Additionally, the RNA binding protein She2p associates with ER membranes in different sucellular fractionation assays and it does so independently of polysomes (thus, ongoing translation), mRNA and the Myo4p/She3p complex. During *in vitro* binding assays, recombinant She2p binds to flotation purified ER microsomes and protease treated membranes suggesting that She2p is not tethered to ER via a protein factor. Finally, She2p was found to have an inherent membrane binding activity since it directly associated with synthetic lipid vesicles in flotation assays. She2p attaches to liposomes also in presence of its RNA ligand excluding the possibility of an unspecific binding via its basic mRNA binding moiety.

In summary, these data indicate that mRNA trafficking and ER inheritance are coordinated processes in *S. cerevisiae* and that She2p is the factor that tethers localizing mRNPs to the ER membranes. Consistent with this observation there is a growing number of examples from higher eukaryotes for a connection between membrane and mRNA transport. This in turn suggests that it is not a yeast specific phenomenon but rather might be a common theme throughout all kinds of eukaryotic species.

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8. Abbreviations

aa	amino acid
ab	antibody
Amp	ampicillin
ATP	adenosine triphosphate
ß-MeEtOH	beta-mercaptoethanol
BSA	bovine serum albumin
bp	basepair
°C	degree centigrade
C. elegans	*Caenorhabditis elegans*
CIP	calf intestine phospatase
clonNAT	nourseothricin
CSM	complete supplement mix
C-terminal	carboxy terminal
Da	dalton
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleosid triphosphate
DTT	dithiothreitol
ECL	enhanced chemoluminiscence
E. coli	*Escherichia coli*
EDTA	ethylenediaminotetraacetic acid
ER	endoplasmic reticulum
et al.	*et alii* (from Latin, “and others”)
5-FOA	5-Fluoroorotic acid
g	gram
x g	relative centrifugal force (rcf)
G418	Geniticin
GFP	green fluorescent protein
GST	glutathione *S*-transferase
h	hour
HA	hemagglutinin
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO endonuclease	homothallic switching endonuclease

IPTG	isopropyl-beta-D-thiogalactoside
k	kilo
kb	kilo basepairs
KH-domain	heterogeneous nuclear (hn)RNP K-homology domain
l	litre
LB	Luria Bertani
LE	localization element
LN_2	liquid nitrogen
µ	micro
m	milli
M	molar
mA	milliampere
MC	mitochondrial cloud
min	minutes
METRO	messenger transport organizer
mRNA	messenger ribonucleic acid
n	nano
NE	nuclear envelope
NP-40	Nonidet P-40 (Igepal-CA-630)
NTP	nucleoside triphosphate
nt	nucleotide
OD	optical density
ORF	open reading frame
p	picot
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEG	polyethylene glycol
pH	potential of hydrogen
PI	phosphatidylinositol
PIP	phosphoinositol
PS	phosphatidylserine
RNA	ribonucleic acid
RBP	RNA binding protein

RNP	ribonucleoprotein
rpm	revolutions per minute
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
s	second
S	sedimentation coefficient (Svedberg)
SHE	Swi5p-dependent HO expression
TCA	trichloroacetic acid
TMD	transmembrane domain
Tris	trishydroxymethylaminomethane
tRNA	transfer ribonucleic acid
UTR	untranslated region
V	volt
WCE	whole cell extract
wt	wild type
YEP	yeast extract peptone
YNB	yeast nitrogen base
YRMs	yeast rough membranes

9. Publications

Schmid M, Jaedicke A, Du TG, Jansen RP. Coordination of endoplasmic reticulum and mRNA localization to the yeast bud. *Curr. Biol.* 2006 Aug 8; 16(15):1538-43.

Du TG, **Schmid M**, Jansen RP. Why cells move messages: The biological functions of RNA localization. *Semin. Cell Dev. Biol.* 2007 Apr; 18(2):171-7.

Hutzler J, **Schmid M**, Bernard T, Henrissat B, Strahl S. Membrane association is a determinant for substrate recognition by PMT4 protein O-mannosyltransferases. *Proc. Natl. Acad. Sci. U S A* 2007 May 8; 104(19): 7827-32.

Lange S, Katayama Y, **Schmid M**, Burkacky O, Bräuchle C, Lamb DC, Jansen RP. Simultaneous transport of different localized mRNA species revealed by live-cell imaging. *Traffic* 2008 Jun 5; [Epub ahead of print]

Du TG, Jellbauer S, Müller M, **Schmid M**, Niessing D, and Jansen RP. Nuclear transit of the RNA-binding protein She2p is required for translational control of localized *ASH1* mRNA. *EMBO Reports* 2008 Jun 20; [Epub ahead of print]

Conferences/Posters:

Schmid M, Jaedicke A, Jansen RP. Coordination of endoplasmic reticulum and mRNA localization to the yeast bud. RNA 2006 - The Eleventh Annual Meeting of the RNA Society. University of Washington, Seattle, USA. 06/2006

Schmid M, Jaedicke A, Jansen RP. Coordination of endoplasmic reticulum and mRNA localization to the yeast bud. EMBO/FASEB Workshop on Intracellular RNA Localization and Localized Translation, Il Ciocco, Italy, 07/2007

Talk:

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