Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians-Universität München

Role of nuclear RNP assembly in cytoplasmic mRNA localization

Tung-Gia Du
aus
Saigon

München
2007

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

München, am …….

……..

(Tung-Gia Du)

Dissertation eingereicht am	6.9.2007
1. Gutachter	Prof. Dr. Ralf-Peter Jansen
2. Gutachter	Prof. Dr. Roland Beckmann
Mündliche Prüfung am	31.10.2007

Table of contents

1 Introduction

1.1 The yeast Saccharomyces cerevisiae

Saccharomyces cerevisiae is the most well known and commercially significant yeast species. As “brewer’s yeast”, it has long been utilized to ferment sugars of rice, wheat, barley, and corn to produce alcoholic beverages. The baking industry takes advantage of *Saccharomyces cerevisiae*’s ability to produce carbon dioxide, which is useful to expand dough. Moreover, yeast is often taken as a vitamin supplement because of its high content of proteins, B vitamins, niacin, and folic acids.
In science, *Saccharomyces cerevisiae* is, along with *E.coli*, one of the most studied model organisms. Yeast has the advantage of being a eukaryotic organism, so the results of genetic studies with yeast are more easily applicable to human genetics. Thus, many proteins important in human biology were first discovered by studying their homologs in yeast. Important processes such as gene regulation, cell cycle regulation, recombination, mitosis, meiosis, nuclear import/export can be examined in this unicellular organism. Because of the short generation time, yeast can be easily cultivated. Importantly, many sophisticated genetic tools such as inducible expression systems, deletion- and epitope-tagging cassettes have been developed in the past decade, which makes yeast a convenient and powerful model system to study eukaryotic cellular processes.

1.2 The yeast life cycle

A yeast, by definition, is a unicellular fungus that reproduces primarily by budding, which is the production of a small outgrowth, the bud from the parent cell. Thus, budding is an asexual method of reproduction. Yeasts have both, budding haploid and diploid stages. In nature, and when nutrients are available, yeast reproduces asexually mainly in the diploid stage. Budding starts at late G1-phase. At the end of M-Phase, the emerged daughter bud has reached the size of the mother cell. The subsequent cell division results in two cells, termed “mother cell” and “daughter cell”. Upon nutritional starvation, diploid cells may undergo meiosis and revert to the haploid stage by sporulation. After meiosis, the formed tetrade consists of usually four ascospores, two of which with the mating type a and two with mating type α.

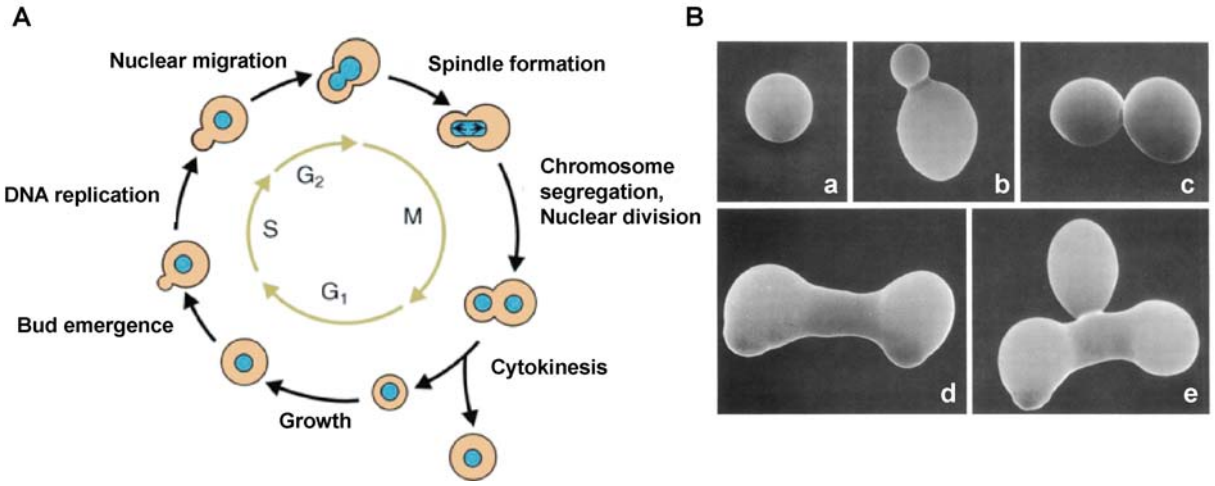

Figure 1: The yeast life cycle. A. The cell cycle of Saccharomyces cerevisiae (Source: Lodish, 1999). Yeast cells multiply asexually by budding. At the end of G1, a bud emerges from the mother cell. Prior to cytokinesis, the daughter bud has reached size of the mother cell. After cell division, the resulting cells grow in G1 until reaching the appropriate size for bud formation. B. Morphology of *S. cerevisiae* cells (Source: Herskowitz, 1988). Upper panel shows an unbudded cell in G1 (a) and cells with different bud sizes (b, c). Mating of a- and α-haploids leads to formation of a diploid (a/α) zygote (d). The zygote is able to produce diploid (a/α) daughter cells by budding. Bud emerges often at the neck (e).

When nutrients are available, the spores germinate and the resulting cells either may multiply asexually as haploids or may serve as a gamete. In yeast, this sexual process is termed “mating” and occurs when two haploid cells with different mating types fuse and form a diploid (a/α) zygote. Cells of each haploid type produce a secreted mating-factor. These mating type-specific pheromones, termed a- and α-factor, act to synchronize the cell cycle of the mating partners and to prepare cells for mating (Herskowitz, 1988).

1.3 Mating type switching

One interesting feature, which occurs in budding yeast, is the phenomenon of mating type switching. After cytokinesis of a haploid cell, mating type switching occurs only in mother cells but not in daughter cells. This is due to the asymmetrically distributed activity of the *HO*-endonuclease.

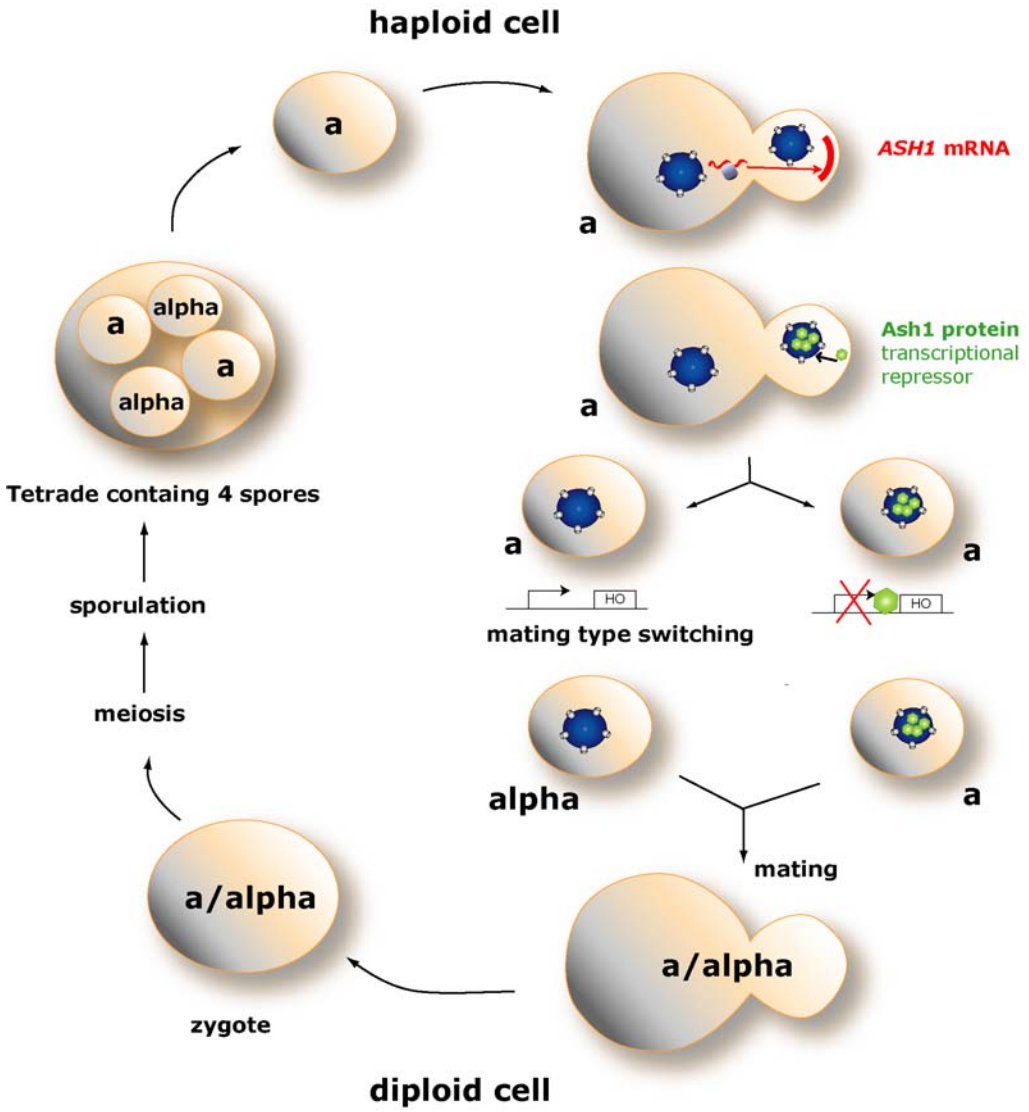

Figure 2: Mating type switching. A diploid yeast cell can undergo meiosis and sporulation when nutrients are limited. This leads to the formation of a tetrade containing four ascospores. After breakdown of the ascus, the spores germinate when nutrients are available. Two haploid a- and α cells can mate to form a diploid zygote. The entry into the diploid phase is facilitated by a phenomenon called mating type switching. After division of a haploid cell, only the mother but not the daughter cell can switch the mating type. This asymmetric cell division is caused by the bud localization of the *ASH1* mRNA.

Mating type switching requires three gene loci on yeast chromosome III, the Mating Type (*MAT*) locus and two silent loci *HML* and *HMR* (*Homothallic Mating Type Copy Left*/*Right*). The mating type of a yeast cell is determined by the alleles of the mating type locus (Haber, 1998). In haploid cells, expression of one of the two alleles leads to cells with either mating type a or α, whereas diploid cells express both alleles (Mating type a/α).

MATα encodes for two proteins termed α1p and α2p. α1p and transcription factor Mcm1p are responsible for the activation of α-specific genes (Shore and Sharrocks, 1995). In contrast, α2p and Mcm1p serve to repress a-specific genes (Wolberger, 1998). The *MATa*-locus encodes for two proteins, of which only A1p is known to have a biological function. A1p and α2p form a heterodimer, which is required to repress haploid-specific genes (Li et al., 1995). Consequently, there is no expression of α-specific genes in a-cells because α1p and α2p are missing, whereas through the activation by Mcm1p, a-specific genes are expressed (Bruhn and Sprague, 1994)
There are two additional copies called HML and HMR, which are positioned upstream and downstream of the *MAT*-locus, respectively. These regions are under the control of silencer sequences, which by binding of Sir1p-Sir4p (silent information regulator) leads to hypoacetylated heterochromatin and consequently, to transcriptional inactivation (Grunstein, 1998). Mating type switching occurs when either *HMLa* or *HMLα* is recombined into the transcriptionally active *MAT*-locus by gene conversion (Hicks and Strathern, 1977; Strathern et al., 1982). Thus, the *MAT*-locus is replaced by the genetic information of the opposite mating type. This recombination event is initiated by a double-strand break, catalyzed by the haploid-specific *HO* endonuclease. Because expression of *HO* at the end of G1-phase occurs only in haploid mother cells, just a half of the cells of a colony can statistically undergo mating type switching (Nasmyth, 1993). In diploid cells, binding of the heterodimer A1p/α2p inhibits *HO* expression (Herskowitz, 1992). Yeast strains used for biological studies in laboratories have lost their ability to change mating types due to a point mutation in the *HO* gene. These strains are called heterothallic and are more accessible to genetic manipulations because of a stable haploid phase.

1.4 Control of *HO* expression

The transcription activation program of *HO* is cell cycle regulated. The expression occurs only transiently and starts during late mitosis, when Cdk1p is inactive and ends during late G1-phase, when Cdk1p is reactivated (Nasmyth, 1993). The *HO* promoter can be divided in two regions: a distant upstream region called URS1 ("Upstream Regulatory Sequence"), which regulates mother cell expression specificity, and a proximal region called URS2 that controls *HO* cell-cycle regulation (Nasmyth, 1993). *HO* transcription depends on the ordered recruitment of several

cell-cycle dependent transcription factors to these promoter regions. The zinc-finger protein Swi5p is one of these factors, which is, except of G1-phase, expressed throughout the cell cycle. However, in S-phase, Swi5p starts to accumulate in the cytoplasm until Cdc14p dephosphorylates it in late anaphase. As a result, Swi5p enters nuclei of both, mother and daughter cell and binds to two sites of URS1 regions within the *HO* promotor. This event triggers the recruitment of the SWI/SNF chromatin-remodelling complex to URS1 and URS2. Subsequently, SAGA complex is recruited to the promoter. The ATP-dependent acetylation of Histone H3 by SAGA and SWI/SNF leads to the remodelling of nucleosomes so that a second transcription factor SBF can bind to URS2 region of the *HO* promoter. In a subsequent step, SBF recruits the Mediator complex to URS2 and to the TATA box. Reactivation of Cdk1p in the end of G1 finally leads to recruitment of RNA polymerase II and the general factors TFTTB/TFIIH for transcription initiation (Bhoite et al., 2001; Cosma et al., 1999; Krebs et al., 1999). However, the highly concerted recruitment of all these transcription factors does not occur in daughter cell nuclei. Therefore, only a differential control of this *HO* promotor in mother and daughter cell nuclei can result in progeny with opposite mating types.

In 1996, the isolation of mutants with daughter cells defective in *HO* repression identified the *ASH1* gene (*Asymmetric synthesis of HO*). It encodes for Ash1p, a 66-kDa zinc-finger transcriptional repressor. In *ASH1* mutants, the daughter cells were able to switch mating type as well. Thus, Ash1p is the factor that inhibits *HO* transcription through its asymmetric accumulation in only daughter nuclei in late anaphase (Bobola et al., 1996; Sil and Herskowitz, 1996). It is also required for pseudohyphal growth (Chandarlapaty and Errede, 1998). Ash1p contains a region that is highly homologous to the zinc-finger domain of the erythroid cell nuclear protein GATA-1 (Bobola et al., 1996; Sil and Herskowitz, 1996). All GATA-like factors bind to GATA motifs, which leads to either activation or repression of transcription. The YTGAT consensus sequence was identified within the *HO* promoter, which mediates the binding of Ash1p. This motif is related to the canonical (A/T)GATA(A/G) sequence bound by most GATA factors and is present at least 20 times within the URS1 region of the *HO* promoter (Maxon and Herskowitz, 2001).

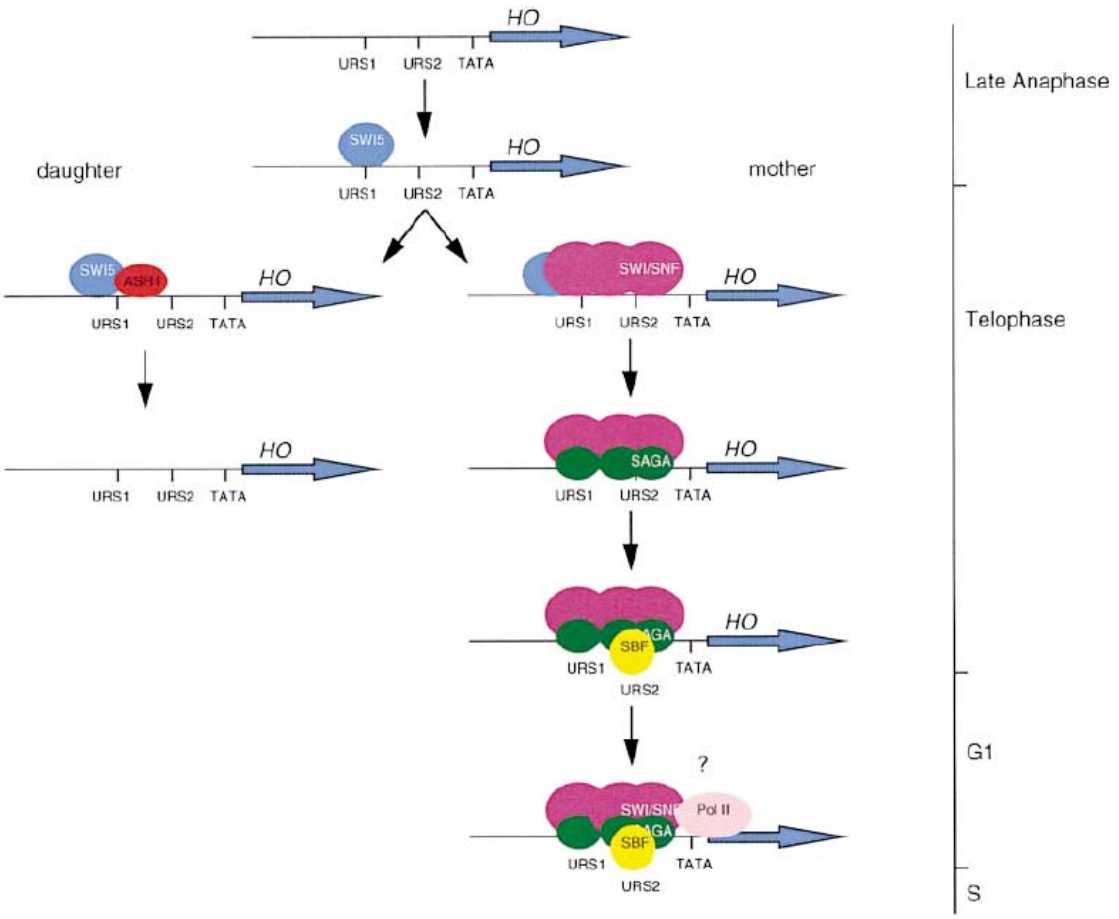

Figure 3: Transcriptional control of the *HO* promoter in *S. cerevisiae* is cell cycle regulated (Source: Cosma 2002, 1999). After inactivation of Cdk1p in late anaphase, transcription factor Swi5p enters the nuclei of mother and daughter cells. Swi5p binds to URS1 of the *HO* promoter. A subsequent binding of the transcriptional repressor Ash1p to URS2 blocks the recruitment of SWI/SNF complex, and leads to inhibition of *HO* transcription. In mother cell nuclei of haploids, because Ash1p is not present, SWI/SNF can bind to URS2 and recruits the SAGA complex to the promoter. Histone acetylation and subsequent nucleosome remodelling allow transcription factor SBF to access and bind URS2 region. In a following step, the Mediator complex associates with URS1 and TATA. Finally, when Cdk1p is reactivated, RNA Polymerase II and additional factors are recruited in order to initiate transcription.

The C-terminal domain of Ash1p mediates DNA binding to the YTGAT consensus of the *HO* promoter, whereas the NH_2-terminus serves to repress *HO* transcription (Maxon and Herskowitz, 2001). The asymmetric control of *HO* expression, which is caused by the sorting of Ash1p to daughter cells, explains why only haploid mother cells can undergo mating type switching.

1.5 Localization of *ASH1* mRNA in S. cerevisiae

1.5.1 *SHE* genes

Mother cell-specific expression of *HO*-endonuclease is due to the asymmetric distribution of its transcriptional repressor Ash1p (Bobola et al., 1996). The asymmetric accumulation of this cell fate determinant in only daughter cell nuclei is mediated by the products of five genes, termed *SHE1–SHE5* (Swi5p-dependent *HO* expression), each of which has a specific function (Jansen *et al,* 1996). They were identified in a genetic screen for factors that are required for asymmetric *HO*-expression in yeast cells (Jansen et al., 1996). Interestingly, *SHE* genes have no influence on SWI regulation but rather are responsible for the sorting of Ash1p. Deletion of a *SHE* gene results in the loss of asymmetric distribution. As a result, the determinant Ash1p is present in both, mother and daughter cell nuclei.

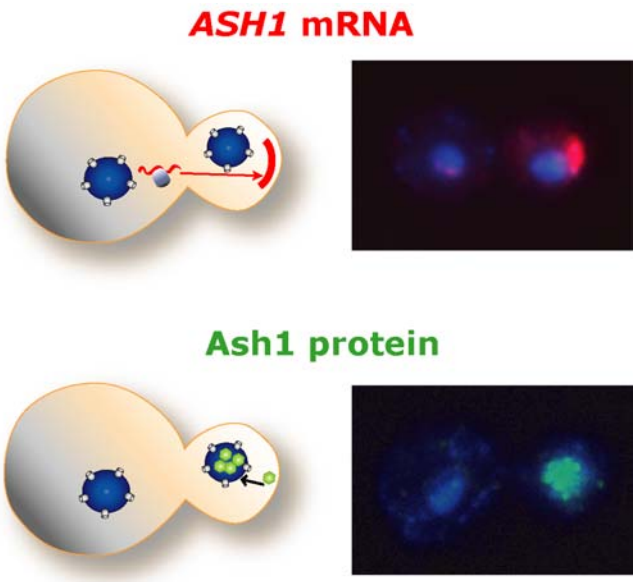

Figure 4: Asymmetric distribution of Ash1p is generated by RNA localization. *Swi5p* activates *ASH1* expression in late anaphase. The *ASH1* mRNA undergoes nuclear export and is subsequently transported to the tip of growing cells by a She-dependent machinery. At the target site, it is anchored and translated into the Ash1 protein, which subsequently enters the nucleus of only the daughter cell to act as a transcriptional repressor of HO expression. Because of this asymmetric sorting, *HO* expression and mating type switching is restricted to only mother cells. *In situ* stain of *ASH1* mRNA with Cy3-labelled antisense oligonucleotides in a WT cell (upper right). Immunofluorescence of Ash1p-myc9 in a WT cell (lower right). Nuclei are stained with DAPI (blue).

Because repression of *HO* occurs in both progeny resulting from cell division, none of the cells is able to switch the mating type. All She-proteins are themselves localized asymmetrically. Epitope-tagged She1-She4p all localize to a crescent at the cortex of the daughter bud, except of She5p, which localizes first to the tip of an emerging bud but then stays at the mother-bud neck. Because Ash1p appears in nuclei of daughter cells at much later stages of the cell cycle when the She-proteins are no longer localized, it is very unlikely that Ash1p is directly targeted by protein transport (Chang and Drubin, 1996). Thus, the asymmetric distribution of Ash1p is achieved by the localization of its corresponding *ASH1* mRNA to the bud tip in late anaphase (Long et al., 1997). This derives from observations that in *she*-mutants, *ASH1* mRNA is mislocalized. In contrast to wild type cells, *she*-mutants are not able to localize *ASH1* mRNA to the tip of daughter cells. Mutants of *SHE1-SHE4* display a mislocalization of *ASH1* mRNA throughout the cytoplasm, except in *she5* mutant where *ASH1* mRNA was found accumulated at the mother-bud neck (Long et al., 1997; Takizawa et al., 1997). Thus, each of the *SHE* gene products is essential for targeting the *ASH1* message to the bud tip.

1.5.2 *ASH1* mRNA – the cargo

Figure 5: Localizations elements (LE) of the *ASH1* mRNA. Three of the localization elements, namely E1, E2A and E2B, are located within the *ASH1* coding sequence. The E3 element spans the stop codon and the first 100 nucleotides of the 3' untranslated region (UTR).

In order to identify *cis*-acting sequences responsible for the localization of the *ASH1* mRNA, fragments of *ASH1* were inserted into a reporter mRNA and the cytoplasmic distribution of these chimeric mRNAs was determined by *in situ* hybridisation (Chartrand et al., 1999; Gonzalez et al., 1999). Four localization elements (LE) or zipcode elements required for bud tip localization of *ASH1* mRNA have been identified. They are termed E1, E2a, E2b and E3 (Chartrand et al., 2002; Chartrand

et al., 1999) or according to a different nomenclature, U1, U2 and U3 (Gonzalez et al., 1999). Each of the LEs alone is sufficient to direct bud tip localization of a heterologous reporter RNA (Chartrand et al., 2002). E1, E2A and E2B are located within the coding sequence whereas E3 spans from the end of the open reading frame into the 3'-UTR. Interestingly, in most cases, LEs are located in the 3' UTR of a localized RNA, whereas all the *ASH1* LEs are located within or in part of the coding sequence. The artificial transposition of these elements to the 3'-UTR lead to an increased Ash1p synthesis suggesting that the *cis*-acting elements within the coding sequence may serve to slow down translation during transport. Thus, this molecular translation retardation mechanism may contribute to the establishment of Ash1p asymmetry in yeast (Chartrand et al., 2002). RNA secondary structure prediction suggests that all *ASH1* LEs form extensive stem-loop structures (Chartrand et al., 2002; Chartrand et al., 1999; Gonzalez et al., 1999). All *cis*-elements are recognized by several *trans*-acting factors. She2 is the major RNA binding protein because of its ability to recognize all four LEs of the *ASH1* mRNA (see below). It can bind the LEs in a range of K_d~100-400 nM (Niessing et al., 2004). A recent study has identified three-dimensional conserved RNA motifs required for recognition by She2p. These motifs consist of two loops separated by a short stem of 4 base pairs, with a conserved cytosine in one loop and a conserved CGA triplet in the other loop, both on opposite strands of the RNA loop-stem-loop structure. Mutations within this three-dimensional motif decreases the interaction with She2p, and results in loss of *ASH1* localization (Olivier et al., 2005). In an independent approach, Jambhekar and co-workers used a high-throughput selection method to map localization elements in RNA targets. A predicted single-stranded core CG dinucleotide appears to be an important component of the RNA-protein interface although other nucleotides contribute in a context-dependent manner. Thus, the extensive sequence and structural plasticity suggest that the She-complex recognizes a precise 3D structure in its RNA target (Jambhekar et al., 2005)

1.5.3 Other localized mRNAs

In a study using DNA micro-array analysis, several other bud-localized RNAs have been identified. Among them is *IST2*, which encodes a putative ion channel with unknown function (Entian et al., 1999; Takizawa et al., 2000). *IST2* mRNA

localization to the cortex of daughter cells created a higher concentration of Ist2 protein in the bud compared with that of the mother cell, and this asymmetry was maintained by a septin-mediated membrane diffusion barrier at the mother-bud neck. In a recent study, a complex peptide-sorting signal located at the extreme C-terminus was identified suggesting that there is an additional sorting of Ist2p acting independent of the targeting achieved by *IST2* mRNA. This novel sorting mechanism to the plasma membrane does not require She-mediated mRNA transport into daughter cells. Thus, such a redundant "backup" mechanism may help to sort the protein in addition to RNA localization. A microarray-based screen identified a set of 22 additional mRNAs, all of which become localized to bud tip of in a She-dependent manner (Shepard et al., 2003). These messages encode a wide variety of proteins, including several involved in stress responses, cell wall maintenance and membrane proteins. However, the biological significance of localizing these RNAs remains to be elucidated because asymmetric distribution of several of these proteins also occurs in the absence of mRNA transport.

1.5.4 She1/Myo4p – a yeast class V myosin motor

She1p, also called Myo4p, is the motor protein that mediates the active transport of the *ASH1* RNP along actin filaments to the tip of daughter cells (Bertrand et al., 1998; Haarer et al., 1994; Jansen et al., 1996; Münchow et al., 1999). In budding yeast, Myo4p and Myo2 belong to class V unconventional myosins (Titus, 1997). Both non-processive motors localize to the bud tip during bud formation (Karpova et al., 2000; Lillie and Brown, 1994; Schott et al., 1999). Myo2p functions in the polarized transport of secretory vesicles (Govindan et al., 1995; Johnston et al., 1991; Lillie and Brown, 1994; Pruyne et al., 1998; Schott et al., 1999), inheritance of the vacuole and the Golgi apparatus ((Catlett et al., 2000; Catlett and Weisman, 1998; Rossanese et al., 2001), and is required to set up the orientation of the mitotic spindle (Yin et al., 2000). Myo4p in contrast, is the only yeast motor protein with a specific role in RNA localization (Long et al., 1997; Reck-Peterson et al., 2000), but in addition is also involved in the inheritance of cortical ER (Estrada *et al*., 2003). There is now strong evidence suggesting that both Myo4p-dependent processes are tightly coordinated (Aronov et al., 2007; Schmid et al., 2006).

1.5.5 The adapter protein She3

The association of Myo4 motor protein with *ASH1* mRNA is dependent on additional factors She2p and She3p (Kruse et al., 2002; Münchow et al., 1999; Takizawa and Vale, 2000). She3p binds to the coiled-coil region of Myo4p's tail domain and to the RNA binding protein She2p. Thus, She3p serves as an adapter that docks the myosin motor onto an *ASH1*–She2p ribonucleoprotein complex. In a two-hybrid (Böhl et al., 2000), and a three-hybrid approach (Long et al., 2000), Myo4p was shown to interact with the NH_2-terminus of She3p. Moreover, sucrose density gradients demonstrated cosedimentation of Myo4p together with She3p, suggesting a tight and permanent association of both proteins (Böhl et al., 2000). The C-terminus of She3p provides the binding to She2. Interestingly, She3p might have an influence on *ASH1*-She2p interaction. In gel-shift assays, binding of She2p to *ASH1* LEs was enhanced in the presence of She3p (Böhl et al., 2000). Takizawa and co-workers independently suggested a cooperative binding of She2/She3 in the cytoplasm. According to their data, they hypothesized that binding of She2p to *ASH1* mRNA may induce changes in the RNA in a way that enables She3-Myo4 complex to associate (Takizawa and Vale, 2000).

1.5.6 She2 – the RNA binding protein

She2p is the key protein in the assembly of the *ASH1* ribonucleoprotein complex. This small 28 kDa RNA-binding protein directly interacts with *ASH1 cis*-acting localization elements and associates with the adapter She3p. Although the primary sequence does not reveal any canonical RNA-binding motifs, She2 is able to bind to each of the localization elements *in vivo* and *in vitro*. This was clearly demonstrated with electrophoretic gel mobility shift assays (Böhl et al., 2000) and with filter binding experiments (Niessing et al., 2004) where purified recombinant She2p displayed specific binding to *ASH1 cis*-acting localization elements. A crystal structure of She2p was determined and revealed that it forms symmetric homodimers. Moreover, dimer formation is required for RNA binding activity (Niessing et al., 2004). Two independent studies have demonstrated the interaction of She2p with the C terminus of She3p (Böhl et al., 2000; Long et al., 2000) suggesting that She2p is required to

interface the Myo4p-She3p complex to *ASH1* mRNA. Disruption of *SHE2* abolishes Myo4p's association with the mRNA (Jansen et al., 1996; Münchow et al., 1999). She2p is also required to mediate the association of She3p to *ASH1* mRNA (Böhl et al., 2000). This suggests that She2 is the factor acting directly on *ASH1* mRNA. Moreover, this association is independent of She3p and Myo4p. The site of RNA binding was initially determined to be located at the N-terminus of She2p. The deletion of the first 70 amino acids of She2p resulted in an accumulation of this mutant in the nucleus (Kruse et al., 2002). This was the first evidence indicating that She2p's export is dependent on RNA binding. Seven amino acids within the N-terminal region of *SHE2* have been reported so far to be required for RNA binding activity. Mutations, resulting in amino acid substitutions N36S, R43A, R44A, R52A, R52K, R63A, and R63K lead to a loss of RNA binding and consequently, to defective mRNA localization (Gonsalvez et al., 2004; Niessing et al., 2004). Moreover, block of mRNA export caused the accumulation of She2 in the nucleus as well (Kruse et al., 2002). This indicates that She2 can enter the nucleus for the binding of its RNA target and thus is able to shuttle between the nucleus and cytoplasm.

1.5.7 She4 – a putative myosin chaperone

She4p was initially characterized as a protein involved in receptor-mediated endocytosis, organization of the cortical actin cytoskeleton and growth at elevated temperatures (Wendland et al., 1996). Nevertheless, deletion of *SHE4* leads to defects in *ASH1* mRNA localization (Jansen et al., 1996). She4p belongs to the protein family containing UCS domains as it shares a 400 residue conserved region that is also present in *Caenorhabditis elegans UNC45* (Epstein et al., 1974) and *Podospora anserina CRO1* (Berteaux-Lecellier et al., 1998). UCS proteins appear to ensure proper folding of myosin heads so that they can perform their ATP-dependent actin-based motor functions. In yeast, She4p was shown to associate with yeast class I and class V myosins. She4p binds directly to motor domains of class V myosin Myo4p and class I myosin Myo5p through its UCS domain (Toi et al., 2003; Wesche et al., 2003). *In vivo*, She4p is essential for the function and localization of Myo3p, Myo4p, and Myo5p (but not of Myo2p) and for colocalization of class I myosins with cortical actin patches. This suggests that in yeast, She4p may be

required for the structural integrity and/or the regulation of the motor domain of unconventional myosins.

1.5.8 She5/Bni1p and Bud6p

RNA localization also requires the establishment of a polarized cytoskeleton. She5/Bni1p is a formin involved in the organization of actin cytoskeleton. She5p has been reported to promote nucleation of barbed-end actin polymerization (Evangelista et al., 1997; Pruyne et al., 2002). Cells of a *she5/bni1*Δ fail to localize *ASH1* mRNA, which accumulate at the bud neck (Long et al., 1997). This observation is consistent with a defect in promoting polymerization of actin fibres at the bud tip. A second protein required for actin filament organization to the bud is Bud6p, which directly binds to She5/Bni1p (Evangelista et al., 1997; Tong et al., 2001). Because both bud-specific proteins populate the cortex at the bud tip, mislocalization of *ASH1* mRNA in *she5/bni1* or *bud6/aip3* cells is probably caused by the loss of specific mRNA anchorage at the bud tip. This suggests that the *ASH1* RNP is able to migrate to the bud but finally fails to remain at the bud tip. Thus, it was hypothesized that Bud6p/Aip3p and Bni1p/She5p may be factors required to maintain the transcript at the cortical bud cap. (Beach and Bloom, 2001; Beach et al., 1999)

1.6 *Trans*-acting factors of *ASH1* mRNA

The Myo4p/She3p/She2p heterotrimeric complex is essential for *ASH1* mRNA trafficking to the bud tip. These proteins are thought to constitute the cytoplasmic core RNP, also termed ‘locasome’ (Beach and Bloom, 2001; Bertrand et al., 1998; Darzacq et al., 2003) since all of the She-proteins co-localize with each other and with the transported *ASH1* mRNA (Böhl et al., 2000; Gonsalvez et al., 2004; Takizawa and Vale, 2000). Our understanding toward how RNA transport is mediated mechanistically in yeast is now becoming even more complex because there is a growing body of evidence suggesting that RNA localization is also linked to the process of ER inheritance (Aronov et al., 2007; Schmid et al., 2006). In addition, there are auxiliary factors, which associate with *ASH1* only transiently or their association is required for functions other than the active cytoplasmic transport such as translational control. In the past years, a set of *ASH1 trans*-acting factors have

been identified and characterized, giving rise to the evidence that it requires more than just a functional motor complex to target a transcript effectively.

1.6.1 Khd1p

Khd1p is an RNA binding protein (KH-domain protein 1) with homology to hnRNPK, and it has been first reported to be required for efficient localization of *ASH1* mRNA. The N-element, a *cis*-acting region spanning the first 800 nucleotides of the *ASH1* coding sequence, is responsible for the association with Khd1p *in vivo* (Gonzalez et al., 1999; Irie et al., 2002). In a more recent study, Khd1p was shown to interact with localization element E1 of *ASH1* mRNA (Paquin et al., 2007). Deletion of *KHD1* had only little effect on *HO* expression, and frequency of mating-type switching was the same as that in a wild type strain. Nevertheless, *KHD1* genetically interacts with a weak mutation in *MYO4*, but a direct interaction with any of the She proteins remains to be shown. Interestingly, the level of Ash1p was decreased, when over-expressing Khd1p*,* and *ASH1* mRNA was not efficiently localized compared to wild type, suggesting a role in anchoring and/or translational control (Irie et al., 2002). Consistent with its role as a translational regulator, Khd1p has recently been reported to interact with the C-terminal domain of translation-initiation factor eIF4G1. Interestingly, deletion of this interaction domain leads to increased translation of an *ASH1* reporter mRNA *in vivo* suggesting that Khd1p may act to reduce translation initiation during transport. Moreover, Khd1p interacts with Yck1p (yeast casein kinase) when reaching the plasma membrane. The phosphorylation by this kinase may possibly mediate the release of Khd1p from *ASH1* mRNA, which subsequently leads to local activation of translation at only the target site (Paquin et al., 2007).

1.6.2 Puf6p

Tandem affinity purification of *ASH1* mRNP and mass spectrometry identified a novel 75 kDa yeast protein (Gu et al., 2004). Puf6p is a member of the PUF-family with highly conserved RNA-binding proteins such as Pumilio in *Drosophila*. Puf proteins are defined by the presence of several repeats of the Pumilio homology domain (Pum-HD), which confers RNA binding activity (Wang et al., 2002). In general, Puf

proteins have been reported to bind to sequences in the 3'-UTR encompassing a so-called UGUR tetranucleotide motif and thereby to repress gene expression by affecting mRNA translation or stability. Thus, all known Puf-proteins (Puf1-Puf6p) in yeast are all involved in the posttranscriptional regulation of mRNAs (Gerber et al., 2004). Puf6p has been shown to interact with a PUF consensus sequence, a conserved UUGU element within the 3'-UTR of the *ASH1* mRNA. Deletion of *PUF6* or mutations of UUGU elements of the mRNA increased the intracellular Ash1p concentration. Moreover, overexpression of Puf6p resulted in a reduced amount of synthesized Ash1p. Thus in a *puf6Δ* strain, asymmetric localization of both Ash1p and *ASH1* mRNA were significantly reduced, suggesting a role for Puf6p in translational repression (Gu et al., 2004). Consistent with its role in translational regulation, Puf6p was reported to colocalize with the *ASH1* mRNP *in vivo* although it is a predominantly nuclear protein. This suggests that this protein needs to shuttle between the two compartments in order to function in cytoplasmic translational control.

1.6.3 Loc1p

The RNA binding protein Loc1p is localized exclusively to the nucleolus but nevertheless, affects the asymmetric localization of both, *ASH1* mRNA and Ash1p (Long et al., 2001). Loc1 was identified in a three-hybrid screen using the *ASH1* E3 element as a bait. Band mobility shift assays and immunoprecipitation followed by RT-PCR of myc-tagged Loc1p clearly demonstrated the binding to the E3 LE *in vitro* and *in vivo.* Nevertheless, Loc1p is also a constituent of the 66S pre-rRNA complex (Harnpicharnchai et al., 2001). Its function in assembly and export of 60S ribosomal subunit has been shown more recently (Urbinati et al., 2006). Thus, consistent with its important role in ribosome biogenesis, *loc1*Δ strains display a severe slow growth phenotype at 30°C and abnormal cell morphology (Stephan Jellbauer, personal communication). Nevertheless, deletion of *LOC1* resembles the phenotype of a *she*-mutant because it has also a significant influence on *ASH1* mRNA localization and consequently, on the asymmetric sorting of Ash1p (Long et al., 2001). One reason why this protein was not identified in the *SHE*-screen might be due to its location near the centromer. Thus, cloning of a fragment containing a centromeric region is difficult since plasmids with two centromers are highly unstable when transformed

into yeast cells (Ralf-Peter Jansen, personal communication). It is still elusive whether both processes, RNA localization and ribosomal biogenesis, are really linked or whether they mutually affect each other.

1.7 The biological functions of mRNA localization

A special feature of the eukaryotic cell is the subdivision into compartments with specific cellular functions. Each of the cellular compartments requires a subset of proteins in order to maintain their full function. One way to direct specific proteins to their appropriate target sites within a subcellular region is the signal-peptide mediated sorting of proteins. However, many localized mRNAs encode proteins that lack such peptide sorting signals. Hence, the subcellular distribution of these proteins is therefore entirely determined by the localization of its corresponding transcript. There are several reasons why mRNAs rather than their protein products become targeted. In multiple rounds of translation, one mRNA molecule can give rise to several protein molecules. Thus, mRNA localization should be more cost efficient than protein transport (Du et al., 2007; St Johnston, 2005). Secondly, mRNA localization not only targets the protein to a specific site within the cell, but also spatially prevents its expression at another unwanted region. This becomes important during early development when the correct localization of cytoplasmic determinants is crucial in order to set up embryonic pattern. Therefore, localized mRNAs are often found in oocytes and early embryos where the pattern of morphogens regulates important developmental processes (Fig. 6B). In *Drosophila melanogaster*, several mRNAs localize to distinct regions of the oocyte. For instance, *bicoid* mRNA localizes to the anterior pole of the oocyte where it is translated after fertilization. Bicoid protein forms the highest concentration at the anterior. The specification of the anterior in the embryo requires the local on-switch of target genes mediated by localized Bicoid protein (Ephrussi and St Johnston, 2004). Localization of *oskar* mRNA to the opposite pole initiates formation of pole plasma at the posterior end of the *Drosophila* oocyte, which is important for the development of primordial germ cells at later stages (Mahowald, 2001). *nanos* is a second mRNA localized to the posterior pole where it is needed to setup posterior structures.

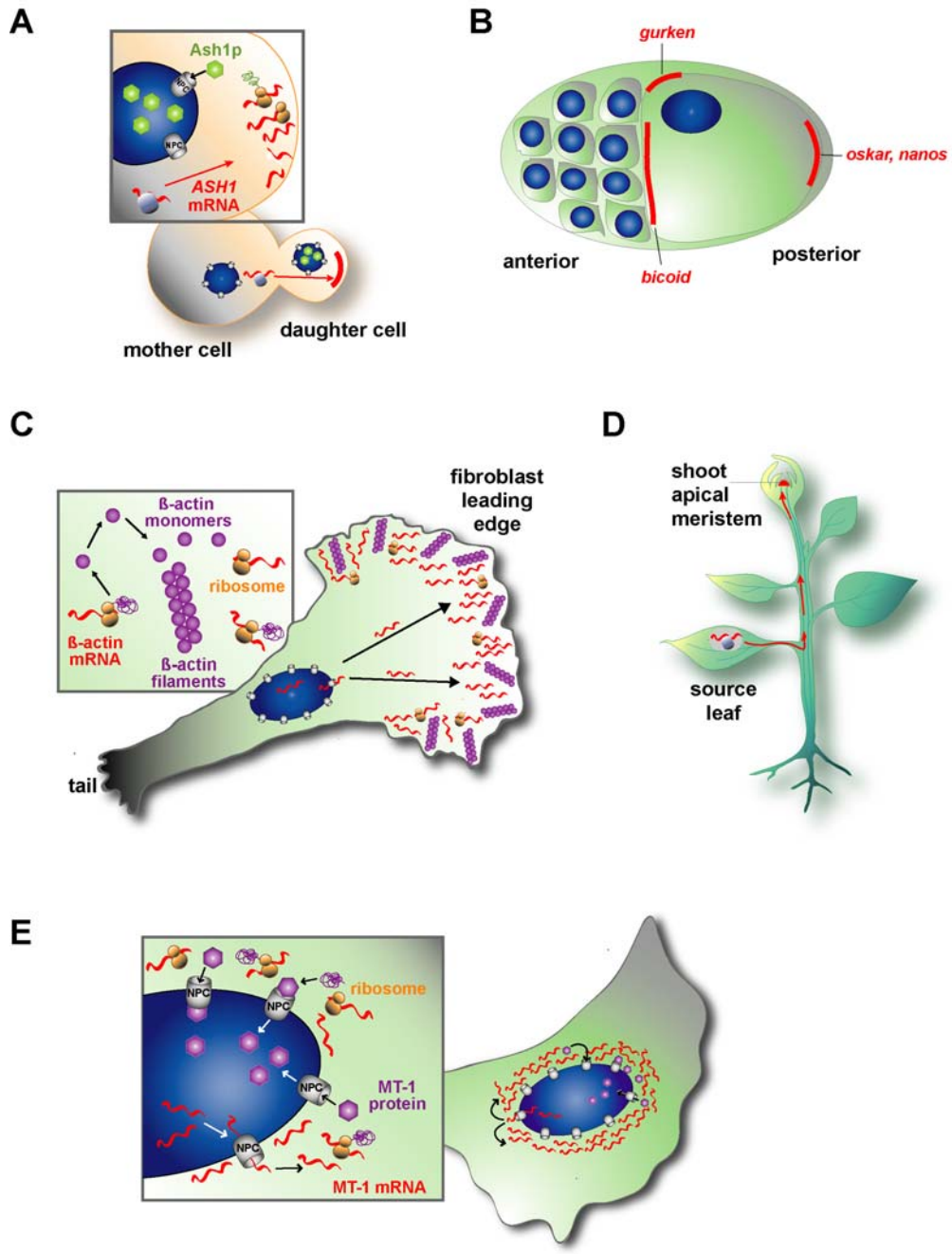

Figure 6: Biological functions of RNA localization. A. Asymmetric distribution of cell fate determinants. In *S. cerevisiae, ASH1* mRNA is localized to the tip of daughter cells prior to cell division. B. Establishment of morphogen patterns in oocytes and early embryos. Several mRNAs are localized in the *Drosophila* oocyte: Localization of *oskar*, *nanos* mRNA and bicoid define the anterior-posterior axis of the embryo. *gurken* mRNA is localized by a two-step mechanism to the anteriodorsal corner of the oocyte and establishes the dorsoventral axis. C. Protein isoform sorting and local assembly of complexes. In a chicken embryonic fibroblast, only ß-actin mRNA but not α-actin is targeted to the leading edge where it leads to local assembly of ß-actin filaments. D. Long distance RNA transport in plants helps to adjust to developmental and environmental stimuli. E. Facilitated protein targeting. Metallothionein (MT-1) mRNA localizes to the perinuclear cytoplasm, which results in a more efficient MT-1 protein import into the nucleus (Source: Du *et al*., 2007).

The dorsoventral axis of the *Drosophila* embryo is defined by the localization of *gurken* mRNA to anteriodorsal corner of the oocyte, where it encodes for a transforming growth factor α (TGFα) like signalling protein.

RNA localization can also contribute to the asymmetric distribution of cell fate determinants. Asymmetric cell divisions are widespread in the eukaryotic kingdom. This is often achieved by the distribution of mRNAs upon cell division. The asymmetric sorting of yeast Ash1p to only daughter cells (Fig. 6A) is just one example. RNA localization also has a role in the asymmetric division of a neuroblast in *Drosophila* embryos. Neuroblasts usually divide into an apical ganglion mother cell (GMC) and a new neuroblast daughter at the basal. One important cell fate determinant is *prospero* mRNA, which is localized to cell cortex of the basal side of the GMC (Broadus et al., 1998). The RNA binding protein Staufen and the adapter Miranda mediate this transport process (Schuldt et al., 1998; Shen et al., 1998). Interestingly, Prospero protein and its mRNA are both transported by Miranda suggesting that RNA localization serves to facilitate but not accomplish the asymmetric sorting of a cell fate determinant. A second cell fate determinant in the GMC is *insecutable*, which is also redundantly targeted to the apical cortex in a dynein-dependent fashion (Hughes et al., 2004). Localization of this mRNA serves to regulate apicobasal polarity and spindle length. In *Xenopus* oocytes, the localization of *VegT* mRNA to the vegetal hemisphere serves as a germ layer determinant. *VegT* mRNA encodes a T-box transcription factor required for mesendodermal development.

In order to create polarity in somatic cells, mRNAs often serve as a template for the local synthesis of proteins. This facilitates the spatial assembly of multifactor complexes. In motile cell types, β-actin mRNA is transported to the leading edge of lamellipodia (Condeelis and Singer, 2005). Local activation of translation and subsequent polymerization into actin filaments serve to create protrusive force and cell motility. The zipcode binding protein 1 (ZBP1) is required for the transport of β-actin mRNA (Ross et al., 1997). The spatial regulation of β-actin translation during transport leads to synthesis of the protein only at the target site (Hüttelmaier et al., 2005). Interestingly, only ß-actin but not α- or γ-actin mRNA become localized. Thus, this could be a sophisticated mechanism that allows the specific sorting of isoforms and consequently, prevents the formation of unwanted isoform heteromers (Condeelis and Singer, 2005). The mislocalization of ß-actin causes abnormal cell

morphology and other effects such as the increase of metastatic potential in tumor cells (Shestakova et al., 1999). In order to establish protrusive force, transcripts of all seven subunits of the actin-related protein 2/3 (ARP2/3) complex are localized to the leading edge of fibroblasts as well (Mingle et al., 2005). This complex provides the nucleation of actin filaments and consequently, is required in a spatial context to establish cell motility (Machesky and Gould, 1999). The synaptic plasticity of developing neurons is established in a similar fashion. For instance, transcript of Calcium/Calmodulin dependent Kinase II (CaMKII α) is localized to dendrites (Mayford et al., 1996). There, directed protein synthesis can lead to rearrangement of synapses, which is important for higher brain functions such as learning and memory. mRNA localization may also contribute to the sorting of proteins into various organelles. Usually, protein sorting is provided by peptide signals, which directs the translated protein to the target organelle. However, there is increasing evidence that some mRNAs are already localized to the vicinity of organelles to facilitate and maximize the import of the corresponding proteins. There are several reports on mRNAs, which display perinuclear localization (Fig. 6E). For instance, transcripts encoding for Metallothionein-1 (MT-1) and transcription factors c-FOS and c-MYC accumulate at the nuclear periphery. These transcripts have also been reported to associate with the perinuclear cytoskeleton in order to become effectively imported into the nucleus (Levadoux et al., 1999; Mahon et al., 1997; Veyrune et al., 1996). Sorting of a subset of nuclear-encoded proteins to mitochondria involves mRNA localization. In yeast, for instance, the 3'-UTR of *ATM1* and *ATP2* mRNAs direct these transcripts to the vicinity of mitochondria. The latter transcript encodes a subunit of the mitochondrial ATP synthase. Interestingly, impaired *ATP2* mRNA sorting correlates with a severe respiratory deficiency indicating a link between mRNA localization and protein function (Corral-Debrinski, 2007; Corral-Debrinski et al., 2000; Margeot et al., 2002; Sylvestre et al., 2003). Local enrichment of transcripts at mitochondrial-bound polysomes may ensure an effective cotranslational import into these organelles.

There is less known about RNA localization in the plant kingdom than in animal cells but a role for RNA as a long-distance information molecule is emerging in plant biology. Over the last decade, there is a growing body of evidence that the phloem high-pressure translocation stream, in addition to substrate delivery, can act as an information pipeline that allows the transport of small RNAs, such as siRNAs and

miRNAs, but also of mRNA transcripts. The intercellular and long distance transport of RNA molecules over makes it possible that developmental and physiological processes are coordinated at level of the whole plant (Fig 6D). Support for the concept of RNA-based long distance signalling was provided by the identification of a unique set of mRNAs isolated from phloem sap (Ruiz-Medrano et al., 1999; Ruiz-Medrano et al., 2001). *In situ* RT-PCR analysis revealed that the phloem stream has the capacity to mediate transport of several messenger RNA transcripts, such as *FT*-mRNA, *PFP-LeT6* mRNA or *CmNACP* mRNA, over very long distances to the apical meristem (Kim et al., 2001; Ruiz-Medrano et al., 1999; Xoconostle-Cazares et al., 1999). Some of these transcripts have a role in developmental processes such as flowering. But how long distance transport of mRNAs is linked to a specific process remains to be determined in more detail.

1.8 Initiation of mRNA localization

Cytoplasmic RNA localization is based on interactions between *cis*-acting sequences and multiple *trans*-acting factors. This dynamic process involves the formation of large RNP complexes that are continually modified through binding and release of protein partners (Arn et al., 2003; Mowry, 1996; Ross et al., 1997). Indeed, large RNP granules have been visualized during RNA transport (Barbarese et al., 1995; Bertrand et al., 1998; Krichevsky and Kosik, 2001; Rook et al., 2000; Wilkie and Davis, 2001). However, it is not yet clear how and when such protein factors associate with localized RNAs. Formation of a localized RNP is arguably an early event in the localization pathway and recent findings have suggested that the process could already initiate in the nucleus (Farina and Singer, 2002). A growing number of *trans*-acting localization factors have been identified as predominantly nuclear proteins or nucleo-cytoplasmic shuttling proteins. These proteins might interact with specific transcripts in the nucleus and could either mark them for localization and/or escort them to their ultimate destination in the cytoplasm. In *Drosophila* for example, Sqd, a member of the hnRNP (heterogeneous nuclear RNP) family, is required for *gurken* mRNA localization to the dorsal pole during oogenesis (Norvell et al., 1999). Sqd has been suggested to bind *gurken* mRNA in the embryonic nucleus. In mammals, recognition of MBP (myelin basic protein) mRNA by hnRNP A2 first occurs in the nucleus (Ainger et al., 1997; Hoek et al., 1998). The

formed MBP mRNP complex then exits the nucleus and localize to the myelin compartment of oligodendrocytes (Izaurralde et al., 1997; Munro et al., 1999). Similarly, the cytoplasmic RNA-binding protein ZBP (zipcode-binding protein), which is involved in β-actin mRNA localization in chicken fibroblasts and neurons, was shown to shuttle through the nucleus by means of specific nuclear import and export signals and to bind β-actin mRNA at its transcription site (Hüttelmaier et al., 2005; Oleynikov and Singer, 2003; Ross et al., 1997; Zhang et al., 2001). Finally, the strictly nuclear dsRNA-binding protein Loc1 is required for the efficient localization of *ASH1* mRNA to the yeast bud tip (Long et al., 2001). She2p was proposed to shuttle between nucleus and cytoplasm (Kruse et al., 2002). In sum, this favours the idea that nuclear and cytoplasmic steps in the RNA transport pathway are linked.

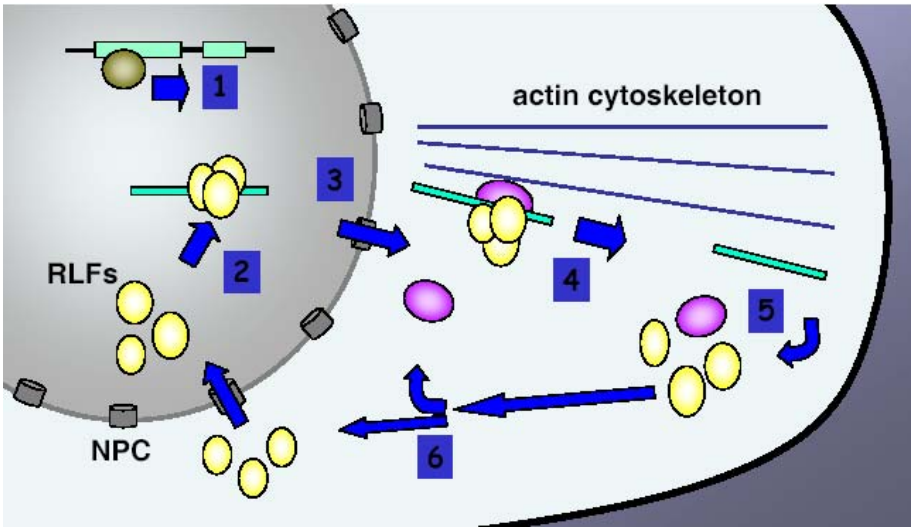

Figure 7: Model of RNA localization factors (RLFs) shuttling between nucleus and cytoplasm. RLFs associate with transcripts designated for cytoplasmic RNA localization shortly after transcription and form a localized RNP or “locasome” (1). The resulting nuclear RNP is exported through the Nuclear Pore Complexes (NPC). After recruitment of additional factors such as adapters and motor proteins, the resulting transport competent RNP complex travels along polarized cytoskeleton e.g. actin filaments (4) to the target site where it is anchored and locally translated (5). The released RLFs can enter the nucleus for a second round of RNA binding (6).

1.9 Aim of this work

There is an increasing line of evidence that the 'nuclear history' of an mRNA has an important role in the determination of its fate in the cytoplasm (Farina and Singer, 2002; Giorgi and Moore, 2007; St Johnston, 2005). In yeast, little is known about when and how such localized RNP complexes are formed. Following the route of *ASH1* mRNA and its subcellular localization upon various conditions should reveal where localized RNPs might be assembled. One aim of this work was the investigation of the subcellular distribution She2p and of all other *trans*-acting factors in order to understand their chronological recruitment to the RNA during RNP assembly. This also includes the biochemical purification of nuclear She2 bound to its target RNA. She2 was reported to be a nucleocytoplasmic shuttling protein (Kruse et al., 2002). In a second approach, cytoplasmic retention of She2 should reveal the biological significance of its shuttling nature. Moreover, following this strategy should give an explanation, which of the cytoplasmic processes in the context of RNA localization is affected when blocking She2's import into the nucleus.

2 Results

2.1 Purification of a specific antibody directed against She2p

At the beginning of this thesis, no specific antibody directed against She2p was available commercially and therefore all biochemical and microscopic approaches had been performed with strains harbouring epitope tagged She2 protein. The disadvantage of an epitope tag becomes obvious if we consider that especially the binding properties of an RNA binding protein could be sterically constricted or inhibited, which in the end could lead to artificial results. In order to facilitate the biochemical characterization of She2p we decided to generate a rabbit polyclonal antibody against full length She2p.

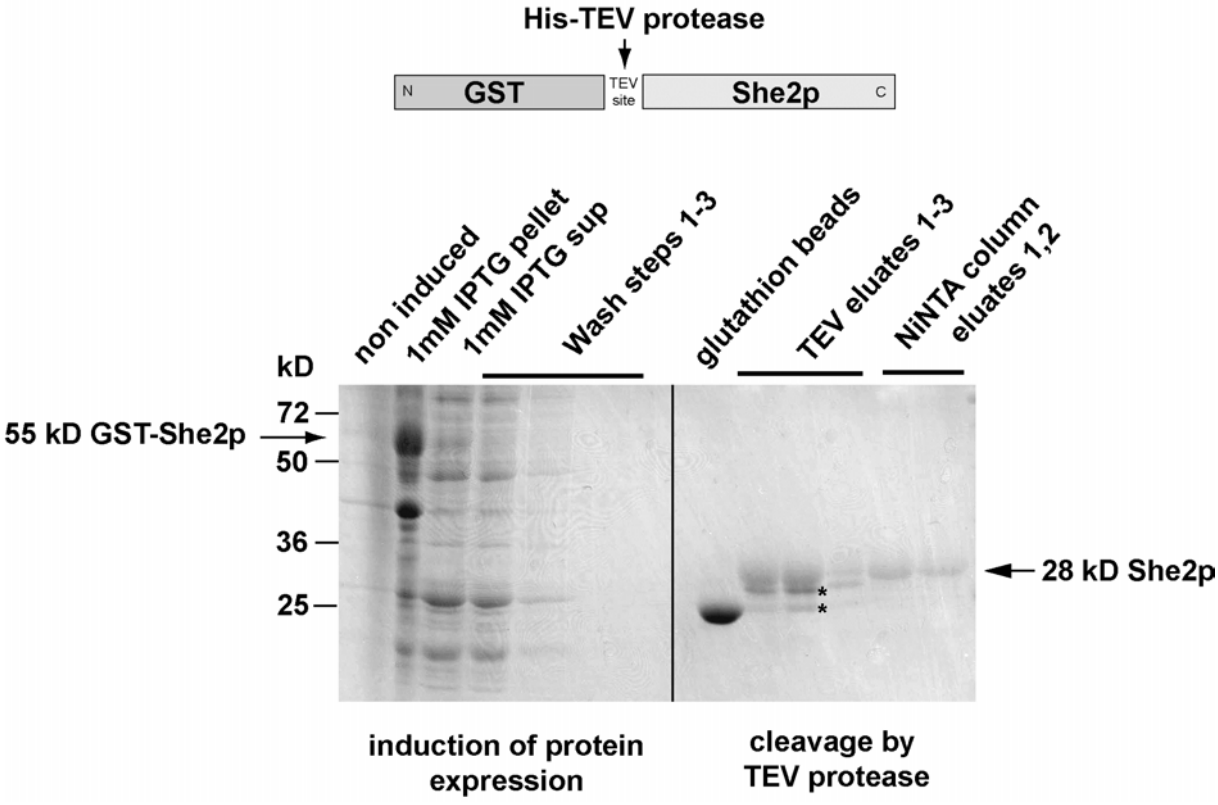

Figure 8: Expression and purification of recombinant She2p in *E. coli*. Expression vector RJP20 (pGEX-TEV-She2) contains a cleavage site for TEV-Protease. Expression of 55 kDa GST-She2p with 1 mM IPTG (left panel). Soluble fraction of the cell extract was applied for binding to Glutathione beads (right panel). She2p was eluted by treatment with TEV-protease (marked by asterisks). Pure She2-antigen was obtained after removal of His-tagged TEV-protease with NiNTA.

To get rid of unspecifically cross-reacting antibodies in the serum it was necessary affinity purify the antibody. Therefore, purified She2-antigen was produced in high amounts. She2p was recombinantly expressed in *E. coli* from a pGEX-T expression vector and was then purified in two subsequent steps. Upon induction of the GST-She2p fusion protein, only a small fraction was soluble, whereas most of the produced protein was in inclusion bodies. The correctly folded GST-She2 protein in the soluble fraction was applied for binding to glutathione beads. To avoid increased production of antibodies directed against GST the fusion protein contains a recognition site for the TEV protease to cleave off the desired She2 protein. To further increase purity of the antigenic material, the engaged His-tagged protease was removed by an additional step using Ni-NTA sepharose. Rabbits immunized with purified She2p produced specific antibodies already after the third bleeding (data not shown). Further purification of the yielded serum by affinity chromatography visibly increased specificity of the antibody.

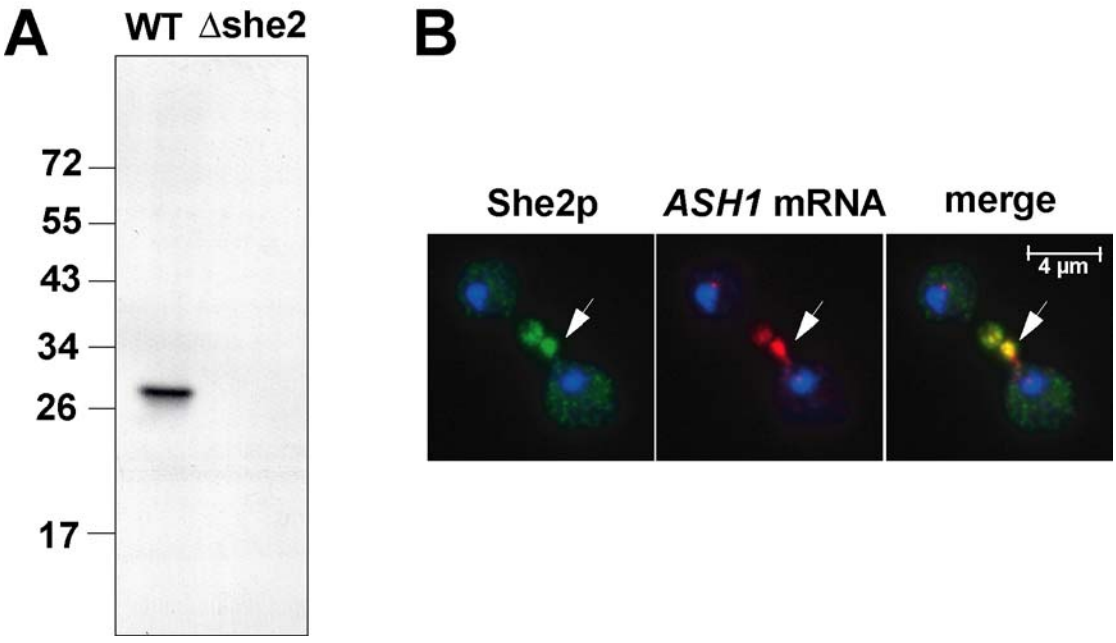

Figure 9: Specificity of purified She2-antibody. A. Western blot analysis using purified α-She2 directed against 28 kDa She2p detects a single band in a wild type strain (WT), but not in a *she2Δ* strain. B. Representatives of WT cells in a simultaneous stain of She2p (green signal) using immunofluorescence (1st purified α-She2/2nd Alexa488 goat α rabbit)and *ASH1* mRNA (red signal). *In situ* hybridisation (FISH) was performed using TexasRed labelled *ASH1*-oligonucleotides. Overlap of She2p and *ASH1* mRNA signals (merge, yellow signal) at the tip of daughter cell (arrowhead) indicates colocalization. Nuclei were stained with DAPI (blue).

As displayed in figure 9A the purified antibody detects She2p as a single band with the expected size of 28 kDa in a western blot whereas in a *she*2Δ strain no bands show up. In both lanes, no cross-reaction by the antibody was detectable. The remarkable specificity makes this polyclonal antibody highly suitable for immunofluorescence (IF) microscopy. In figure 9B, functionality of the antibody was assayed in a dual stain of She2p and the transported *ASH1* mRNA. The purified antibody detects She2 in emerging daughter cells (green signal). The merged picture clearly shows the colocalization of She2p with its target mRNA.

2.2 She2 is a nucleo-cytoplasmic shuttling protein

Although no well-known RNA binding motifs are present within the She2 protein, it can bind to all localization elements of the *ASH1* mRNA (Böhl et al., 2000). In theory, the first binding of She2p to its target RNA may already occur shortly after transcription in the nucleus. This suggests that She2p is able to enter the nucleus. After binding, there are two possible scenarios in order to leave the nucleus, either dependent on the export of its mRNA or dependent on protein export. With a new antibody in hands, it is now possible to address this question without the use of epitope tagged She2p. Precedence for the scenario that She2p enters the nucleus at least temporarily and become exported in an RNA-dependent fashion was provided by a previous study using a thermosensitive mutant of the mRNA export factor Mex67 (Kruse et al., 2002). In a complex together with Mtr2p, Mex67 is essential for the export of bulk poly(A)+ mRNAs since it provides the passage of mRNPs through the nuclear pore complex. In the temperature sensitive mutant *mex67-5*^{ts}, mRNA export is strongly impaired when shifted to its non-permissive temperature at 37°C (Segref et al., 1997). After 5 minutes at elevated temperatures, the mutant cells start to accumulate poly(A)+ mRNA in the nucleus. The nuclear accumulation of poly(A)+ mRNA can be monitored by *in situ* hybridisation with fluorescently labelled oligo-dT probes. Figure 8B shows that at permissive temperature (26°C) mRNA export proceeds as can be observed by the cytoplasmic staining of exported mRNA. However, cells show accumulated poly(A)+ mRNAs in nuclei after shift to elevated temperature (37°C). Since mRNA export is a highly essential process, a single deletion of *MEX67* is not viable. The temperature sensitive mutant *mex67-5* strain

was generated by 'shuffling out' the wild type *MEX67* copy (*URA* selectable plasmid) with a plasmid containing the mutant *mex67-5* mutant allele (*TRP* selectable plasmid) using 5'-FOA (5'-Fluorootic Acid) containing plates. 5'-FOA is converted to a toxic compound (5'-fluorouracil) by cells expressing a functional URA3 gene. Thus, on 5'-FOA containing media, mutant strains can be selected that have lost the wild type copy (URA plasmid) but instead carrying the mutant plasmid (TRP plasmid).

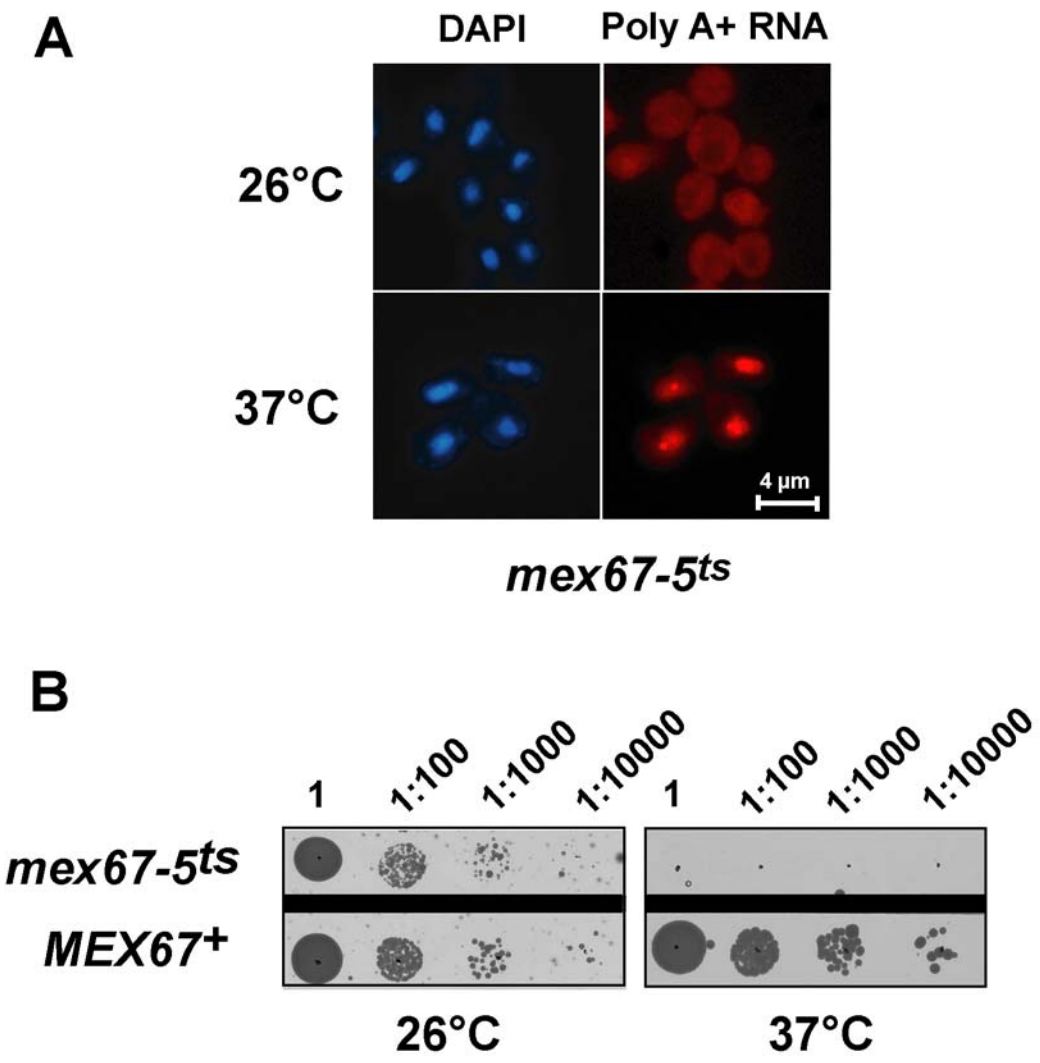

Figure 10: The thermosensitive mutant *mex67-5*. A. Cells of strain RJY612 (*mex67-5*) were fixed and stained by *in situ* hybridisation with Cy3-labelled oligo-dT probes directed against poly(A)+ mRNA. Cells display a cytoplasmic staining of poly(A)+ mRNA at 26°C (upper panel), after shift to non-permissive temperature (37°C), poly(A)+ mRNA accumulate in nuclei (lower panel). Nuclei were stained with DAPI (left panels). B. Temperature sensitive growth of mutant strain RJY612 (*mex67-5*) and respective wild type control RJY646 (MEX67+) in drop test at different dilutions (OD 1-1:10000). Plates incubated at 26°C (left panel) or 37°C (right panel) for 2 days. At 37°C, *mex67-5* mutant is not viable. In contrast, growth of *MEX67+* control strain is not affected.

In order to determine whether the resulting strains are temperature sensitive, growth of both, *mex67-5* and *MEX67+* control strain were analysed in a drop test under permissive and restrictive conditions (Figure 10B). The thermosensitive *mex67-5* strain is not able to grow at 37°C whereas in contrast, growth of the control strain (*MEX67+*) remains unaffected. To investigate nuclear/cytoplasmic distribution of She2p in a *mex67-5* temperature sensitive mutant, cells of this strain were grown logarithmically at 26°C and fixed for immunofluorescence before and after a shift to the restrictive temperature at 37°C for one hour. Staining of She2p was then performed using the purified She2-antibody. Under permissive conditions, She2p localizes to the bud tip like in wild type. In contrast, cells of the same culture display an accumulation of She2p in nuclei after temperature shift to restrictive conditions (Figure 11). Hence, while following its target mRNAs She2p is trapped in nuclei upon inhibition of mRNA export. This suggests that She2 is able to enter the nucleus and under normal condition is exported in mRNA-dependent fashion. Taken together, these results confirm a nucleo-cytoplasmic shuttling for She2 as was proposed in previous studies performed with myc-tagged She2p (Kruse et al., 2002).

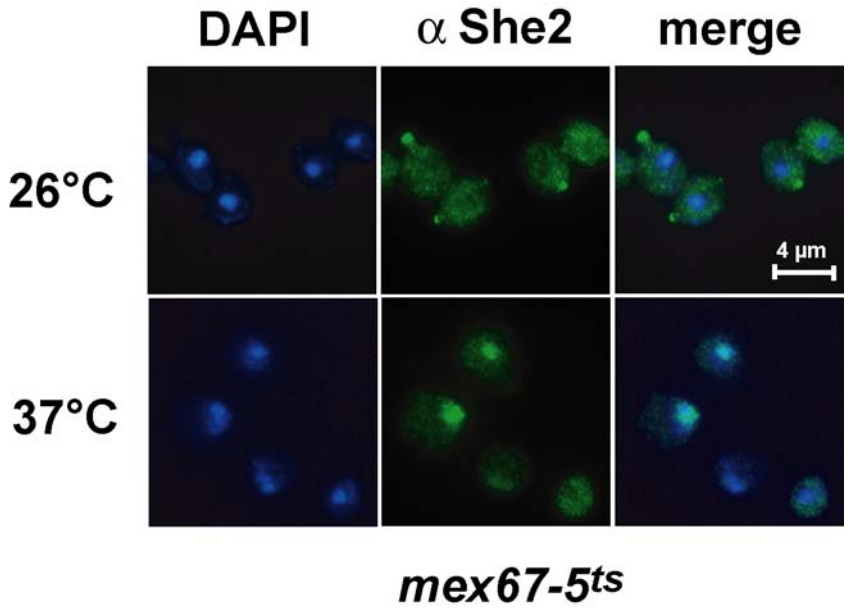

Figure 11: She2p accumulates in nuclei upon inhibition of mRNA export. Cells of strain RJY612 (*mex67-5*) in indirect immunofluorescence. She2 was stained with 1st purified α-She2 and 2nd Alexa488 goat α rabbit antibodies (green signal, middle panels). Nuclei were stained with DAPI (blue, left panels). Merged signals (right panels). Prior to a temperature shift at 26°C, cells display localization of She2p to the bud tip (upper panels). Temperature shift of the same culture to 37°C for one hour resulted in a nuclear staining of She2p (lower panels).

2.3 A subpopulation of She2p is nuclear

The accumulation of She2p under conditions where mRNA export is blocked suggests a nuclear interaction of She2p with *ASH1* mRNA. Indirect immunofluorescence of wild type cells displays an overall distribution of She2 with the majority of the protein in the cytoplasm. However, this indicates that at least a portion of She2p might be present in the nucleus. In order to gain biochemical evidence on nuclear She2p, intact yeast nuclei were isolated by subcellular fractionation using differential centrifugation (Hurt et al., 1988). After cell breakage, the nuclei containing cell fraction (crude nuclear pellet, CNP) was separated from cytoplasmic material and cell debris by two steps of differential centrifugations. The resulting CNP was loaded onto a Ficoll/sucrose density step gradient ranging from 1.2 to 2M sucrose in order to purify the nuclear extract further (see Fig. 12). All fractions collected were analyzed in a western blot. As shown in figure 12A pure and intact nuclei accumulate into fraction IV through sedimentation, which corresponds to the interphase between 1.5 and 1.8M sucrose. The presence of nuclei in this fraction was verified with an antibody directed against a subunit of RNA polymerase I (Rpa49p). Because Rpa49p is a soluble nuclear marker, it is also detectable in lighter fractions due to broken nuclei. However, fraction IV is devoid of the soluble cytoplasmic marker Pgk1p (phosphoglycerate kinase). In contrast, also a subpopulation of She2p is cofractionating with intact nuclei. The presence of She2p in nuclear fractions has been shown independently with other biochemical fractionation methods. In 2-step and 5-step gradients, which were used for the floating of ER membranes, She2p has been observed to cosediment with intact nuclei as well (Schmid et al., 2006) supporting the evidence that She2p is at least partially nuclear. The nuclear interaction of She2p with *ASH1* mRNA in nuclei was assessed by immunoprecipitation of She2p using only purified nuclear material. In a subsequent step, the immune pellet was probed for the presence of bound *ASH1* mRNA by RT-PCR (IP-RT). As a control, subcellular fractionation and the subsequent IP-RT reaction were in parallel performed also with a *she2Δ* strain in order to exclude that She2p or *ASH1* mRNA was precipitated with beads only. Immunoprecipitation of She2p was performed with the purified She2-antibody coupled to magnetic Protein G-beads. Using wild type nuclear fractions, She2p was successfully precipitated with the antibody (Fig. 12B).

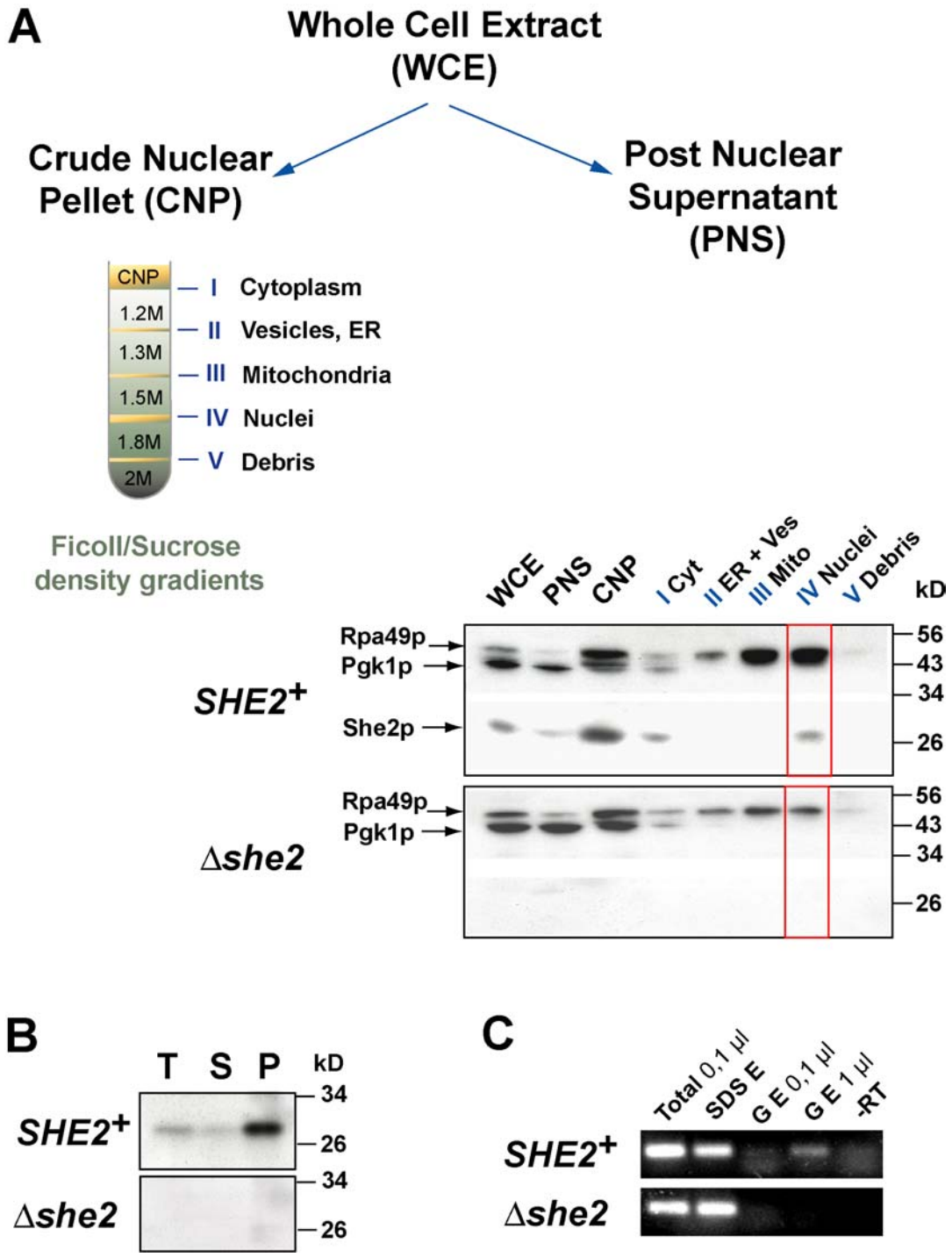

Figure 12: Nuclear RNA-protein interaction of She2p and *ASH1* mRNA. A. Subcellular fractionation of WT (SHE2+, upper panel) and control strain RJY126 (*she2Δ*, lower panel). Western blot analysis: In WT a subpopulation of She2 cofractionates with Rpa49p (Fraction IV, red box). Same fraction is devoid of cytoplasmic marker Pgk1p. B. Immunoprecipitation of She2p in a western blot. She2p was successfully immunoprecipitated (immune pellet, P) out of nuclear fraction IV (total, T, supernatant S). C. RT-PCR with primers against *ASH1* E3 localization element. E3-signal was only detected in an elution with 100 mM Glycine-HCl (GE 1µl and dilution 0.1 µl) of WT (SHE2+, upper panel) but not in control (*she2Δ,* lower panel). Elution with 10% SDS (SDS). Control without Reverse Transcriptase (-RT)

A portion of the immunoprecipitated material was subjected to RT-PCR reactions. In a mild elution step using 100 mM glycine-HCl at low pH, the She2 antigen could be specifically released from the antibody. This ensures that only She2-associated but not DNA or unspecific mRNA attached to magnetic beads is detected in the subsequent RT-PCR. This step is critical since nuclei are full of nucleic acids. In a second elution step using SDS, all unspecific bound material attached to the beads is washed off. In a wild type strain, RT-PCR reaction with primers amplifying the E3 localization element reveals the presence of *ASH1* mRNA in the glycine-elution. Hence, the detected *ASH1* mRNA was bound to She2p precipitated from nuclear fractions. Under the same conditions, *ASH1* PCR product was not detected in the glycine elution of the control (*Δshe2*) suggesting that amplification of *ASH1* E3 is dependent on the presence of She2p. Unspecific bound *ASH1* mRNA/DNA contaminants were detected in the SDS elution of both, wild type and control strain. Taken together, these results suggest a nuclear interaction of She2p with *ASH1* mRNA. This let me hypothesize that She2p enters the nucleus in order to fulfil an early recognition and binding to *ASH1* mRNA.

2.4 Subnuclear accumulation of She2p upon inhibition of mRNA export

A closer look at microscopic data of She2p revealed that the accumulation upon mRNA export block is not within the chromatin-rich regions of the nucleoplasm. She2 signals did not overlap with corresponding signals of chromosomal DNA stained by DAPI (4',6-Diamidino-2-phenylindol) but shows a crescent-like structure next to this region. To address the question if accumulation of She2p might be located within other subnuclear regions such as the nucleolus, a costain of She2p with Nop1p was required (Aris and Blobel, 1988). Thus, cells of thermosensitive *mex67-5* strain and the corresponding wild type control (*MEX67*$^{+}$) were grown logarithmically at 26°C. *ASH1*, which was under the control of a GAL1 promoter was overexpressed for one hour and then shifted to non-permissive temperatures. In the wild type control, bud tip localization of She2p remains unaffected upon temperature shift. In contrast, *mex67-5* temperature sensitive mutant displays increased accumulation of She2p in nuclei. Moreover, in an immunofluorescence double staining accumulated She2-signals largely overlapped of with signals of the nucleolar antigen Nop1p (Fig. 13, merged panel, yellow signal). In summary, the inhibition of mRNA export does not

allow She2p to leave the nucleus but leads to its accumulation at sites of the nucleolus. This supports the idea that under normal conditions nuclear She2p may transit through the nucleolus.

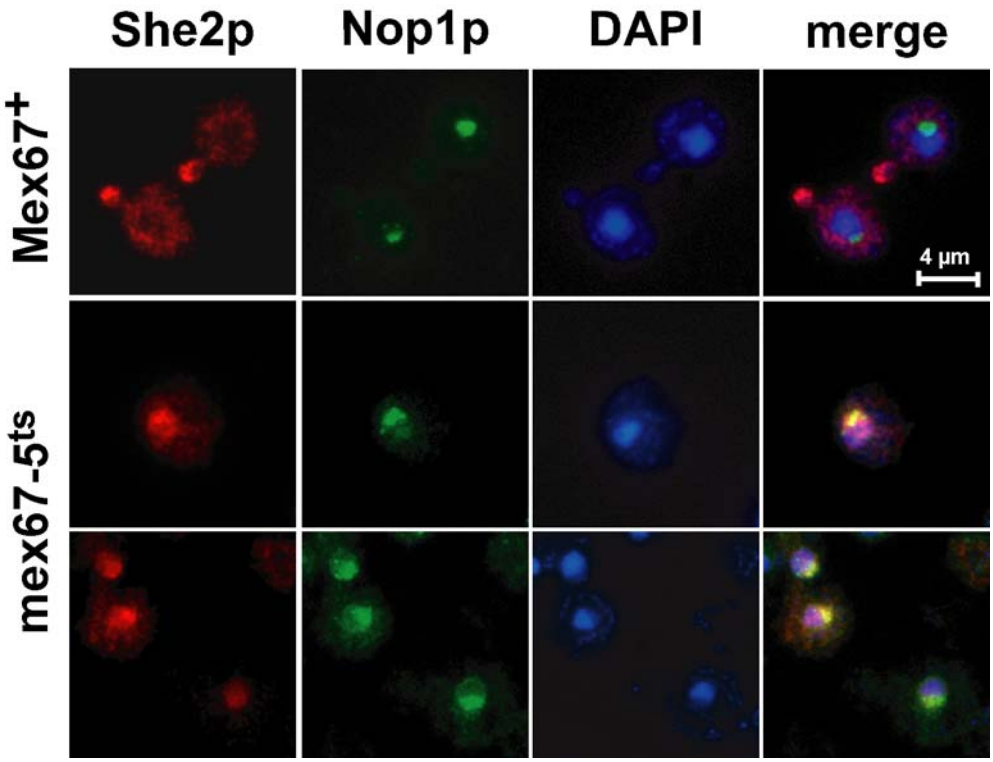

Figure 13: She2p accumulates in nucleoli upon block of mRNA export. Representative cells of thermosensitive strains RJY1149 (*mex67-5^{ts}, GAL1-ASH1*) and corresponding WT control RJY2239 (*MEX67^{+}, GAL1-ASH1*) in an immunofluorescence double staining of She2p and Nop1p. Overexpression of *ASH1* was induced by 4% Galactose, and cells shifted to restrictive temperatures for one hour. Fixed cells were stained for She2p (red signal) with 1st purified α-She2 and 2nd Alexa594 goat α rabbit antibodies and Nop1p (green signal) with 1st α-Nop1p and 2nd Alexa488 goat α mouse antibodies. Nuclei were stained with DAPI (blue signal). Wild type control (top panels) displays normal localization of She2p at the bud tip. Overlap of She2p and Nop1p signals *mex67-5^{ts}* (lower panels) indicate colocalization (merge, yellow signal).

2.5 The export of She2p is dependent on the binding to its target mRNA

Previous data suggested that She2p protein is exported in an mRNA-dependent manner. It is also known that a deletion mutant with an N-terminal truncation of She2p (She2pΔN70) leads to an increase in nuclear accumulation (Kruse et al., 2002). Furthermore, a genetic screen identified certain arginine and asparagine

residues within the NH_2-terminus of She2p, which are essential for RNA binding activity (Gonsalvez et al., 2003; Niessing et al., 2004). In order to assess if some of these specific mutations in She2p would alter the nuclear/cytoplasmic distribution, mutants having lost the ability to bind *ASH1* mRNA were investigated by indirect immunofluorescence. Since it has been reported that single amino acid substitutions N36S or R63K are sufficient to cause the loss of *ASH1* mRNA localization (Gonsalvez et al., 2003), we generated these RNA binding mutants by *in vitro* mutagenesis. To ensure a complete loss of RNA-binding activity, also a double substitution She2-N36S-R63K was generated also (Maria Schmid).

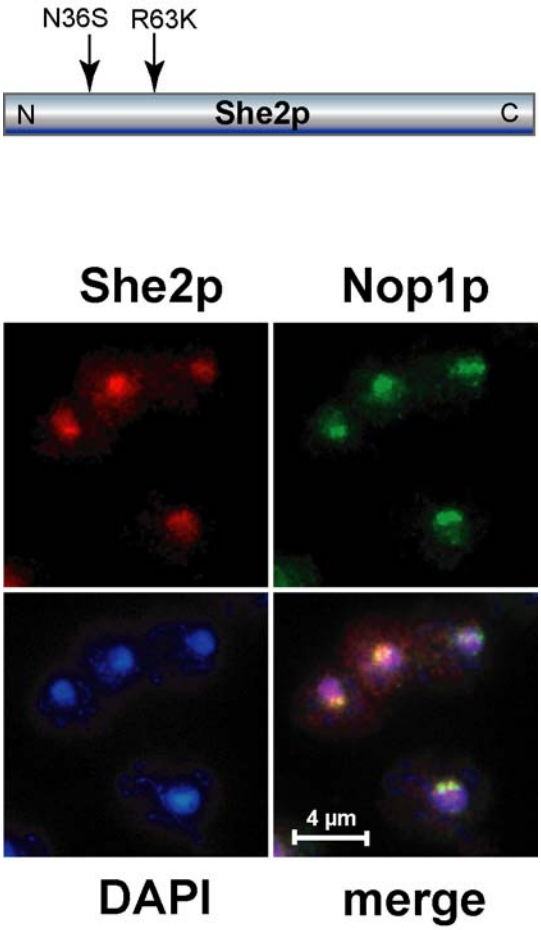

Figure 14: RNA binding mutant of She2p accumulate in nucleoli. Representative cells of strain RPY2838 (*she2Δ*, She2-N36S-R63K) in indirect immunofluorescence. Nucleocytoplasmic distribution was assessed in a dual stain of She2p and Nop1p. She2p (red signal) was stained with 1st purified α-She2 and 2nd Alexa 594 goat α-rabbit antibodies. Nop1p (green signal) was stained with 1st α-Nop1p and 2nd Alexa 488 goat α-mouse antibodies. Nuclei were stained with DAPI (blue signal). Overlap of She2 and Nop1-signals in the merged panel (yellow signal) indicate colocalization.

When expressing She2-N36S, She2-R63K and She2-N36S-R63K mutants instead of She2p the ability to become localized at the tip of daughter cells was lost. Moreover, *ASH1* mRNA was mislocalized in all She2-mutants analyzed. In contrast to a previous study, which was performed with myc-epitope tagged She2p (Gonsalvez et al., 2003), the single amino acid substitution in the She2-N36S mutant was sufficient to accumulate She2p in nuclei (Schmid et al., 2006). The She2-R63K mutant showed just a very weak nuclear accumulation, whereas the mutant phenotype was strongest in the double mutant She2-N36S-R63K. Interestingly, nuclear accumulation of this mutant displays the staining of a nucleolar crescent similar to that observed for She2-accumulation upon block of mRNA export. A double stain of She2p with nucleolar marker Nop1p clearly shows that signals of both proteins largely overlap (Figure 11, yellow signal, merge). Importantly, nucleolar accumulation in this case was not caused by the inhibition of mRNA export. Instead, nuclear retention of this mutant may be due to the loss its specific RNA-binding activity. Indeed, when addressing the binding affinity of this mutant in filter binding experiments She2-N36-R63K mutant revealed a strong decrease compared to wild type She2p, which was able to bind *ASH1* E3 RNA with a binding constant of K_d=147 nM (Marisa Müller, personal communication). The binding of this mutant to an unrelated RNA stem loop of the human immundeficieny virus (HIV-I TAR RNA) was also abolished excluding that RNA binding could be responsible for nucleolar accumulation. Therefore, She2's association with the nucleolus is probably independent of RNA binding activity. However, the export of She2 requires the export of *ASH1* mRNA. This suggests that RNA binding activity is a prerequisite for She2p to become exported into the cytoplasm together with its RNA target.

2.6 Following She2p in vivo

In order to rule out artefacts based on staining or fixation procedures, Green fluorescent protein (GFP) was used to study She2's cellular distribution also in living cells. So far, tagging cassettes used for a C-terminal fusion with GFP on the genome have not resulted in a functional She2 protein (Andreas Jaedicke, personal communication). A large GFP-tag at the C-terminus may probably interfere with She2-function. Therefore, GFP was fused to the NH_2-terminus of She2p. GFP-She2p fusion was provided on a plasmid using the endogenous promotor and the 3'

untranslated region of the *SHE2* gene. To avoid interference or inhibition of RNA-protein or of other protein-protein interactions, an additional flexible linker that consists of the amino acid sequence PPGPP was placed in between the two proteins. Based on structural data, it has been reported that She2p forms dimers (Niessing et al., 2004). To investigate functionality of GFP-She2p, the constructed plasmid was transformed into *she2Δ* background to exclude hybrid dimerisation products between endogenous She2p and GFP-She2p. Cells transformed with a plasmid express GFP-She2p at a size of ~60 kDa as determined by western blot analysis with an antibody directed against either the She2 or the GFP portion. When GFP-She2p instead of She2p was expressed, green RNP particles were visible under the microscope. These green particles showed movement and finally localization at the tip of daughter cells under normal conditions suggesting that transport of GFP-She2 was functional (Figure 15A). In addition, functionality regarding RNA transport was verified by *in situ* hybridization showing bud tip-localization of *ASH1* mRNA in cells expressing GFP-She2p (data not shown).

2.7 Binding of She2 to *ASH1* mRNA occurs at early stages of mRNA maturation

To test if GFP-She2p accumulates in nuclei of living cells upon inhibition of mRNA export the constructed plasmid was transformed into a *mex67-5* mutant background. Consistent with previous results, GFP-She2p localizes to the tip of daughter cells under permissive conditions, whereas upon arrest of mRNA export, GFP signals visibly accumulated in nuclei. This suggests that the 60 kDa GFP-She2p, even though it is bigger in size is also able to enter the nucleus. In order to address the question if binding of She2 to *ASH1* mRNA occurs at earlier stages of RNA processing, it would be necessary to test if temperature sensitive mutants of other mRNA export factors such as Yra1p and Sub2p are able to accumulate GFP-She2p in the nucleus. Yra1p is an essential and conserved mRNA export factor that directly interacts with the mRNA transport complex Mex67p/Mtr2p (Strasser and Hurt, 2000). Sub2p has been reported to be a splicing factor required for nuclear mRNA export through its interaction with Yra1p (Strasser and Hurt, 2001). In the process of RNA export, both proteins act upstream of the final mRNA exporter complex Mex67p/Mtr2p.

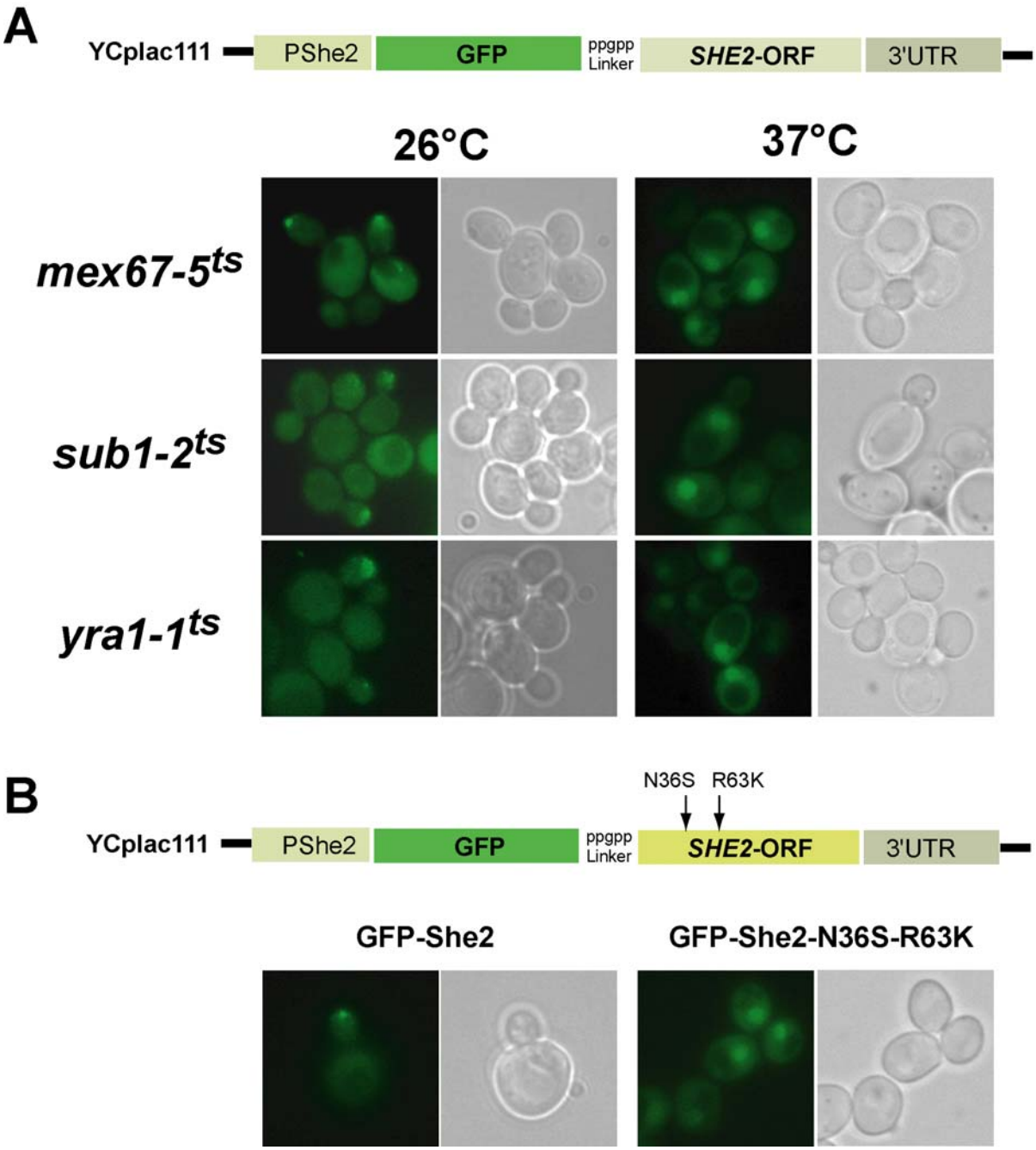

Figure 15: Nuclear accumulation of GFP-She2p in living cells. A. Nuclear/cytoplasmic distribution of representative cells of thermosensitive strains RJY2198 (GFP-She2p, *mex67-5, GAL1-ASH1*), RJY2354 (GFP-She2p, *sub2-85, GAL1-ASH1*) and RJY2273 (GFP-She2p, *yra1-1, GAL1-ASH1*) expressing GFP-She2. At permissive temperature (26°C), all strain show bud tip localization of GFP-She2p (left block). Nuclear accumulation was observed only after shifting temperature to restrictive conditions (37°C) for two hours (right block). Cells in DIC (right). B. The RNA binding double mutant GFP-She2-N36S-R63K was expressed instead of She2p in living cells (RJY2785). GFP-She2 localizes to the bud tip (left). In contrast, the corresponding GFP-She2-N36S-R63K mutant displays sequestration of GFP signal in nuclei. Note that mRNA export was not inhibited in these cells.

To test whether She2 export depends on Sub2p or Yra1p, thermosensitive mutants *sub2-85* and *yra1-1* were generated by 'shuffling out' the wild type copy with 5'-FOA containing media as was previously described for the *mex67-5*^{ts} strain. The resulting temperature sensitive strains expressed and localized GFP-She2 under permissive conditions (Figure 15A, left panels). However, after a shift to restrictive temperatures, accumulation of GFP-She2 was also detectable in nuclei of a *sub2-85* and *yra1-1* thermosensitive cells (Figure 13, right panels). This can only be the case if She2-binding occurs at very early stages prior to the recruitment of Yra1p. Because Yra1 and Sub2p are recruited to nascent mRNAs already during transcription elongation (Aguilera 2005), binding of She2p to *ASH1* mRNA may occur co-transcriptionally, probably at transcription sites. Indirect immunofluorescence has already shown that She2-N36S-R63K mutant is defective in RNA binding and thus leading to its accumulation in nuclei. In order to address if accumulation of this RNA binding mutant occurs in living cells as well, it was provided with a GFP-tag. In the wild type control, cells expressing GFP-She2 display bud tip localization of the GFP signal. However, GFP-She2-N36S-R63K RNA binding mutant visibly accumulates in nuclei of living cells (Fig. 15B).

2.8 She2 dimerisation is necessary for localization

Based on biochemical and structural data (Niessing et al., 2004) and on observations coming from two-hybrid interactions of She2p with itself (Böhl et al., 2000), it has been suggested that She2p forms a homodimer. Furthermore, a serine at position 120 was reported to be essential for dimer formation. Thus, amino acid substitution S120Y results in only monomeric She2p, which is also defective in *ASH1*-localization. To assess whether monomeric She2p has an altered nuclear/cytoplasmic distribution, the dimerisation mutant She2-S120Y was generated by site directed mutagenesis and expressed in *she2Δ* cells. Western blot analysis and IF microscopy revealed that monomeric She2 is expressed, and is ubiquitously distributed throughout the cell. In contrast to wild type She2p, the monomer does not show any localization at the tip of daughter cells suggesting that binding of She2p to She3p/Myo4p complex is impaired and dependent on dimerisation. Moreover, the dimerisation mutant does not accumulate within the nucleus as was observed previously in RNA binding mutants of She2p. It has been reported for monomeric

She2 that specific binding to each of the *ASH1* localization elements is lost (Marisa Müller, personal communication). According to these data, one would assume that the dimerisation mutant would also show an altered distribution similar to that observed for the RNA binding mutant. Apparently, nuclear accumulation is not strictly linked to the loss of RNA binding activity. This could be the case if only dimerised She2p is actively imported into the nucleus. Alternatively, only the dimer but not the monomer of She2p is kept by an unknown nuclear factor when RNA binding is lost.

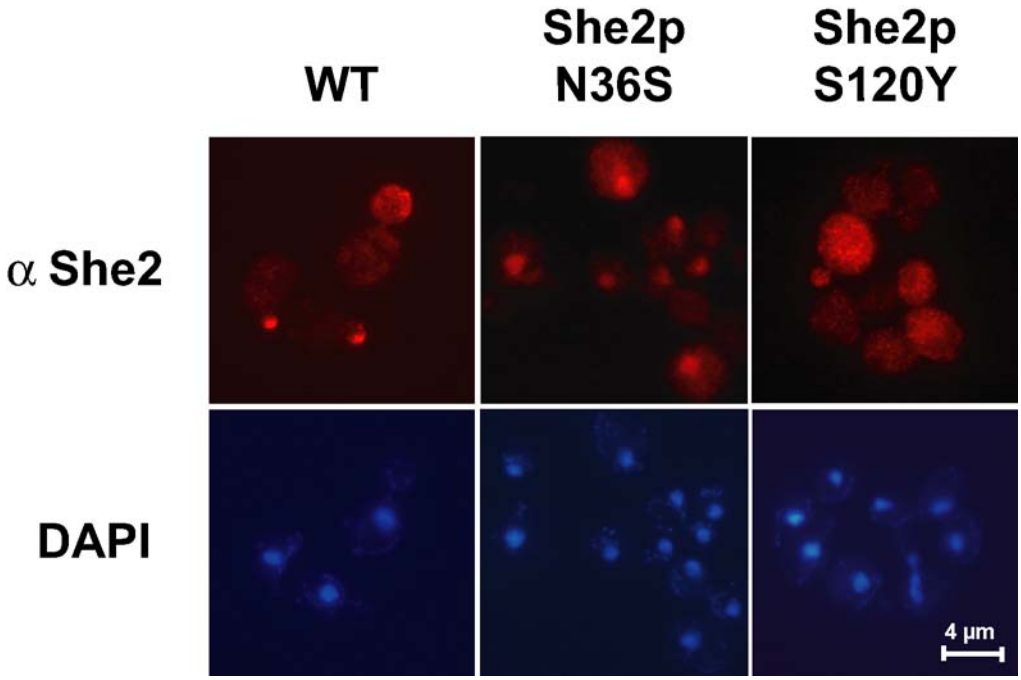

Figure 16: Monomeric She2p is ubiquitously distributed throughout the cell. Cells of strain RJY358 (WT, left), RJY2783 (She2-N36S RNA-binding mutant, middle) and RJY2960 (She2-S120Y dimerisation mutant, right) stained by the She2 antibody in indirect immunofluorescence. Note that She2-S120Y mutant does not localize to the tip of daughter cells. Accumulation of monomeric She2 in nuclei like in the RNA binding mutant was never observed.

2.9 Inhibition of mRNA export in a *mex67-5^{ts}/Δrrp6* mutant leads to accumulation of *ASH1* mRNA in the nucleolus

To understand why She2p is visibly associated with the nucleolus, it was necessary to investigate if *ASH1* mRNA also has its transit through this region. It was previously reported that when the 3'-Untranslated Regions (3'-UTR) of various RNAs were fused to U1A-hairpins, only the *ASH1*-3'UTR was able to direct a U1A-GFP reporter into the nucleolus under conditions where mRNA export was blocked. The same

construct using *PGK1*-3'UTR resulted in a filling of the nucleoplasm (Brodsky and Silver, 2000). Most likely, the cis-element within the *ASH1*-3'UTR contains the required information to direct it into the nucleolus. This observation supports the idea that localized mRNAs such as *ASH1* mRNA may assemble into a RNP complex in the nucleolus where many kinds of RNPs are formed. To test whether this hypothesis is valid, cells of *mex67-5* mutant were subjected to inhibition of mRNA export. The cellular localization of *ASH1* mRNA was investigated by a dual colour *in situ* hybridisation with labelled antisense oligonucleotides against *ASH1* (red) and *ITS2* (green). *ITS2* oligonucleotides directed against the internal transcribed spacer (*ITS*) region of ribosomal RNA precursors were used as to mark the position of the nucleolus. In addition, these cells expressed *ASH1* under the control of an inducible *GAL1* promotor. A short pulse of *ASH1* expression was induced by the addition of 4% galactose prior to a temperature shift. In cells of a wild type control (*MEX67+*), *ASH1* mRNA is localized to the bud tip (Figure 15, upper panel).The bright red dots within the DAPI-stained region of chromosomal DNA are most likely sites of *ASH1* transcription as their presence is strictly correlated to the induction by galactose. Because ribosome processing predominantly occurs in nucleoli, they were clearly stained by *ITS2* antisense oligonucleotides (green). The observed accumulation of *ASH1* mRNA in *mex67-5* cells was not nuclear nor nucleolar but only visible as red dots at transcription foci. A possible explanation for this was already given by previous observations made for heat shock RNAs. It has been suggested that block of nuclear mRNA export in mutants such as *mex67-5* leads to hyperadenylated transcripts, which as a result accumulate at sites of transcription (Hilleren and Parker, 2001; Libri et al., 2002). Retention of the transcripts requires components of the nuclear exosome. Thus, the additional deletion of the non-essential nuclear exosome component Rrp6p in combination with an export mutant would eliminate transcript retention within foci (Hilleren et al., 2001; Thomsen et al., 2003). In order to analyze subnuclear localization of released *ASH1* transcripts, a *mex67-5/Δrrp6* mutant was generated, and tested under conditions where mRNA export is blocked. After a shift to its restrictive temperature, cells of this mutant have lost transcript retention at genomic foci. Instead, the *ASH1* mRNA accumulated visibly in an area of the nucleus largely devoid of DAPI stain but considerably overlapping with the *ITS2* stain.

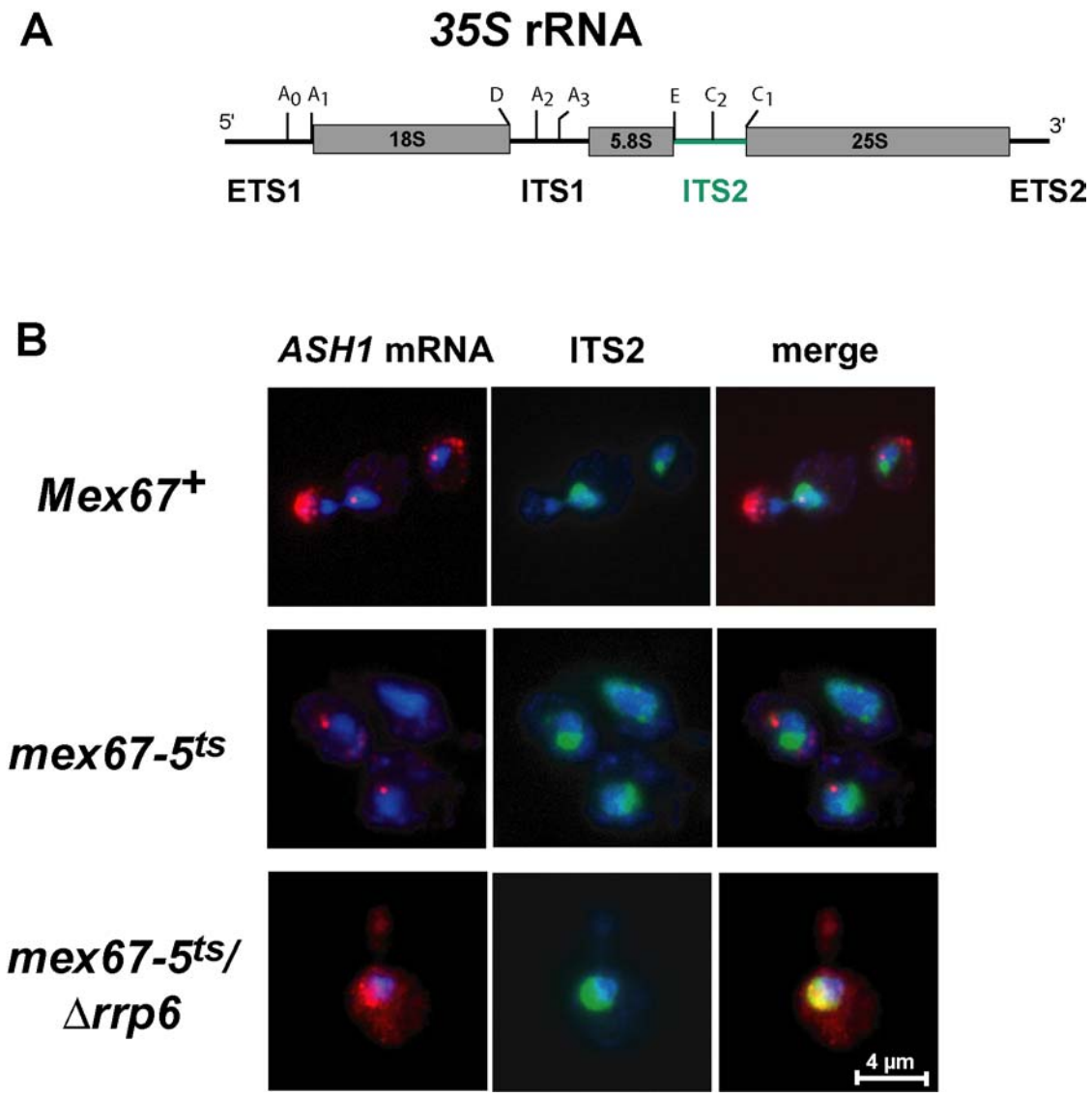

Figure 17: In the *mex67-5/Δrrp6* mutant, *ASH1* mRNA accumulates in nucleoli upon block of mRNA export. A. Schematic representation of 35S rRNA containing *ITS* (Internal Transcribed Spacer) and *ETS* (External Transcribed Spacer) regions with cleavage sites for rRNA processing. Fluorescently labelled antisense oligonucleotides directed against *ITS2* was used as nucleolar marker, which marks the position of 35S, 33S, 32S and 27S rRNA intermediates in the 90S and 60S preribosome. B. Double *in situ* hybridisation performed for strains RJY2239 (*MEX67*$^{+}$, top panels), RJY1149 (*mex67-5,* middle panels) and RJY2849 (*mex67-5/Δrrp6*, bottom panels). Cells were grown at 26°C; *ASH1* expression was induced by addition of 4% galactose for half an hour prior to temperature shift to restrictive conditions at 37°C. WT control shows normal localization (top panels). Cells of *mex67-5* only show stain of transcription foci (red dots) in chromatin-stained region. In cells of *mex67-5/Δrrp6* double mutant *ASH1* stain overlaps with *ITS2* signal (yellow signal, merge).

This suggests that *ASH1* mRNA is accumulating in the nucleolus when released from transcription sites. The same experiment was performed using a *mex67-5/Δrrp6* strain that was additionally deleted for *SHE2*. In this case, *ASH1* mRNA was not accumulating in the nucleolus upon RNA export block. This indicates that the nucleolar accumulation of the *ASH1* transcript requires the presence of She2p. In summary, the microscopic data on subnuclear localization of both, the *ASH1* mRNA and its corresponding binding protein She2p provide the first evidence that components of a localized RNP have at least a transient passage through the nucleolus.

2.10 *Trans*-acting factors Puf6 and Loc1 are nucleolar proteins

The PUF family protein Puf6 is an RNA binding protein found in tandem affinity purification (TAP) with She2 as bait protein. It has been reported to bind PUF consensus sequences in the 3' UTR of *ASH1* mRNA and therefore suggested to act as a repressor of translation (Gu et al., 2004). The cellular distribution of myc-tagged Puf6p was assessed by indirect immunofluorescence. Cells expressing Puf6p-myc9 show the typical crescent like staining of the yeast nucleolus (Figure 16A). In yeast, Nhp2p has been reported to be a component of box H/ACA small nucleolar ribonucleoprotein particles (snoRNPs), which have a key role in the synthesis of eukaryotic ribosomes (Henras et al., 2004). In a dual stain, Puf6p signals colocalize with the nucleolar marker Nhp2p (Fig. 18A). This is also consistent with a study on the global analysis of protein localization in yeast (Huh et al., 2003). Interestingly, this *trans*-acting factor with impact on cytoplasmic *ASH1* transport is a predominantly nucleolar protein suggesting that the nucleolus might have a role in the assembly of localized RNPs. However, Puf6p has been suggested to shuttle as well. In a previous study, which used the MS2-system to follow RNA localization *in vivo,* Puf6p was found to colocalize with cytoplasmic *ASH1* particles. Therefore, it has been proposed to travel with the localized RNP to prevent its translation during transport (Gu et al., 2004). Nevertheless, bud tip localization of Puf6p was never observed with indirect immunofluorescence even when *ASH1* was highly overexpressed. This could be due to the different experimental setups. However, this observation cannot exclude a putative shuttling behaviour of Puf6p.

A three-hybrid *in vivo* RNA binding assay identified Loc1p as an RNA binding protein that acts in *ASH1* mRNA localization. IP-RT and band-shift assays have demonstrated the binding of Loc1p to the E3 element of *ASH1* mRNA (Long et al., 2001). Subcellular localization of myc-tagged Loc1p was analyzed using indirect immunofluorescence. Like Puf6p, Loc1p clearly shows a subnuclear crescent indicating that it could be also a nucleolar protein. Colocalization with the nucleolar marker protein Nhp2 (Henras et al., 2001, see above) was confirmed in a dual stain (Fig. 18B). Although Loc1p is essential for the efficient localization of *ASH1* mRNA, it was recently published to be also required for the assembly and nuclear export of the 60S ribosomal subunit (Urbinati et al., 2006). Consistent with this function, it has been shown to colocalize with the nucleolar marker Nop1. Loc1p has been previously suggested to be a stationary nucleolar protein because shuttling for this protein has not been observed (Long et al., 2001). However, it is not clear why nucleolar Loc1 has such an impact on cytoplasmic *ASH1* mRNA localization. But interestingly, three of four known RNA binding proteins involved in *ASH1* mRNA localization reveal a connection to the nucleolus. One of them, She2p, is a nucleo-cytoplasmic shuttling protein whereas Puf6p and Loc1p are mainly nucleolar proteins. *ASH1* mRNA transits the nucleolus as well supporting the evidence that nuclear formation of localized RNPs may occur within this subnuclear compartment.

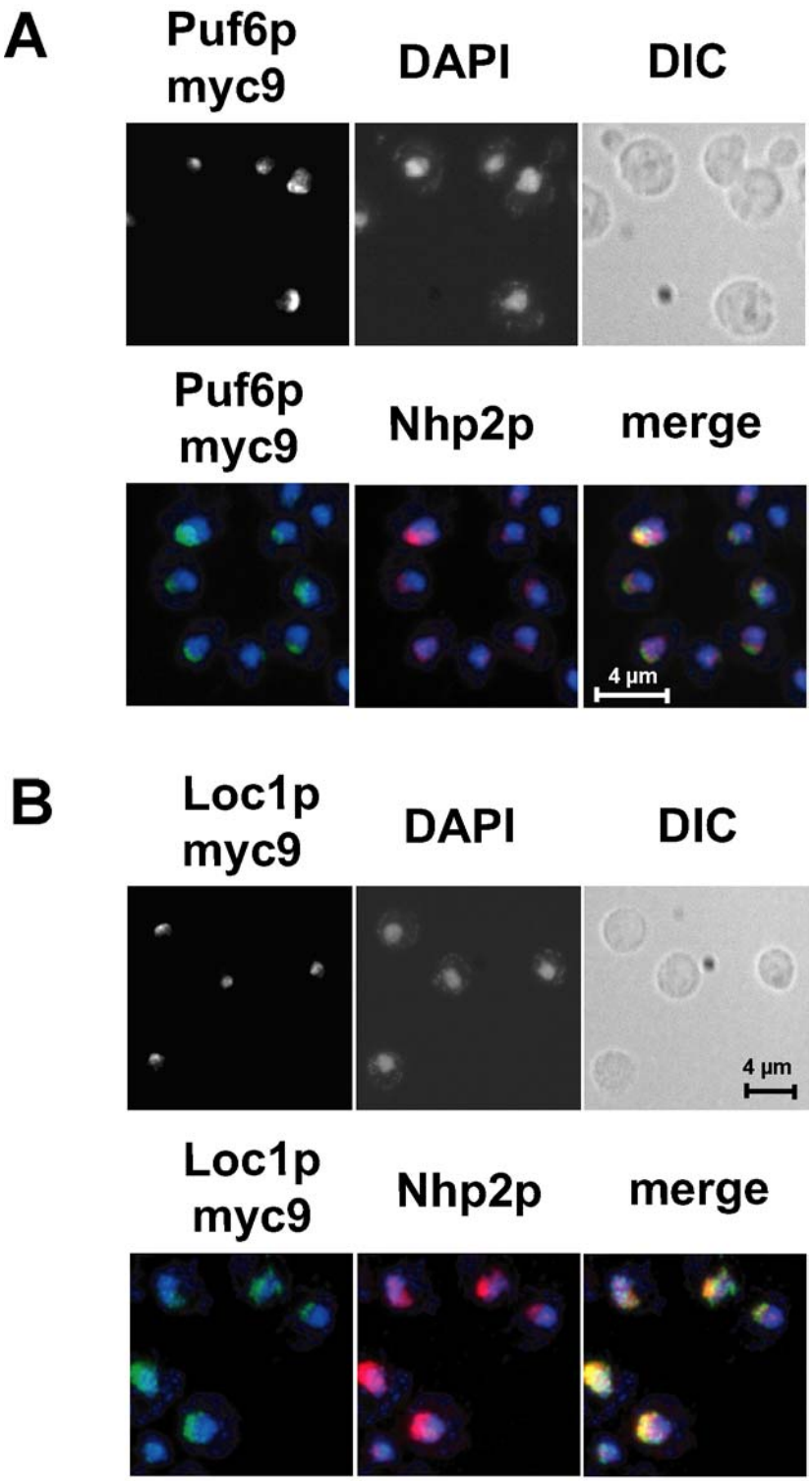

Figure 18: **Puf6 and Loc1 are nucleolar proteins.** Subcellular distribution of Puf6 (A) and Loc1p (B) in IF staining of strains RJY2663 (Puf6p-myc9) and RJY915 (Loc1p-myc9). Myc-tagged proteins were stained with 9E10 α-myc (left panels), nuclei were stained DAPI (upper panels, middle), DIC (upper panels, right). Colocalization of Loc1p and Puf6 with the nucleolar marker protein Nhp2 was assessed in an immunofluorescence double stain (A, B, lower panels). Merged signals indicate colocalization with the nucleolar marker (lower panels, right).

2.11 Khd1p does not accumulate in the nucleolus upon block of mRNA export

The presence of cis-acting localization elements within the coding sequence of *ASH1* mRNA reflects the putative role in the regulation of its translation (Chartrand et al., 2002). Khd1p, a protein with three hnRNPK homology (KH) RNA-binding domains, is another trans-acting factor, which has been reported to bind only to the E1 localization element and to participate in *ASH1* mRNA translational control (Irie et al., 2002; Paquin et al., 2007). To address the question if nucleo-cytoplasmic shuttling is also evident for Khd1p cellular distribution was analyzed in a *mex67-5* mutant under conditions of mRNA export block. Prior to inhibition of mRNA export *ASH1,* expression was induced using a strong *GAL1* promotor. Under permissive conditions, Khd1p was found distributed throughout the cell. Although *ASH1* was strongly overexpressed, bud tip localization of Khd1p could not be observed. Regardless, in cells shifted to non-permissive temperature, a substantial fraction of Khd1p accumulated in the nucleus.

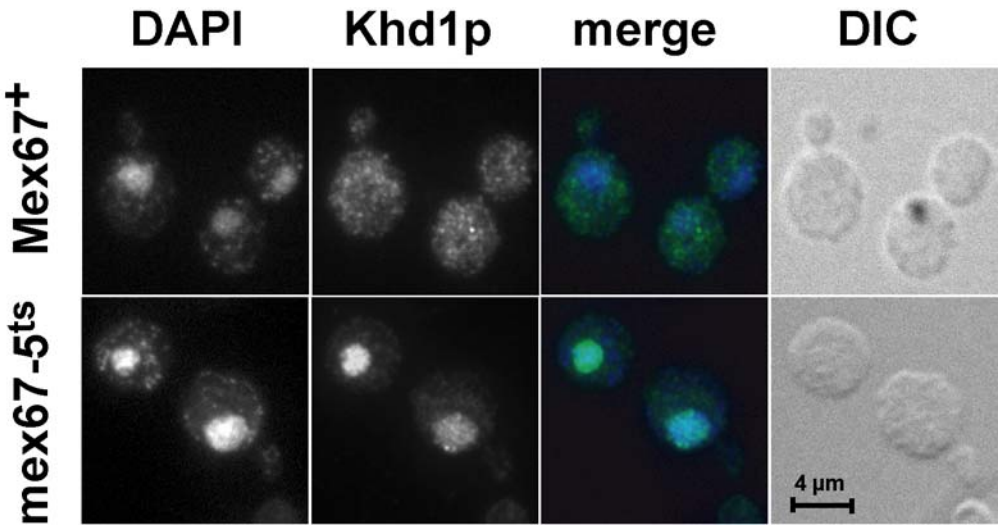

Figure 19: Khd1p is a nucleo-cytoplasmic shuttling protein. Indirect immunofluorescence showing cellular distribution of HA-tagged Khd1p in thermosensitive strain RJY2645 (Khd1p-HA_6, *mex67-5*, *GAL1-ASH1*, lower panel) and control strain RJY2646 (Khd1p-HA_6, *mex67-5*, *GAL1-ASH1,* upper panel). Cells were grown logarithmically in raffinose- containing medium at 26°C. *ASH1* was induced by addition of 4% Galactose for 1 hour prior to temperature shift to 37°C for 2 hours. Left panels show staining of Khd1p-HA_6 with 1st 16B12 α HA and 2nd Alexa488 goat α mouse antibodies. DAPI staining (middle) and DIC (right panels). Khd1p staining is cytoplasmic under permissive conditions (upper left panels). In *mex67-5* cells, Khd1p accumulate in nuclei at its restrictive temperature of 37°C.

Hence, a second *trans*-acting factor of the *ASH1* mRNA shows characteristics of a nucleo-cytoplasmic shuttling protein. However, nuclear accumulation of Khd1p does not resemble that of She2p. While She2p accumulated opposite of DAPI-stained region, the nuclear signal of accumulated Khd1p clearly overlaps with DAPI-signal. Thus, not all shuttling RNA-binding proteins involved in *ASH1* localization accumulate in the nucleolus upon block of mRNA export. Similar observation has also been reported for the La-motif containing RNA-binding protein Sro9. The accumulation of this shuttling protein results in a filling of the nucleoplasm (Susanne Röther, personal communication). Nevertheless, Khd1p's ability to shuttle may reflect the requirement to bind *ASH1* mRNA already in the nucleus. This early association may help to assemble into an mRNP, which is competent for cytoplasmic translational control.

2.12 She2p does not physically interact with other RNA localization factors

To determine if She2 is physically interacting with one of above-mentioned factors, cells expressing myc-tagged Loc1, Khd1 and Puf6 proteins were subjected to co-immunoprecipitation with monoclonal 9E11 α-myc antibody coupled to magnetic Protein G-beads and checked for the presence of She2p. The immunoprecipitated myc-tagged proteins were eluted with 100 mM glycine and SDS, and collected fractions were analyzed in a Western blot. In all strains tested, She2p was only present in the input material (Total) and the corresponding supernatants but not in immune pellets (Fig. 20). Thus, She2 is not co-immunoprecipitating with myc-tagged Loc1p, Khd1p and Puf6. Co-immunoprecipitation performed vice versa and TAP-purifications of each of the proteins (Stephan Jellbauer, personal communication) have not revealed any direct protein-protein interaction between She2 and all other *trans*-acting factors involved in RNA localization. Since the association of these auxiliary *trans*-factors with the She2-containing core RNP is probably only temporary, the precipitated complex might not be stable enough to endure the experimental procedure. Alternatively, association of these *trans*-factors may be not dependent on a direct interaction with She2. However, preservation of the RNP under these conditions is also unlikely, thus leading to the loss of indirectly associated factors.

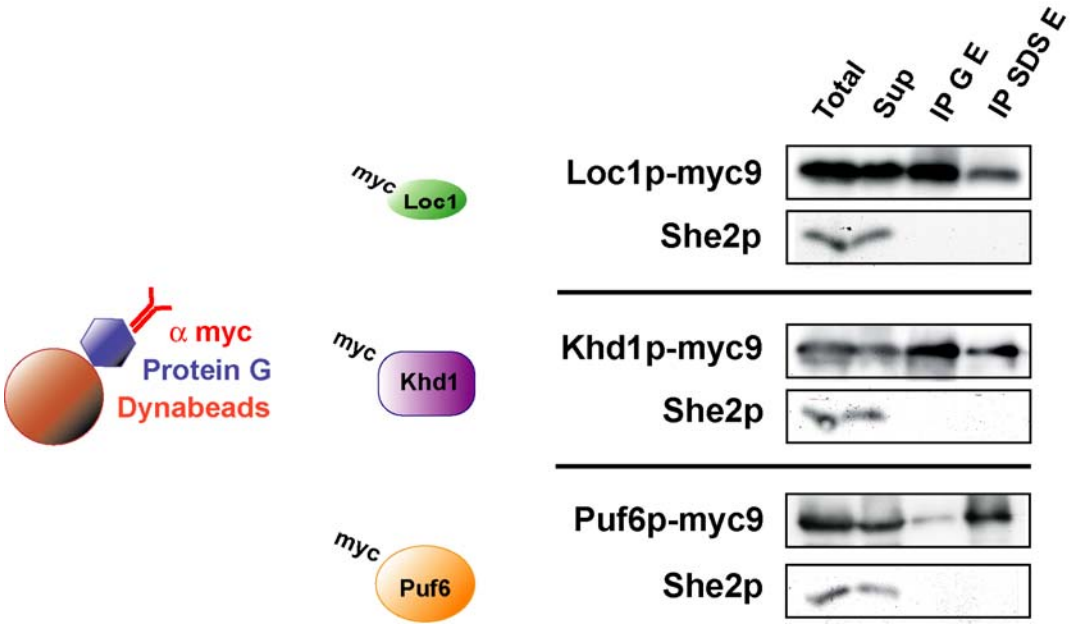

Figure 20: Co-IP of myc-epitope tagged RNA localization factors. Strains RJY915 (Loc1-myc9, upper panel), RJY2662 (Khd1p-myc9, middle panel) and RJY2663 (Puf6p-myc9, lower panel) were immunoprecipitated with 9E11 α myc antibody coupled to magnetic Protein G-beads. Aliquots of total cell extract (Total), supernatant (Sup) and immune-pellets were eluted with 100 mM glycine (IP G E) and SDS (IP SDS E). Western blot probed with 9E10 α-myc and purified α-She2 antibody. She2p signal was visible in totals and in supernatants but not in immune pellets.

2.13 Nuclear Export of *ASH1* mRNA does not require She2p

The previous results support the idea that the early recognition and binding of all RNA localization factors may occur already in the nucleus. Subsequently, the assembled nuclear RNP is exported to the cytoplasm in an RNA dependent fashion. To address the question whether the export of *ASH1* mRNA requires the presence of She2p, *ASH1*-localization was assessed in a strain lacking She2p. *ASH1* expression driven by the *GAL1* promoter was induced by the addition of galactose for only half an hour. When following the distribution of *ASH1* mRNA by *in situ* hybridisation, *ASH1* signal was not found within the nucleus but instead was accumulating in a region surrounding the nucleus (Fig. 21). Apparently, *ASH1* transcripts can undergo the mRNA export process independent of She2p. Because the recruitment of *ASH1* mRNA to the She3/Myo4-transport machinery requires She2p, the exported message is not transported to the bud tip. Consistent with a previous study, which followed movement of the *ASH1-MS2* mRNP *in vivo*, the exported particle was also exported

but stayed in close proximity to the nuclear envelope, probably attached to perinuclear ER (Schmid et al., 2006). Moreover, in more than 50% of *she2Δ* cells imaged, only smaller and less bright particles were observed, possibly due to formation of less stable or smaller mRNPs in the absence of She2p (Bertrand et al., 1998). Thus, integrity and bud tip transport, but not the export of the *ASH1* mRNP, are dependent on the RNA binding protein She2.

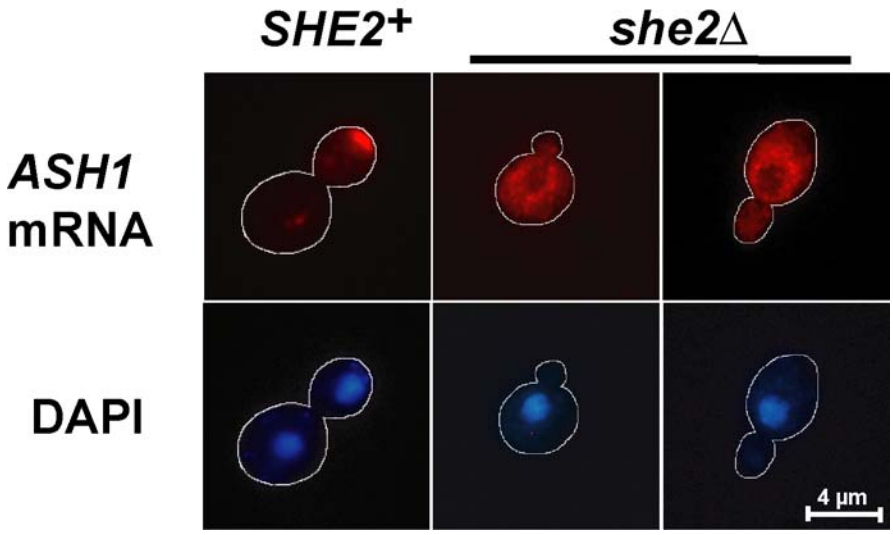

Figure 21: Perinuclear localization of *ASH1* mRNA in *she2Δ* cells. Cells of strain RJY2239 (*GAL1-ASH1,* SHE2+) and RJY2416 (*GAL1-ASH1*, *she2Δ)* stained for *ASH1* mRNA by *in situ* hybridisation with TexasRed labelled antisense *ASH1* oligonucleotides (upper panels). A pulse of *ASH1* transcription was induced with 4% galactose prior to fixation of cells. Nuclei were stained with DAPI (lower panels). Wild type control shows normal localization to the bud tip (left panels). In absence of She2p, cells accumulate exported *ASH1* mRNA at the nuclear periphery.

2.14 Cytoplasmic retention of She2 protein

Since export of *ASH1* mRNA occurs also in the absence of She2, it should be sufficient when the RNA is recognized and recruited to the transport complex in the cytoplasm. Why should She2p then enter the nucleus at all? To address this question, one would have to exclude She2p from the nucleus and generate a situation that would only allow cytoplasmic binding to *ASH1* mRNA. One way to achieve this would be the use of temperature sensitive mutants that are defective in protein import. Although the 28 kD She2 protein is theoretically small enough to enter the nucleus by passive diffusion, an association with yeast importin-α (Srp1) has

been reported, suggesting that She2 might actively imported into the nucleus (Ito et al., 2001; Kruse et al., 2002). However, a simple block of protein import would also affect other essential factors required for cell viability.

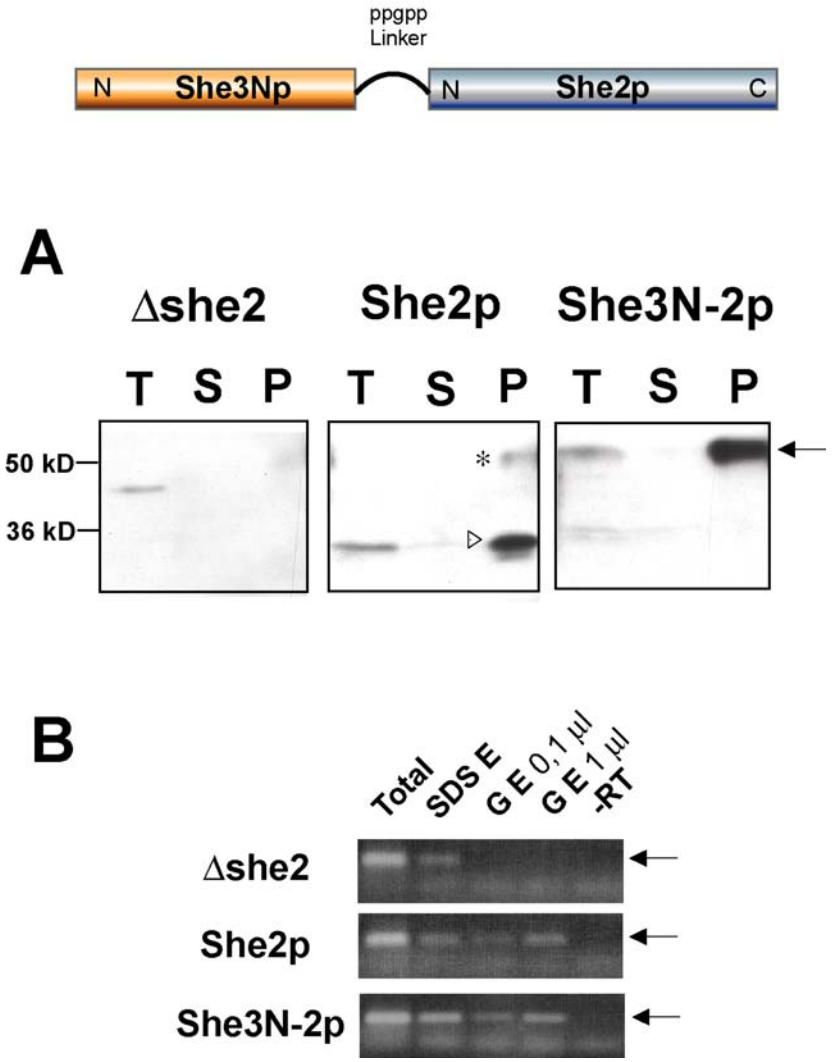

Figure 22: She3-She2 fusion protein is able to bind *ASH1* mRNA in vivo. The NH_2-terminus of She3p was fused to full length She2p with a flexible linker in between. A. Western blot showing immunoprecipitation of She2p and She3N-She2p fusion using purified She2-antibody performed in strains RJY126 (Δshe2, left panel), RJY358 (WT, middle panel) and RJY2414 (She3N-She2p, right panel). T (total input), S (supernatant). 28 kD She2p (white arrowhead) or 55 kD She3N-2p fusion protein (black arrowhead) detected by She2-antibody in the immune pellet (P). The asterisk marks the portion of IgG heavy chain of She2-antibody eluted together with precipitated material.
B. RT-PCR of immunoprecipitated materials as templates. SDS E (elution with 10%SDS), G E (elution with 100 mM glycine-HCl), -RT (control without reverse transcriptase). A specific product was amplified in the elution (G E) of precipitated She2p (middle) and She3N-S2p, but not in the negative control (Δshe2).

A more elegant method to achieve cytoplasmic retention is to tether She2p to the adapter protein She3 artificially. She3 is a protein with cytoplasmic distribution (Huh et al., 2003), which serves as an adapter to link She2p to the Myo4 motor protein. As has been shown by two-hybrid analysis, interaction of the adapter She3p to the motor protein requires the NH_2-terminus of She3p (Böhl et al., 2000), which has a strong binding affinity toward the coiled-coil region of Myo4p (Kruse et al., 2002). Therefore, full-length She2 protein was fused to the first 197 amino acids of She3p's N-terminus in order provide a direct link to the cytoplasmic motor complex. The She3N-She2p fusion was constructed on a plasmid using the promotor and 3' untranslated regions (UTR) of the endogenous *SHE2* gene. When transformed into a *she2Δ*-background the resulting cells express a 55-kDa fusion protein. In order to determine whether binding of the fusion protein to *cis*-elements of the *ASH1* mRNA is functional She3N-She2 fusion protein was precipitated and assessed for bound *ASH1* mRNA in an IP-RT experiment. IP-RT was simultaneously performed in a strain expressing She2p (wild type control) and a *she2Δ* strain (negative control). Both, She2p and She3N-She2p were successfully precipitated using α-She2 antibody coupled to magnetic beads (Figure 22A). The immunoprecipitated material was eluted specifically with glycine at low pH (G E), and then subjected to RT-PCR with primers for *ASH1* E3 element. The detected PCR products in the elution fractions (G E) of precipitated She3N-She2p and She2p indicate the presence of bound *ASH1* mRNA. In the control that was lacking She2p, the respective elution with glycine does not show any signal in RT-PCR, suggesting that She3N-She2 fusion protein is able to bind *ASH1* mRNA *in vivo*.

2.15 She2 artificially tethered to cytoplasmic She3p leads to its nuclear exclusion

In order to test if tethering of She2 to the myosin motor is functional and if the resulting complex leads to a full transport to the bud tip, She3N-She2p mediated localization was assessed by indirect immunofluorescence using the She2-antibody. The fusion protein itself localizes to the bud tip more frequently than was observed for endogenous She2p (data not shown). This could be due to the She3-portion of the fusion protein, which through the direct link results in a higher localization frequency of endogenous She3p.

To further address, whether cytoplasmic retention of She2p could be achieved with this approach She3N-She2p was expressed in a *mex67-5* mutant background and assessed for nuclear accumulation upon arrest of mRNA export. Under restrictive conditions, these cells did not accumulate She3N-She2p in nuclei (Fig. 23). Instead, nuclear exclusion of She3N-She2p was visible by the lack of staining at the region of the nucleus. In contrast, cells of a positive control show accumulation of She2p in nuclei under same conditions. Thus, the high affinity of the She3N-She2 fusion protein toward the coiled-coil region of Myo4p leads to cytoplasmic retention, and unlike the wild type She2 protein cannot be trapped in the nucleus.

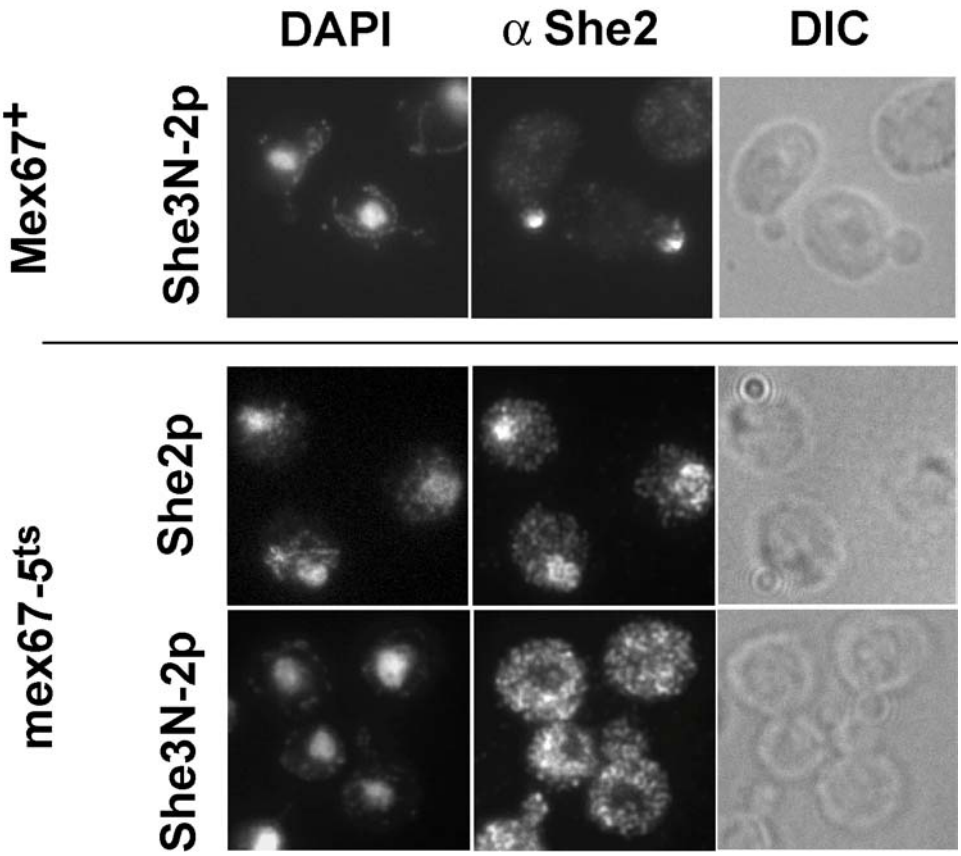

Figure 23: Cytoplasmic retention of She3N-She2 fusion protein. Cellular distribution of She3N-She2p assessed in indirect immunofluorescence. In strain RJY2422 (*MEX67, she2Δ*, She3N-She2p), She3N-S2p is expressed and localized to the tip of daughter cells (upper panel). Cells of control strain RJY2421 (*mex67-5, she2Δ*, She2p) accumulate She2p in nuclei upon inhibition of mRNA export for one hour (middle panel). In contrast, cells of strain RJY2420 (*mex67-57, she2Δ*, She3N-She2p) could not accumulate She3N-She2p in nuclei (lower panels). Note that nuclei are visibly devoid of She2-stain. Nuclei stained with DAPI (left), α-She2 (middle), DIC (right).

2.16 Cells expressing She3N-She2 fusion protein are able to localize *ASH1* mRNA

With a new strain in hands that restricts She2 to the cytoplasmic compartment, it was now possible to address the question if She2's nuclear history has an effect on later stages of RNA localization.

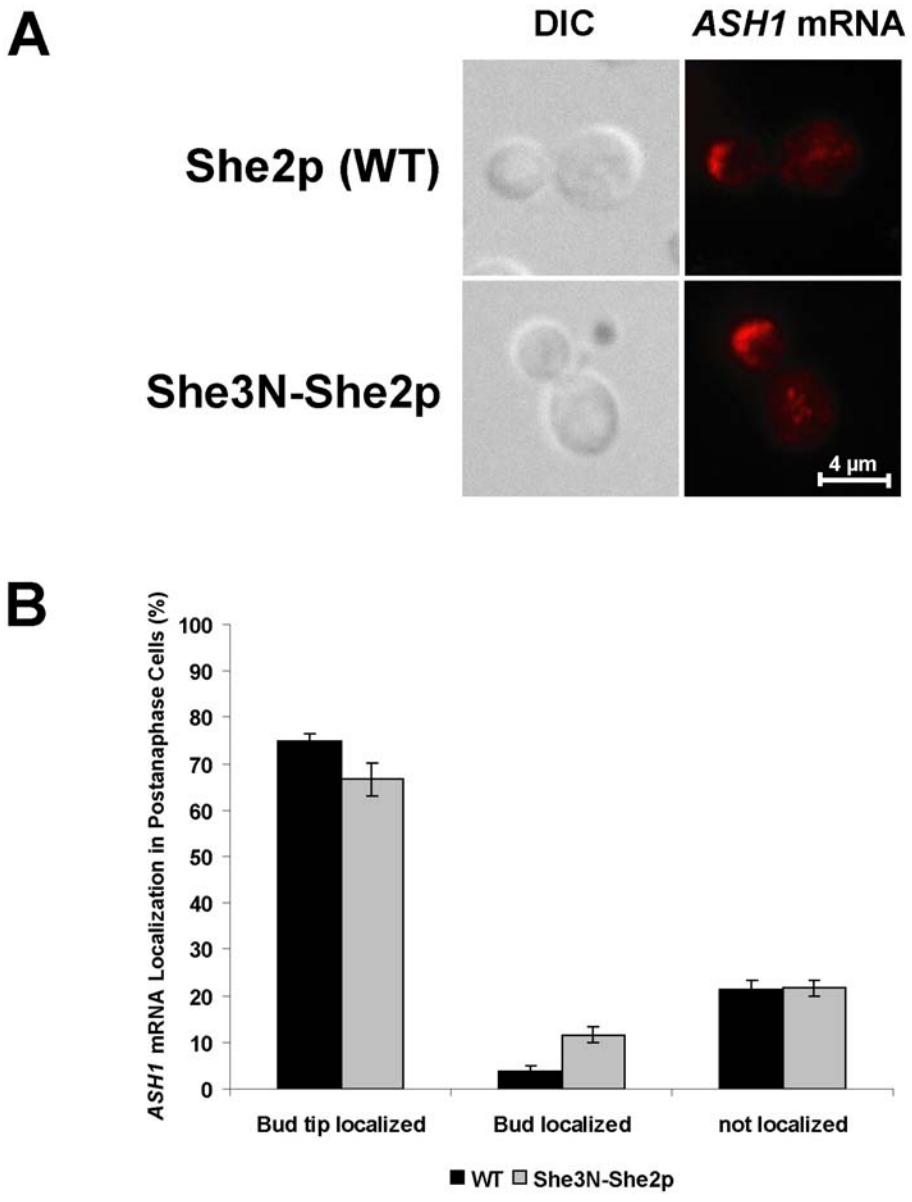

Figure 24: She3N-She2p localizes *ASH1* mRNA with the efficiency of a wild type. A. Representatives cells of strain RJY2997 (*2µ ASH1*, *SHE2*+, upper panel) and RJY2414 (*2µ ASH1*, *SHE3N-SHE2,* lower panel) stained by *in situ* hybridisation show functional *ASH1* mRNA localization. B. Efficiency of *ASH1* mRNA localization was statistically determined by cell counts. Cells of a WT (black) were compared to cells expressing She3N-She2p (grey). 150 postanaphase cells were counted and categorized as either bud tip (75%/65%), bud (3%/12%) or not localized (22/3%).

Although She3N-She2 fusion protein was shown to become localized effectively to the bud tip it remains to be determined if *ASH1* mRNA after its nuclear export is recognized and transported by this artificial locasome. Thus, cells expressing the fusion proteins were examined for functional *ASH1* mRNA localization by *in situ* hybridisation. *ASH1* provided on a high copy plasmid was over-expressed in these cells. Like the wild type She2p, also She3N-She2p fusion protein is fully capable of localizing *ASH1* mRNA to the tip of daughter cells (Fig. 24A). To measure efficiency of the fusion protein mediated transport statistically, *ASH1* mRNA localization was determined by *in situ* hybridisation. The observed localization patterns of late anaphase cells fall into three categories: bud tip localization, bud localization or no localization. Of 150 cells counted, 65% displayed bud tip localization, 12% bud localization and 23% no localization. In comparison, 75% of WT cells showed localization at the bud tip, 3% in the bud and 22% had no localization. Conclusively, *ASH1* mRNA can be localized by She3N-She2p fusion protein and the efficiency of this RNA transport is comparable to that of a wild type.

2.17 Localization mediated by She3N-She2p leads to ineffective sorting of Ash1p into daughter cells

The previous results revealed that She2p does not necessarily have to enter the nucleus in order to fulfil *ASH1*-binding and subsequent localization to the bud tip. Since other mechanisms such as repression of translation during transport or proper anchoring at destination sites may also contribute to an effective sorting of Ash1p, it would be necessary to examine whether translation is affected when She2 is not allowed take its nuclear route. Thus, *ASH1* was tagged with 9 myc-epitopes in order to monitor the cellular distribution of the translated protein (Bobola et al., 1996). Translation of a 95-kDa Ash1-myc9 protein could be confirmed in a Western blot. Cells expressing either She3N-S2p or She2p were analyzed for their ability to sort Ash1-myc9 protein into daughter cells using indirect immunofluorescence. In postanaphase cells, Ash1p-myc9 is clearly visible in daughter cell nuclei. Since Ash1p sorting is not effective in all cells, a portion show Ash1p-myc9 signal in both nuclei. To determine the exact sorting efficiency, the observed Ash1p distribution patterns have been classified as follows.

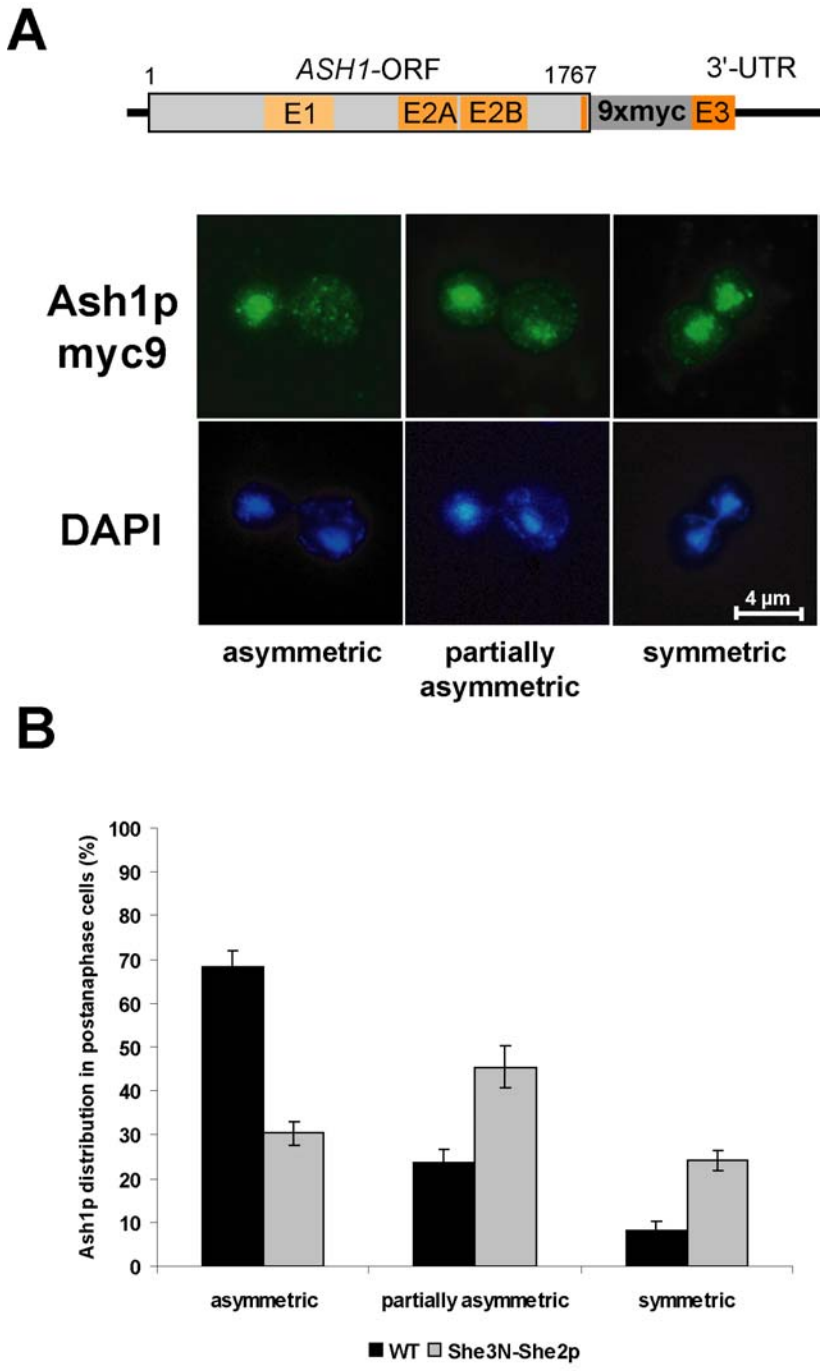

Figure 25: Asymmetric sorting of Ash1p mediated by She3N-She2 fusion protein is ineffective. A. 9 myc-epitopes were inserted between the *ASH1*-ORF and the stop codon. In immunofluorescence pattern of Ash1p distribution fall into three categories: Distribution is either asymmetric (left), partially asymmetric (middle) or symmetric (right). Ash1p staining (green, upper panel) is nuclear. Nuclei were stained with DAPI (blue, lower panel). B. Statistics of Ash1p distribution. 300 cells of strains RJY137 (black bar, WT) and of RJY1462 transformed with plasmid RJP1100 (grey bar, She3N-She2) were counted. WT strain show 68% asymmetric, 24% partially asymmetric and 8% symmetric distribution. She3N-She2 mediated distribution was to 30% asymmetric, 46% partially asymmetric and 24% symmetric distribution.

As depicted in figure 25A Ash1p is either only visible in daughter nuclei (asymmetric distribution) or in both nuclei (partially asymmetric and symmetric distribution). In a wild type background, 60% of counted postanaphase cells localized normally, but 24% showed partially asymmetric and 8% symmetric distributed Ash1 protein. In contrast, Ash1p sorting mediated by She3N-S2p leads to only 30% asymmetric distribution of observed cells. Instead, the majority of cells were impaired in asymmetric Ash1p sorting as 45% showed partially asymmetric and 24% symmetric distribution. Strikingly, cells expressing She3N-S2p effectively localize *ASH1* mRNA but this in the end does not account for an effective sorting of its protein product. This could be the case if the localization of *ASH1* mRNA is 'leaky', meaning that translation occurs prematurely during its transport to the bud tip. Premature translation occurs when for example localized RNAs are not protected by the presence of translational repressors. Taken together, these observations suggest that She2's 'nuclear history' may have a key role in the proper formation of the *ASH1* RNP. This might include either the recruitment of factors important for cytoplasmic translational regulation or to ensure the proper packaging of a localization competent RNP.

2.18 The absence of a nucleolar RNA localization factor leads to an increased rate of Ash1p synthesis

An effective sorting of a protein to its destination site also requires that during the transport process the localized RNA is prevented from the access of translating ribosomes. Premature translation occurs when repression of translation is inefficient, thus leading to an increased rate of translation and in this case, would result in a loss of Ash1p asymmetry. In order to show if nucleolar interactions with *ASH1* mRNA have an influence on cytoplasmic translational control, it would be necessary to follow *in vivo* kinetics of Ash1p-translation in cells lacking either nuclear She2p or one of the nucleolar factors Loc1p and Puf6p. In cells used for the following experiment expression of Ash1p-myc9 was driven by an inducible *GAL1*-promotor. Cells were grown in non-inducible medium containing 2% raffinose and were then induced by the addition of 4% galactose. After induction, samples were taken at different time points in order to determine the overall amount of synthesized *ASH1* transcripts and translated Ash1 protein over time. In a northern blot of a WT strain,

ASH1 transcripts were already detectable 5 minutes after induction of expression. With a delay of about 5 minutes, translated Ash1-myc9 product shows up in a western blot (Fig. 26). To determine the relative intensities of synthesized Ash1 protein at various time points, Ash1p-levels were normalized against the expression levels of actin.

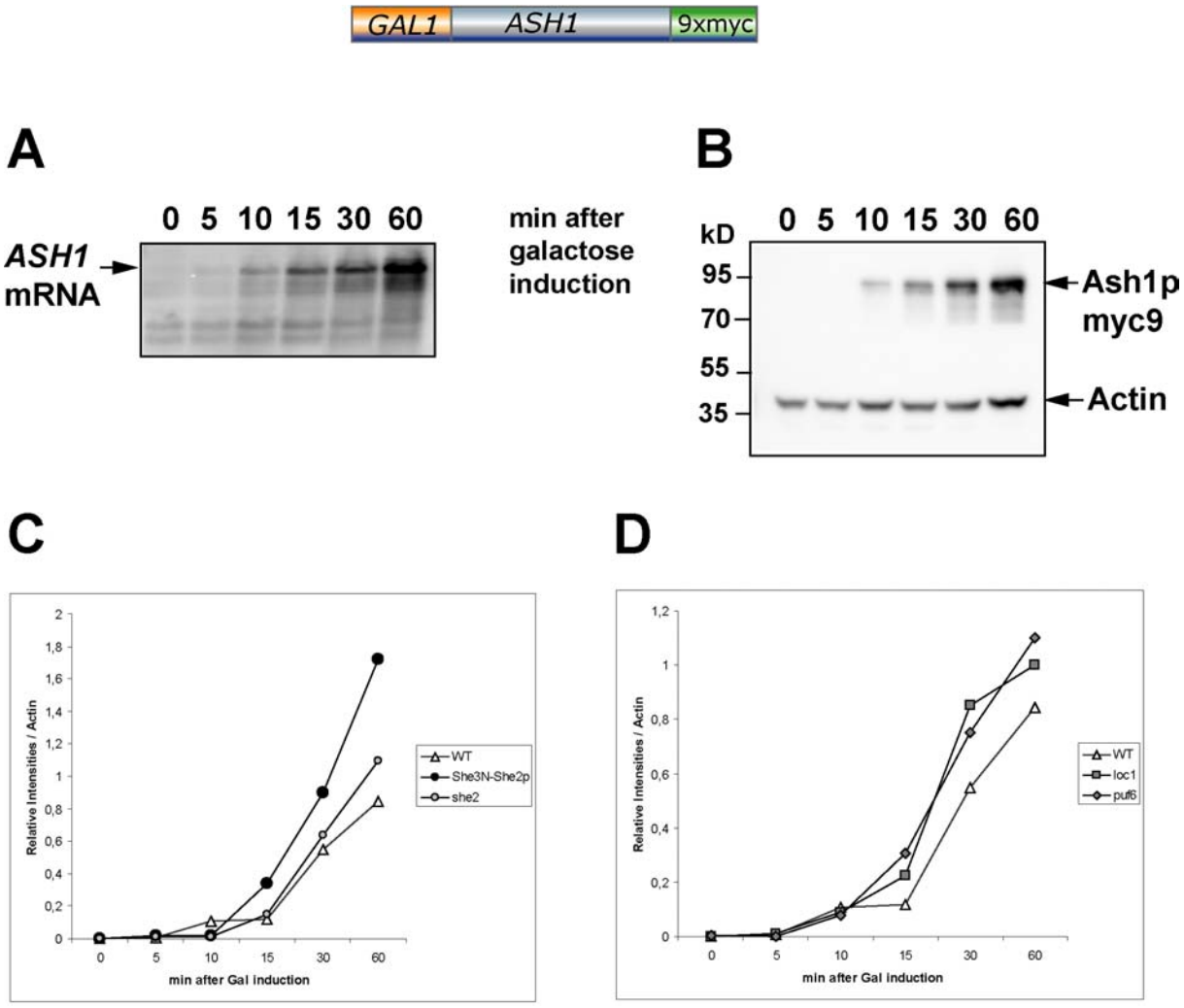

Figure 26: *In vivo* kinetics of *ASH1* mRNA translation. Ash1p-myc9 expressed under the control of an inducible GAL1 promotor. Strains were grown in media containing 2% raffinose and induced by the addition of 4% galactose. (A) Northern blot showing expression levels of *ASH1* RNA at various time points after galactose induction in a wild type strain (RJY280). (B) Samples of corresponding time points of a wild type strain in a Western blot. Intensities of synthesized Ash1p at various time points were normalized against levels of 40 kD Actin. (C) Relative intensities of Ash1-myc versus Actin were plotted over time: Δ-WT (RJY280), ●-She3N-She2 (RJY3269), ○-she2Δ (RJY3164) (D) Relative intensities of Ash1-myc versus Actin were plotted over time: Δ-WT (RJY280), ■-loc1Δ (RJY1362), ♦-puf6Δ (RJY1363)

Cells where *ASH1* transport is mediated by She3N-She2 fusion protein showed increased levels of Ash1p-translation at 15 min after induction. As shown in figure 26C the rate of translation was increased to nearly 2-fold in comparison to wild type cells. This was not corresponding to levels of the *ASH1* mRNA as they showed the same intensities in all strains (data not shown). This would explain why in these cells protein sorting remains inefficient although mRNA localization is fully functional. The rate of Ash1p synthesis in a *she2Δ* was also higher than was observed for wild type cells. Consistent with previous microscopic data, translational control during fusion protein-mediated transport seems to be impaired, which would result in a higher rate of premature translation. This could be the case if upload of a translational repressor did not occur because She2p in these strains was not allowed to traverse the nucle(ol)us. To investigate if nucleolar *trans*-acting factors have an influence on translational control, kinetics of *ASH1* translation were also determined for *loc1Δ* and *puf6Δ* strains. When following relative Ash1p intensities over time, similar changes in kinetics can be observed in both strains analyzed (Fig. 24D). Similar to kinetics observed for She3N-She2 cells both strains show higher intensities of synthesized Ash1p after 15 minutes of induction compared to the wild type. This is consistent with a previous study showing increased levels of Ash1p in *puf6Δ* cells (Gu et al., 2004). This suggests that the loss of translational control is caused by the lack of the nucleolar factor Puf6. Interestingly, *loc1Δ* cells display a similar phenotype suggesting that it might act in translational control as well. This supports the idea that the nucleolus may serve as a kind of scaffold where localized RNAs together with all the *trans*-acting components are correctly assembled into a nuclear RNP. This process might account for efficient sorting of Ash1p in the cytoplasm.

2.19 Loc1p binding to *ASH1* mRNA is dependent on the delivery of She2p

Previous results suggested that She2 is possibly the *trans*-acting factor that recognizes *ASH1* transcript first. Secondly, nucleolar accumulation of *ASH1* was not observed in cells of a *mex67-5/Δrrp6* double mutant when additionally deleted for *SHE2*. This suggests that She2p might be the factor required to guide the *ASH1* message into the nucleolus. This argument is strengthened by the observation that She2's association with the nucleolus occurs in absence of *ASH1*-binding. Thus, if the assembly of localized RNPs should occur in the nucleolus how can a stationary

RNA localization factor such as Loc1p face and bind to *ASH1* mRNA? To test whether *ASH1*-binding of nucleolar Loc1p is dependent on the delivering action of She2p, IP-RT experiments were performed in a wild type (*SHE2*$^{+}$) and in a *she2Δ* strain. Loc1p in these strains was tagged with 9-myc epitopes for a pull down with magnetic beads coupled to 9E11 α-myc antibody. Immunoprecipitated Loc1p-myc9 was eluted with glycine and SDS, and eluates were subsequently analyzed for bound *ASH1* mRNA by RT-PCR with primers amplifying the E3 element. In the wild type strain, RT-PCR shows a signal for E3 element in the specific elution with glycine (Fig. 27). However, the signal for E3 element was significantly decreased in the respective elution of a *she2Δ* strain.

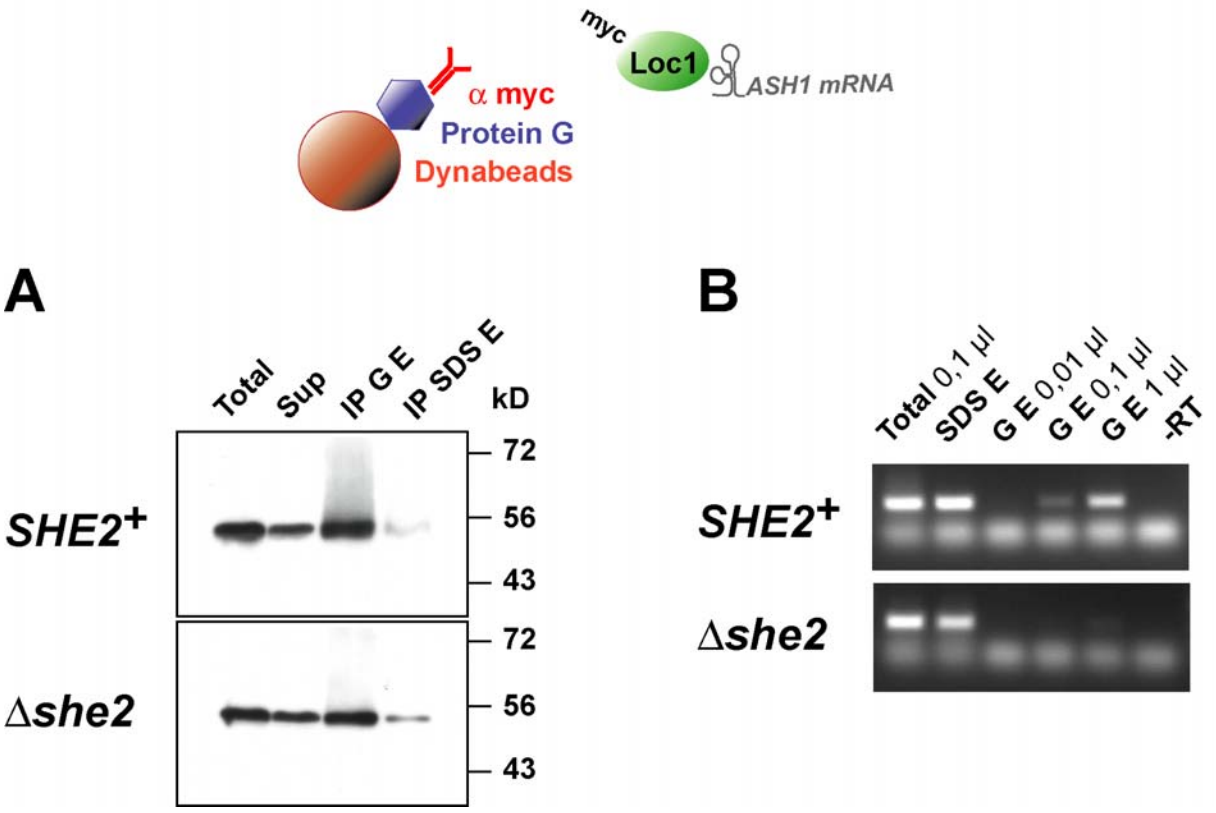

Figure 27: Binding of Loc1p to *ASH1* mRNA is dependent on She2p. IP-RT experiments of strains RJY915 (SHE2^{+}, Loc1p-myc9) and RJY3130 (she2Δ, Loc1p-myc9). Immunoprecipitation of myc-tagged Loc1p was performed with 9E11 α-myc antibody coupled to magnetic Protein G beads. A. Western blot analysis show successful immunoprecipitation of 55 kD Loc1p-myc9 in both stains (Total, Sup=Supernatant, IP G E=Elution with 100 mM glycine-HCl, IP SDS E=Elution with SDS). B. RT-PCR with primer for E3 localization element. SDS E (elution with 10%SDS), G E (elution with 100mM glycine-HCl), -RT (control without reverse transcriptase). E3 element was amplified in the specific elution (G E) of precipitated Loc1p in a *SHE2*$^{+}$ (top) but not in the *Δshe2* strain (bottom).

This observation suggests that binding of the nucleolar *trans*-acting factor Loc1p to the *ASH1* mRNA might dependent on the presence of She2. Interestingly, *ASH1*-binding of the nucleo-cytoplasmic shuttling protein Khd1p is not dependent on She2p as was also confirmed in independent experiments (Gonçalo Rebelo de Andrade, personal communication). This could be explained by the fact that unlike Loc1p, Khd1p's association with *ASH1* mRNA is not dependent on She2 because localization of this protein is not restricted to the nucleolus.

2.20 A direct binding of She3p to *ASH1* may be involved in cytoplasmic tethering to the motor complex

The She2p-*ASH1* RNP complex, once exported to the cytoplasm, is recruited to the myosin motor Myo4p by the adapter She3p in order to provide active transport to the bud tip (Böhl et al., 2000; Gonsalvez et al., 2004). Since the She3N-She2 fusion protein already provides a direct artificial link to Myo4p, it would be interesting to see if endogenous She3p in these cells is dispensable. Therefore, the fusion protein was tested for its ability to localize *ASH1* in a background devoid of endogenous She3p. Cells of a strain expressing She3N-She2p in a *she3Δ/she2Δ*-background were investigated in indirect immunofluorescence and *in situ* hybridisation. In a dual staining, signals of She3N-She2p and Myo4-myc largely overlap at the bud tip (Fig. 26A) indicating that the fusion protein was functionally tethered to the myosin motor complex. Interestingly, this machinery completely fails to localize *ASH1* mRNA when endogenous full length She3p is absent. Apparently, binding of *ASH1* by the She2-portion alone is not sufficient to provide localization of *ASH1* mRNA. This suggest that a portion of She3 protein that is missing is these mutants is required in order to effectively tether RNA to the myosin motor complex or to ER membranes (Schmid et al., 2006), which in the end would allow a functional transport to the bud tip. In order to test if the missing half of She3p contributes to *ASH1* mRNA binding *in vivo*, one would have to be able to express the C-terminus of She3p alone and test its ability to restore localization in this mutant. Thus, She3-C-terminus was cloned into a p413-*GAL1* vector that allows the induction of expression in a galactose-containing medium. In order to monitor expression and localization of this protein, it was provided with 6 HA-epitopes at the NH_2-terminus.

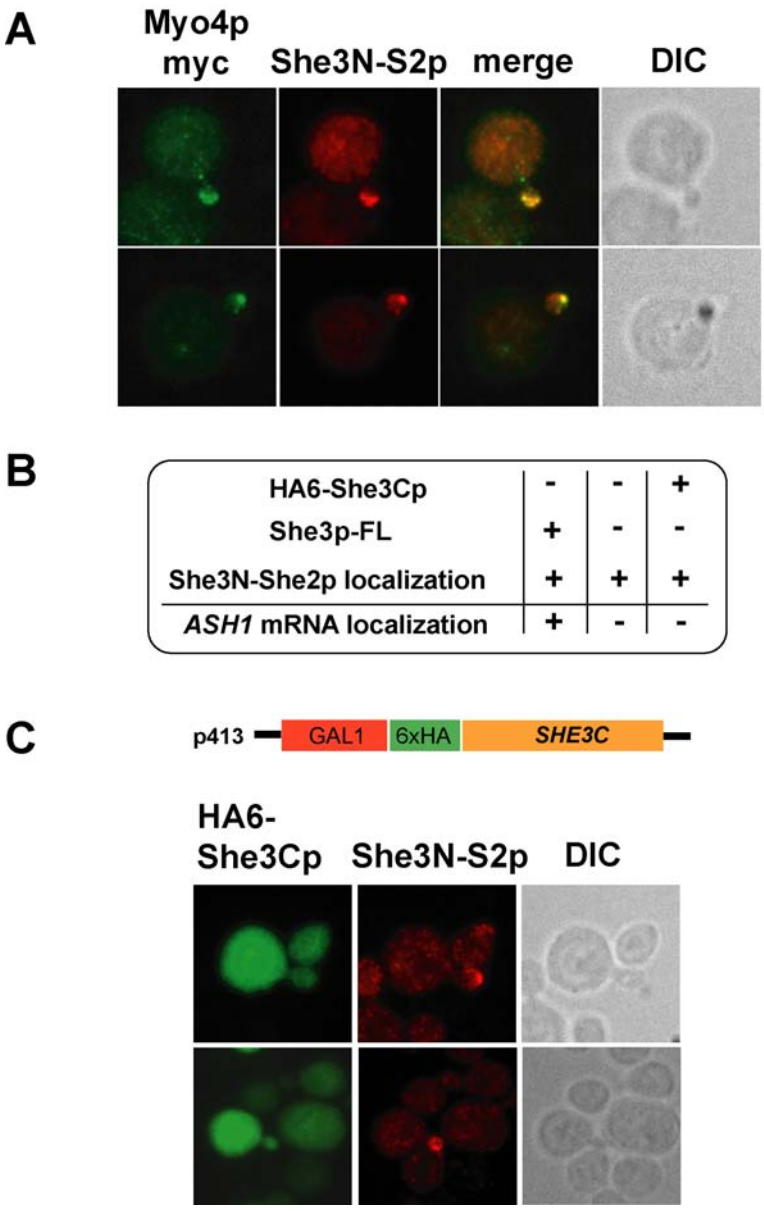

Figure 28: The C terminus of She3 contributes to cytoplasmic binding of *ASH1* mRNA. A. The fusion protein is successfully tethered to the myosin motor. Cells of strain RJY3270 (She3N-She2p, *she2Δ, she3Δ*) in indirect immunofluorescence. In dual stain of She3N-She2p and myc-tagged Myo4p, signals colocalize at the bud tip (merge). B. Table showing *ASH1*-localization analyzed by *in situ* hybridisation of strains expressing She3N-She2p in a background with either endogenous She3p (left lane, She3p-FL, RJY2414), no endogenous She3p (middle lane, RJY3270) or with an overexpressed C-terminal portion of She3p (right lane, HA6-She3Cp, RJY3271). *ASH1* localization was only observed when full length She3p was present. Note that She3N-She2 is localized to the bud tip in all strains. C. Cells of strain RJY3271 (She3N-She2p, *GAL1-HA6-She3*C, She3N-She2p, *she2Δ, she3Δ*) showing overexpression of HA6-She3Cp in indirect immunofluorescence. HA6-She3Cp is strongly expressed by the *GAL1* promotor and is ubiquitously distributed throughout the cell (left panel). Although fusion protein is localized to the bud tip (middle panels) *ASH1* localization in these cells could not be rescued.

A strain expressing She3N-She2 fusion protein in a *she3Δ/she2Δ*-background was additionally transformed with the constructed plasmid. Cells were grown in galactose containing medium to provide induction of expression. Western blot analysis identified the expression of a 30-kDa HA6-She3Cp protein. As depicted in figure 26C indirect immunofluorescence shows that overexpressed HA6-She3Cp was ubiquitously distributed throughout in the cells. A concentration of signals at the bud tip or along actin fibres could not be observed, may be due to the strong over-expression or due to a missing association. Bud tip localization of She3N-She2p in these cells instead remains unaffected. However, these cells were not able to restore *ASH1*-localization by the overexpression of the C-terminal She3p. Thus in the absence of endogenous She3p, the She3N-She2 fusion protein itself is localized to the bud tip. However, the RNA cargo is was mislocalized in this case. The fusion protein alone can apparently not account for a functional *ASH1* transport indicating that parts of She3p are indispensable for additional *ASH1*-binding or for the formation and remodelling of a functional cytoplasmic transport complex. This provides the evidence that She3p, or at least parts of it, may participate in a cooperative *ASH1* binding and/or is needed to ensure the integrity of the transported *ASH1*-mRNP.

3 Discussion

3.1 Nuclear factors involved in cytoplasmic RNA localization

A large number of *trans*-acting factors involved in RNA localization have been characterized in the past years. Interestingly, many of them are either predominantly nuclear proteins or nucleo-cytoplasmic shuttling proteins. This suggests that nuclear and cytoplasmic steps in the RNA transport pathway are often highly interconnected. This model becomes more and more evident as connections between steps in RNA biogenesis are described (Farina and Singer, 2002; Giorgi and Moore, 2007; Reed, 2003). For example, analyses on *oskar* mRNA localization in *Drosophila* have revealed a requirement for components of the exon junction complex (EJC) such as Mago nashi (Mago) and Y14 (Hachet and Ephrussi, 2001; Mohr et al., 2001). The EJC has been shown to mark the location on a spliced transcript where introns have been removed. In general, components of the EJC are required for nonsense-mediated decay (NMD). Mago-Y14 and the translation initiation factor eIF4IIIA, an RNA DEAD box helicase, are predominantly nuclear proteins and assemble on *oskar* mRNA during nuclear RNP formation (Palacios et al., 2004). In general, many factors involved in the localization of a transcript either have an additional nuclear function such as splicing or are recruited already in the nucleus.

In yeast, we find the situation that two out of four *ASH1* mRNA binding proteins, Loc1 and Puf6, are predominantly nuclear. Loc1p is therefore often regarded as the nuclear component of the localization machinery that is likely needed to mark the *ASH1* mRNA for cytoplasmic RNA transport (Long et al., 2001; Urbinati et al., 2006). Immunofluorescence of Loc1p reveals a strictly nucleolar localization (Fig. 18B). It is essential for the efficient localization the *ASH1* mRNA (Stephan Jellbauer, personal communication). Loc1p is also a constituent of pre-60S ribosomes and required for the assembly and export of the 60S ribosomal subunit (Urbinati et al., 2006). Indirect immunofluorescence have revealed that Puf6p is also a mainly nucleolar protein (Fig. 18A). Puf6p is a member of the PUF family, and has been proposed to function in translational control of *ASH1* mRNA. The recruitment of this RNA binding protein has to occur in the nucleus. To date, there is no evidence showing that Puf6p has a nuclear function other than RNA localization. At the beginning of this work, it also remained controversial whether the RNA-binding proteins, She2 and Khd1 can

shuttle between the nucleus and the cytoplasm. She2 was previously suggested to enter the nucleus and to be exported into the cytoplasm in an RNA dependent manner (Kruse et al., 2002). In a *mex67–5*^{ts} mutant, She2 accumulated in the nucleus when mRNA export is blocked. Moreover, the truncated mutant *she2ΔN70* failed to bind *ASH1* mRNA and therefore accumulated in the nucleus as well. However, all these results remained controversial, as Gonsalvez and colleagues reported that nuclear export of She2 was independent of both mRNA transport and the ability of She2 to bind mRNA (Gonsalvez et al., 2003). This thesis followed the same experimental approach by using a generated and highly specific antibody against the She2 protein. At elevated temperatures, several export mutants investigated displayed an accumulation of She2 due to inhibition of mRNA export (Fig. 15). This suggests that She2's export occurs in an mRNA dependent fashion. This argument is strengthened by the observation that an RNA binding mutant of She2 is not able to leave the nucleus (Fig. 14). The binding affinity of She2 and of the She2-N36S-R63K mutant toward the *ASH1* E3 element was determined by filter binding experiments (Marisa Müller and Dierk Niessing, personal communication). She2p binds to the E3 RNA with a binding constant of K_d=147 nM, whereas the binding affinity of the mutant was depleted. However, accumulation of this mutant in the nucleus especially within the nucleolus could be a result of its unspecific binding to any other RNA. This argument has to be considered as the nucleolus is full of various RNA species such as rRNA and snoRNAs. Thus, in order to address unspecific binding affinity of She2p towards an unrelated RNA of the human immunodeficiency virus was determined (HIV-I TAR RNA). Wild type She2p could bind HIV-I TAR RNA with a low constant of K_d=912 nm. However, it was abolished in the She2-RNA binding mutant. This suggests that the observed nuclear accumulation of this mutant is caused by the loss RNA binding. Thus, RNA binding is a prerequisite for She2 in order to become exported together with its RNA target. Nevertheless, the results are in contrast to observations made by Gonzalvez and colleagues, as they cannot see any nuclear accumulation of different myc-tagged versions of She2 RNA-binding mutants (Gonsalvez et al., 2003). In order to rule out artefacts due to staining procedures it was necessary to show that RNA-binding mutants of She2 accumulate in nuclei *in vivo*. Indeed, GFP-She2-N36S-R63K mutant was sequestered in nuclei of living yeast cells as well (Fig. 15B). Moreover, accumulation of GFP-She2 in nuclei upon inhibition of RNA export further approves the RNA dependent export of She2p

also in living cells (Fig. 15A). In addition, biochemical purification of yeast nuclei (Hurt *et al.*, 1988) by steps of differential centrifugations has revealed that a portion of She2p is nuclear. Cosedimentation of She2 with intact nuclei was also observed in another method used for subcellular fractionation (Schmid et al., 2006). In a second approach, She2 was immunoprecipitated out of fractions containing purified nuclei. The subsequent RT-PCR reaction suggests that in these fractions immunoprecipitated She2 was bound to the E3 element of *ASH1* mRNA (Fig. 12C). This indicates a nuclear association of She2p with *ASH1* mRNA. However, the biochemical purification provided by this fractionation method cannot completely exclude the contamination with ER from the nuclear periphery since there is now evidence suggesting a direct or indirect association of the She2p with ER (Schmid et al., 2006). Thus, the RT-PCR signal emanating from this purification might not be exclusively nuclear. She2p is the major player among all known *trans*-acting factor as it can bind to all localization elements of *ASH1* mRNA. The model of this work suggests that She2p, due to its shuttling nature, enters the nucleus in order to recognize and bind to *ASH1* mRNA co- or posttranscriptionally, probably at transcription sites. Subsequently, it may initiate the assembly of *ASH1* mRNA and its corresponding RNA binding proteins into a nuclear RNP. Once exported from the nucleus, the *ASH1* RNP possibly undergoes a remodelling step in order to recruit the She3/Myo4 motor complex (Böhl et al., 2000; Takizawa et al., 2000). Nuclear recruitment may represent a mechanism by which yeast cells ensure that exported RNPs designated for localization are already loaded with She2p. This would certainly increase the chance for She2-containing RNPs to be recognized and recruited by the cytoplasmic transport machinery. The adapter She3 could have an active role in the cooperative binding of *ASH1* mRNA in the cytoplasm (Böhl et al., 2000; Shepard et al., 2003; Takizawa et al., 2000). However, direct binding of She3p to *ASH1 in vitro* has never been demonstrated because a stable expression of recombinant She3p is to date not possible (Alexander Houck, personal communication). It has been known that the C-terminus of She3p is required in order to tether She2 to the myosin motor complex (Böhl et al., 2000; Long et al., 2000). Preliminary data shown in this thesis suggest that the She3 C-terminus might be involved in the cooperative association of She2p, She3p and A*SH1* mRNA, which is required in order to link the *ASH1* RNP to the myosin motor complex (Fig. 28). In a *she2Δ/she3Δ* strain, RNA transport could not be rescued by an artificial She3N-She2 fusion protein, which lacks the C-terminal

domain of She3p. Thus, this domain of She3 is indispensable in order to provide full transport even when *ASH1* was already linked to the She2 portion of the fusion protein. This indicates that a simple *ASH1*-She2-She3N-Myo4 link is not sufficient to provide functional RNA localization. Probably the assembly of a functional transport-competent locasome is more complex and requires a remodelling step after nuclear export, which involves the C-terminal domain of She3p. Interestingly, *Candida albicans* does not encode any She2p, but orthologs of She3p and Myo4p. Instead, *C. albicans* She3 probably has to fulfil both jobs, RNA binding and subsequent tethering to the myosin motor.

Nuclear binding of *trans*-acting factors and subsequent remodelling of localizing RNPs occurs also in *Xenopus* oocytes (Kress et al., 2004). Here, association of the RNA binding proteins hnRNP I and Vg1RBP/vera with localized *Vg1* and *VegT* RNAs occurs already in the nucleus. After export from the nucleus, the core RNP complex was shown to undergo remodelling and additional factors, including Prrp and XStau, are recruited to provide transport to the target site. This suggests that there are distinct nuclear and cytoplasmic steps in the RNA localization pathway, which initiates in the nucleus rather than in the cytoplasm.

Hence, the finding that *trans*-factors are nucleo-cytoplasmic shuttling proteins gives rise to the evidence that binding of specific RNA-binding proteins in the nucleus may help to target RNAs to their appropriate destinations in the cytoplasm. This comes even more into focus as Khd1p, a second yeast *trans*-acting factor of *ASH1* mRNA, can also shuttle between nucleus and cytoplasm. In a *mex67–5*^{ts} mutant, myc-tagged Khd1p accumulates in nuclei upon inhibition of RNA export as well (Fig. 19). Thus, the export of both proteins, She2p and Khd1p is dependent on the export of its RNA target. The early nuclear association of Khd1p with the *ASH1* transcript may serve to form an RNP competent for cytoplasmic localization. Thus, nuclear RNP assembly might have a crucial impact on the translational control in the cytoplasm. One of the best examples for nuclear assembly of a transport-competent mRNP involves the association of the zipcode binding proteins 1 and 2 (ZBP1/2) with β-actin mRNA. ZBP1 interacts with β-actin mRNA already in the nucleus and mediates its localization neuronal growth cones. Similar to Khd1p, ZBP1 represses translation of β-actin mRNA until it has reached the target site where it is phosphorylated by the Src kinase (Hüttelmaier et al., 2005). A second reason for Khd1p to enter the nucleus might be its putative role in telomere maintenance (Denisenko and Bomsztyk, 2002).

This work provides the first detailed investigations on the subcellular localization of all yeast RNA binding proteins involved in *ASH1* mRNA localization. Each of the factors can bind to motifs in the *ASH1* mRNA, and have at least a transient localization in the nucleus. This supports the idea that RNP assembly initiates early in the nucleus rather than in the cytoplasm. The nuclear formation of a localized RNP may influence the cytoplasmic fate, which includes additional steps of remodelling, transport or translational regulation.

3.2 The Nucleolus, a multifunctional compartment

3.2.1 Ribosome Biogenesis

The subcellular localization of the *ASH1* transcript and of its *trans*-acting factors revealed a surprising connection to a subnuclear compartment, the nucleolus. The nucleolus is primarily the site of ribosome biogenesis in eukaryotic cells. However, several lines of evidence show that it has additional functions, such as regulation of mitosis, cell-cycle progression and proliferation, many forms of stress response and biogenesis of multiple RNPs (Boisvert et al., 2007; Carmo-Fonseca et al., 2000).
Nucleoli form around the tandemly repeated clusters of ribosomal DNA (rDNA) genes: The result is a subnuclear compartment that locally concentrates the transcription and processing machineries required for generating ribosome subunits. In yeast, two independent studies have demonstrated that Loc1p is a component of the processing and export apparatus of the 60S ribosome subunit. *loc1Δ* cells display a defect in the synthesis of 60S ribosomal subunits, resulting in “halfmer” polyribosomes (Stephan Jellbauer, personal communication) (Urbinati et al., 2006). The involvement in the process of ribosome biogenesis may reflect why Loc1p is a stationary nucleolar component. Nevertheless, it still not clear which function it has in RNA localization. Loc1p was identified in a three-hybrid screen and deletion leads to a significant decrease of bud tip localized *ASH1* transcripts. Interestingly Loc1p is able to bind the *ASH1* transcript but is not able to shuttle between nucleus and cytoplasm (Long et al., 2001). Consequently, it is not a component of the locasome suggesting that *ASH1*-Loc1p interaction is temporary (Urbinati et al., 2006). Nevertheless, Loc1p is arguably an interesting link between RNA localization and ribosome biogenesis. As was proposed by the lab of Pamela Silver, Loc1p could for

example be required to load *ASH1* mRNA onto ribosomal subunits. These non-canonical ribosomes become then localized themselves in a repressed state, and are translationally activated once reaching the target site. Certainly, this would explain why the *ASH1* message has its transit through the nucleolus. But until now, there is no clear evidence for such a mechanism. Nevertheless, in order to show if this is the case, one would have to block ribosome export alone and determine if cellular distribution of *ASH1* is altered. This can be achieved in *crm1* (*xpo1*) mutants rendered sensitive to leptomycine B (LMB), which allow the inhibition of NES-Crm1 mediated export (Neville and Rosbash, 1999). However, with a delay of 15 min also poly(A)+ RNA accumulates in nuclei, which makes it difficult to determine if RNA localization is impaired in this mutant. More recently, Mex67-Mtr2 complex was described to function in the export of pre-60S ribosomes. Mutations in the Mex67 loop lead to an accumulation of 60S subunits, but not mRNA, in the nucleus (Yao et al., 2007). Thus, this would be an ideal candidate to differentiate between ribosome and RNA export. Another aspect that remains to be elucidated is whether these two processes mutually influence each other. It remains possible that in *loc1Δ* cells, the observed defect in RNA localization is just a secondary effect caused by defective ribosomes. To address this one would have to determine, which of the two processes is affected first when Loc1p is 'turned off' by a depletion system *in vivo*. Another nucleolar factor involved in RNA localization is Puf6p. Until now, there is no evidence indicating that Puf6p is required for ribosome biogenesis. Nevertheless, it copurifies with pre-60S particles as well (Nissan et al., 2002). Moreover, it provides the link between translation and *ASH1* mRNA localization. Consistent with a previous study (Gu et al., 2004), the deletion of *PUF6* leads to an increase of Ash1p synthesis suggesting a role in translation control (Figure 26).

3.2.2 Assembly of non-ribosomal RNPs

There is increasing evidence that the nucleolus may represent a region where many RNPs other than ribosomal subunits are assembled. Several RNP complexes have been reported to be formed in the nucleolus, such as small nuclear RNPs, RNaseP RNP, telomerase containing particles and signal-recognition particles (Boisvert et al., 2007). This gives rise to the idea that there is also a "non-ribosomal landscape" within the nucleolus. This is best documented for signal-recognition particles (SRPs)

(Alavian et al., 2004; Ciufo and Brown, 2000; Grosshans et al., 2001; Jacobson and Pederson, 1998; Politz et al., 2002; Politz et al., 2000; Sommerville et al., 2005). The SRP complex consists of six proteins and an RNA of ~300 nucleotides. Recent studies have shown that both the RNA and the proteins from the SRP transit through the nucleolus of mammalian cells before SRP export to the cytoplasm. These results indicate a possible function for the nucleolus in the assembly and processing of the SRP complex and a potential association with newly formed ribosomes before their cytoplasmic export. Yeast SRP resembles its mammalian counterpart as it also consists of six proteins (Srp72p, Srp68p, Srp54p, Sec65p, Srp21, and Srp14p) and a single RNA molecule called scR1 (Brown et al., 1994; Hann and Walter, 1991; Mason et al., 2000). There is evidence that assembly of the SRP requires import of all SRP core proteins into the nucleolus, where they assemble into a pre-SRP with scR1. The formed particle is subsequently targeted to the nuclear pores and exported in an Xpo1p dependent fashion (Grosshans et al., 2001). It is likely that the core SRP act as kind of RNA chaperone that facilitate correct folding and stabilization of scR1-RNA. Similar to SRP formation, assembly of non-ribosomal RNPs in general, may also require the import of the RNA and additional *trans*-acting factors. The assembly might occur in the nucleolus. Alternatively, the nucleolus may also function as a checkpoint to control the integrity of RNP complexes. Once the RNPs have matured and passed this quality control, they can leave the nucleolus.

In plants, a number of viruses interact with nucleolar proteins. It has been suggested that many viruses target the nucleolus and its components to favour viral transcription, translation or to alter cell growth and the cell cycle to promote virus replication (Hiscox, 2002). One interesting observation has been reported for the ORF3 protein encoded by the Groundnut rosette virus (GRV), which reflects a functional link between nucleolar activities and development of systemic viral infections. ORF3 is a multifunctional RNA-binding protein that protects viral RNA and is involved phloem-associated long distance RNA movement. ORF3 together with viral RNA assemble into cytoplasmic granules. Interestingly, the nucleo-cytoplasmic shuttling protein ORF3 was found associated with the nucleolus. The nucleolar localization of ORF3 correlates with the ability to transport viral RNA over long distance over the phloem. This supports the idea that in infected cell, viral RNAs and proteins take the route through the plant nucleolus because of its ability to form stable RNP complexes (Kim et al., 2004).

3.2.3 Post-transcriptional modifications

The nucleolus has a pivotal role in covalent RNA modifications. Various RNAs, especially transcripts made by RNA Pol III, such as 5S rRNA, some tRNAs, RNase P RNA, the signal recognition particle (SRP) RNA and now also microRNAs (miRNAs) undergo maturation in the nucleolus (Gerbi et al., 2003). The most common covalent modifications found in rRNA are 2′-*O*-ribose methylation and pseudouridylation. Both modifications are catalyzed by snoRNPs that target the pre-rRNA substrate with conserved motifs called “C/D-box”, which contains a UGAUGA sequence (box C) and a CUGA sequence (box D). The pseudouridylation of rRNA is also catalyzed by a distinct class of nucleolar snoRNPs and involves a ‘guide RNA’ targeting mechanism with two short conserved sequences called boxes H and ACA. Until now, there is no evidence that mRNA is post-transcriptionally modified as well. Nevertheless, there are box C sequences within the *cis*-elements of *ASH1* mRNA. It remains to be determined in the future whether there is any modification in the *ASH1* message. Such modifications could be tools to mark RNAs for localization or to render them into a translational repressed state.

3.2.4 Transit of *ASH1* mRNA through the nucleolus

The first evidence showing a connection of the nucleolus with *ASH1* mRNA comes from a study about mRNA export. In an *in vivo* imaging system, the 3’ Untranslated Regions (3’-UTR) of various RNAs were fused to U1A-hairpins and their cellular distributions has been observed with an U1A-GFP reporter under conditions when mRNA export was blocked. Interestingly, only the *ASH1*-3’-UTR directed the GFP reporter into the nucleolus (Brodsky and Silver, 2000). In an independent approach, our lab examined the accumulation of *ASH1* mRNA with labelled oligonucleotides directed against *ASH1* RNA (Fig. 17). However, upon block of mRNA export in the *mex67-5* mutant only the site of transcription was visible by *in situ* staining, even when *ASH1* was overexpressed. It has been reported previously that defects in RNA export factors such as Rat7p, Gle1p, Mex67p and Rat8p can cause the hyperadenylation during 3’ end formation of nascent transcripts (Hilleren et al., 2001; Jensen et al., 2001; Libri et al., 2002), which as consequence are sequestered at transcription foci. Transcript retention has been shown to require components of the

nuclear exosome (Thomsen et al., 2003). Thus, the additional deletion of *RRP6* in an export mutant has been shown to release transcripts from these foci. Indeed, in an *rrp6Δ/mex67-5* mutant, the *ASH1* signal was no longer detected at transcription site. Instead, the released transcript was found accumulated within nucleoli during inhibition of RNA export (Fig. 15). Controversially, hyperadenylation and transcript retention of U1A-*ASH1*-3'UTR did not occur (Brodsky and Silver, 2000). May be there is a significant difference in Poly(A)-tail length. Another reason could be the high affinity of the U1A-GFP reporter toward the U1A-hairpins, which may force the release from transcription sites. Nevertheless, as one can examine the subnuclear distribution of RNAs, this would be an excellent alternative method to visualize the nuclear route of localized RNAs *in vivo*. The accumulation of transcripts in nucleoli during block of mRNA export could be certainly a general phenomenon. In a previous study, it has been reported that a mutant of the nucleolar protein Mtr3, which is a component of the yeast exosome can cause the accumulation of poly(A)+ mRNA in the nucleolus (Kadowaki et al., 1994; Kadowaki et al., 1995). The authors suggested that all mRNAs may encounter nucleolar components before export, thus supporting the idea that nucleolus may have a role in mRNA export (Schneiter et al., 1995). However, the link to the nuclear exosome may give raise to the possibility that polyadenylated transcripts are subjected to 3'-5' degradation when mRNA export or processing is defective. Thus, the nucleolus may act as a kind of 'trash bin' for mRNAs as well. The nucleolar accumulation was stained with oligo(dT)-probes by in situ hybridisation directed against Poly(A)+ RNA. Whether these polyadenylated transcripts correspond to mRNA or snoRNA, or both is not clear (van Hoof et al., 2000; van Hoof and Parker, 1999).

Thomsen and co-workers reported that the block of mRNA export in *rat7-1/rrp6Δ* mutants leads to the accumulation of heat-shock RNAs *HSP104* and *SSA4* in the nucleolus (Thomsen et al., 2003). In contrast, poly(A)+ mRNA has previously been clearly shown not to accumulate in the nucleolus but to localize to the nuclear periphery in a *rat7-1/rrp6Δ* (Hilleren et al., 2001). Thus, not all mRNAs have their transit through the nucleolus. It remains elusive which of the factors might determine nuclear trafficking and how many RNAs might undergo the transit through the nucleolus. It also remains to be determined in the future whether also other localized RNAs such as *IST2* mRNA follow this nuclear pathway.

3.2.5 Assembly of localized RNPs

This work provides the first evidence on subnuclear localization of the RNA binding protein She2. Upon block of mRNA export, this shuttling protein sequestered at sites of the nucleolus (Fig. 13). To address the question whether this observed effect was due to the RNA binding affinity toward the highest amount of RNA, which is in the nucleolus, it was reasonable to render She2 defective for RNA binding by mutagenesis. Interestingly, mutant She2 cannot leave the nucleus and consequently accumulates in nucleoli without any inhibition of export (Fig. 14). The accumulation in the nucleolus is possibly not a net result of its RNA binding affinity (see above). Thus, the accumulation of She2p in the nucleolus is independent of RNA binding suggesting that She2p may be the factor that brings *ASH1* mRNA into the nucleolus. This is strengthened by the observation that in the absence of She2p, accumulation of *ASH1* mRNA in the nucleolus does not occur. Hence, She2p together with its RNA target can at least transiently enter the nucleolus. Moreover, two additional factors Loc1 and Puf6 are both nucleolar proteins (Fig. 18). This supports the idea that the stationary factor Loc1 is only able to face the *ASH1* transcript when delivered by She2p. Indeed, preliminary IP-RT experiments show that immunoprecipitated Loc1 was bound to *ASH1* E3 RNA (Fig. 27). In an additional deletion of She2p, binding of Loc1 to *ASH1* was significantly reduced. Future experiments would have to address whether also Puf6-*ASH1* binding is dependent on nuclear She2p. Puf6p is the only nucleolar factor so far that has been reported to be also a transient component of the cytoplasmic *ASH1* locasome (Gu et al., 2004). Therefore, Puf6p must have the ability to shuttle even when it is predominantly located in the nucleolus. In yeast, Puf proteins have been proposed as integral factors of several RNPs in order to maintain their integrity and stability (Gerber et al., 2004). This is consistent with its role in translational control during transport. Thus, the proper formation of a localized RNP might also include the recruitment of Puf6p onto *ASH1* mRNA. This would explain the requirement of the *ASH1* message to transit through the nucleolus. The stationary *trans*-acting factor Loc1p may provide a framework for such a temporal and spatial assembly of the *ASH1* RNP in the nucleolus. This narrow time-window could be sufficient to allow the association of Puf6p and other factors required for RNA export with the *ASH1* message. Not all shuttling RNA binding proteins accumulate in the nucleolus upon block of mRNA export. For instance, the La motif-containing protein

Sro9p is known to associate with polyribosomes in the cytoplasm (Sobel and Wolin, 1999). The RNA binding protein Sro9 is cytoplasmic, which is consistent with its putative role in translational regulation. However, upon inhibition of mRNA export in the *mex67-5* mutant, Sro9p accumulates in the chromatin-rich nucleoplasm but not in the nucleolus (Susanne Röther, unpublished data). Moreover, nucleo-cytoplasmic shuttling Khd1p is involved cytoplasmic control but displays a similar subnuclear localization like Sro9p when mRNA export is blocked (Fig. 17). Hence, not all RNA binding proteins are attracted to nucleolar sites when not allowing their nuclear export. However, it is not clear, which of the factors is required for She2's association with the nucleolus. Puf6p would be an ideal candidate because it was initially identified in a TAP-purification of She2p (Gu et al., 2004). Controversially, Immunoprecipitation of myc-tagged Puf6 did not reveal any direct interaction with She2 (Fig. 18). A possible approach to identify direct interacting partners would be the TAP purification with the RNA-binding mutant of She2 because of its nucleolar accumulation. This work favours the idea that the correct assembly of factors into a nuclear *ASH1* RNP may occur in the nucleolus. Alternatively, the nucleolus may also represent a checkpoint to verify the integrity of RNP before export into the cytoplasm. According to the data provided by this thesis, the resulting model suggests that She2 enters the nucleus to bind *ASH1* mRNA already co- or posttranscriptionally and guides the bound message into the nucleolus where formation or further maturation of *ASH1* RNP may occur.

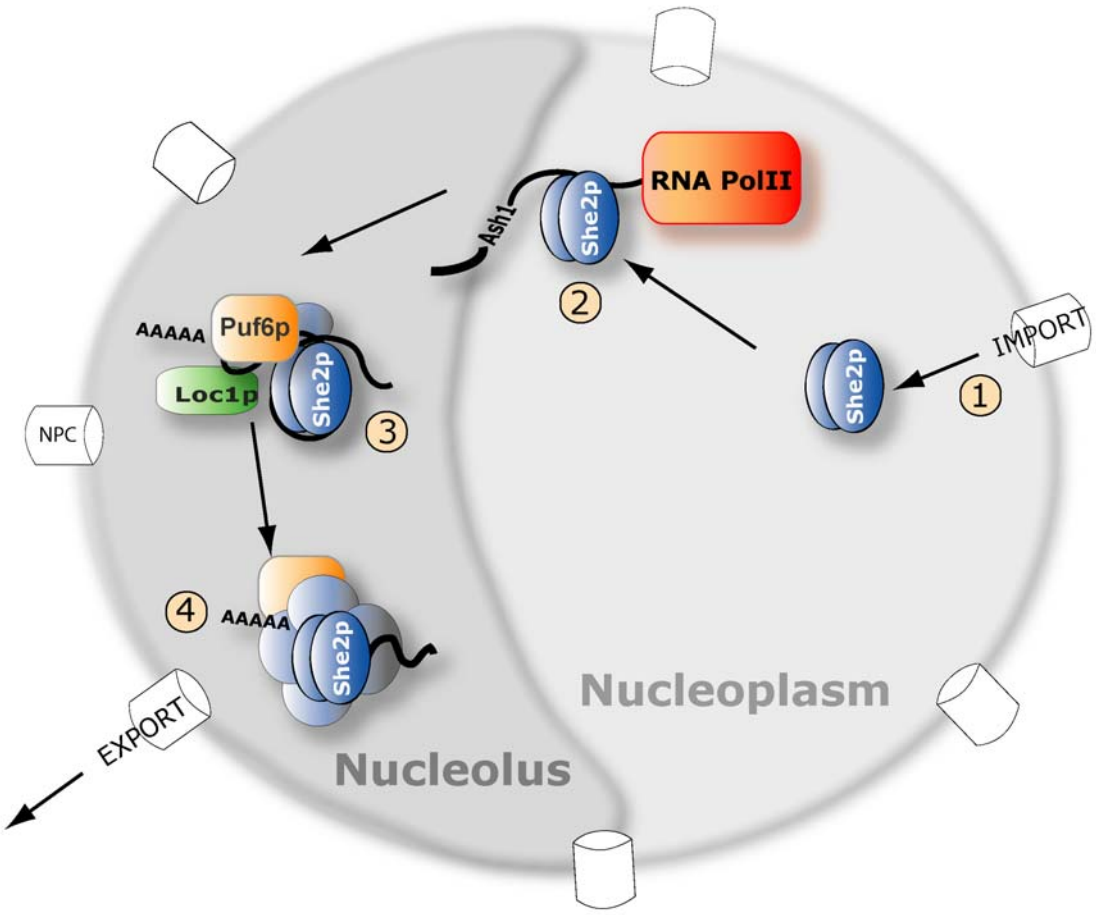

Figure 29: Working model. She2p enters the nucleus either due its small size or by active import (1). She2 binds *ASH1* mRNA at sites of transcription and guides the message into the nucleolus (2). Binding of two additional *trans*-acting factors and subsequent RNP formation and/or maturation might occur in the nucleolus (3). Export of a localization competent RNP into the cytoplasm occurs through the nucleopores (NPC) (4).

Interestingly, a similar phenomenon has recently been observed in mammalians. Two independent studies have provided evidence that in mammalian neurons, Staufen2, a brain-specific RNA-binding protein involved in the localization of CaMKIIα-RNA, can enter the nucleus and behave like a nucleocytoplasmic shuttling protein (Kiebler et al., 2005; Macchi et al., 2004; Miki and Yoneda, 2004). In a mutagenesis approach, Stau2 was rendered incompetent for RNA-binding. Strikingly, mutant Stau2 proteins accumulated in the nucleus and, in particular, in nucleoli. This strongly suggests that Staufen2 can transiently enter the nucleolus. There, the

assembly of Staufen2 into transport-competent RNPs might occur before exported into the cytoplasm. Conclusively, these observations give rise to the evidence that the nucleolus has a role in the assembly or maturation not only of ribosomal subunits but also of localized RNPs. In the cytoplasm, additional cytosolic factors, such as additional RNA-binding proteins and molecular motor complexes, are recruited to these RNPs and transport to the final destinations inside the cell occur

3.3 She2p's 'nuclear history' is required for efficient asymmetric sorting of Ash1p

In order to understand why She2p has to enter the nucleus, we followed a strategy that restricted She2 to take its nuclear route. A mere block of protein import would cause an unwanted amount of secondary effects, as this is a highly essential process. Therefore, a fusion of the proteins She2 and its cytoplasmic adapter She3 should provide a milder way to restrict She2 from entering the nucleus. Indeed, She3-She2 fusion protein was functionally tethered to the myosin motor without disturbing its ability to bind *ASH1* mRNA *in vivo* (Fig. 22). Due to its high affinity toward the Myo4p motor She3-She2 fusion was not able enter the nucleus (Fig. 23). In contrast to wild type She2, nuclear accumulation of this fusion protein was never observed upon the inhibition of mRNA export. Surprisingly, cytoplasmic retention of She2 does not influence its ability to localize *ASH1* mRNA. When keeping She2 in the cytoplasm, it is still able to bind and localize *ASH1* mRNA with the same efficiency as a wild type (Fig. 25). Thus, the nuclear history of She2 is not important for the process of RNA localization itself. But controversially, there was a significant difference when looking at the efficiency of Ash1p sorting. In a strain expressing She3N-She2 fusion protein, the amount of cells, which show symmetric or improper distribution of Ash1p, was significantly higher. There are two possible scenarios leading to this phenotype. The fusion protein due to its artificial nature could not allow proper anchoring of the *ASH1* transcript when reaching the target site. Anchoring of *ASH1* mRNA at the bud cortex has been reported to require remodelling of the trimeric She2-She3-Myo4 complex (Gonsalvez et al., 2004). This was observed in cells where *lacZ-MS2* mRNA was artificially tethered to She2-MS2. In contrast, *lacZ-ASH1*, which displays bud tip localization at all cell-cycle stages in 80% of observed cells, anchoring of *lacZ-MS2* mRNA was inefficient leading to delocalization in post-

anaphase cells because remodelling of She2-MS2/*lacZ-MS2* complex could not occur. Therefore, this study proposed that anchoring might require the transfer of localized *ASH1* mRNA to an unknown anchoring factor, which probably associates with She3-Myo4 complex at the bud cortex. In case of the She3N-She2p mediated transport, efficiency of *ASH1* localization resembles that of the wild type. Importantly, localization efficiencies in this case were determined by *in situ* hybridisation exclusively in post-anaphase cells, thus ruling out a defect in anchoring. Another reason explaining why sorting of Ash1p but not *ASH1* mRNA is inefficient in the fusion protein mediated transport may be premature translation. RNA localization only restricts a protein to a particular region of a cell if the mRNA is not translated until it reaches its destination (St Johnston, 2005). Therefore, many localized mRNAs are subjected to translational control. This involves the integrity of a localized RNP such that it blocks the initiation of translation during their transport. In yeast, two components have been reported so far to control translation of the *ASH1* message, namely Khd1p and Puf6p, both binding directly to the *cis*-elements of the RNA (Gu et al., 2004; Irie et al., 2002; Paquin et al., 2007). The deletion of each of the factors causes prematurely translated Ash1p protein and as a result leads to symmetric distribution. Interestingly, She3-She2 fusion protein mediated transport has led to a similar effect. Premature translation should result in a higher rate of synthesis because regulation of translation is missing. Indeed, when comparing *in vivo* kinetics of Ash1p synthesis in cells expressing She3N-She2 versus the wild type cells, the relative amount of synthesized Ash1 protein was much higher (Fig. 26). This could be due to the artificial nature of the locasome. The link between She2 and She3 could alter the stoichiometric composition of the locasome in such a way that *trans*-factors required for translational control are no longer able to associate with *ASH1* mRNA. But alternatively, these factors should have the potential to bind to the *ASH1* transcript already in the nucleus unless this is not the case because nuclear She2 is absent.

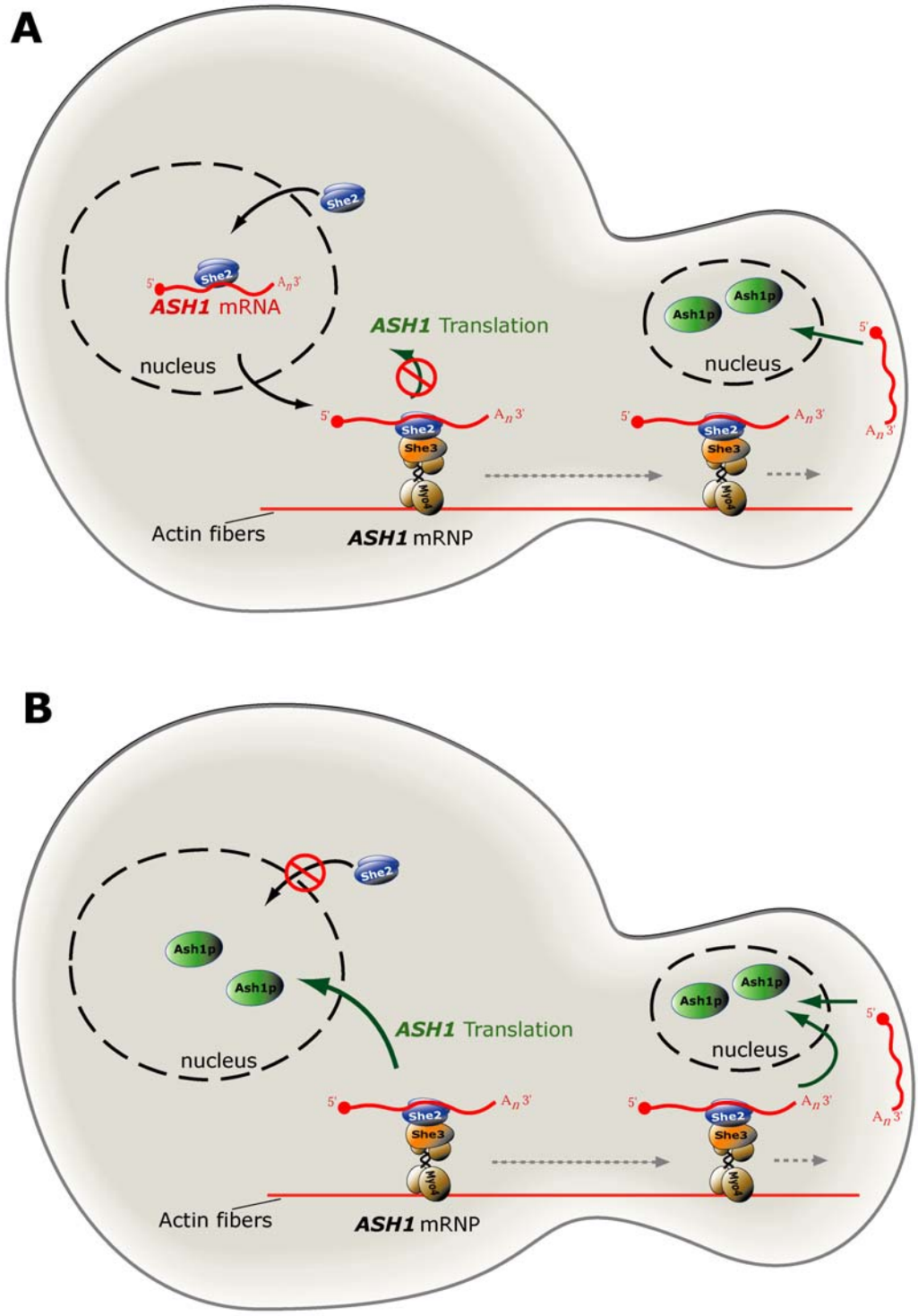

Figure 30: Model of premature translation. A. In wild type cells, She2's nuclear history is important for formation of a transport-competent mRNP. During transport of the *ASH1* mRNP to the bud tip, translation of the transcript is repressed. B. Loss of asymmetric Ash1p sorting is caused by premature translation. Cytoplasmic retention of She2 leads to loss of translational control. Therefore, *ASH1* mRNA is translated prematurely before reaching the target site. As a result, synthesized Ash1p can enter also mother cell nuclei and leads to a symmetric Ash1p distribution.

Hence, She2's nuclear history might have an important role in determining translational control in the cytoplasm (Fig. 30). It remains to be shown how the stepwise assembly of factors occurs mechanistically. The passage of She2 through the nucleolus might have a pivotal role in this process. This could trigger the spatial association of proteins such as Puf6p and Loc1p with the mRNA, and the subsequent formation of an export competent nuclear RNP. Alternatively, She2p may serve as a kind of RNA chaperone that by binding to all cis-acting elements of the RNA facilitates the recruitment of other trans-factors. The involvement of two nucleolar factors in cytoplasmic translational regulation of a localized RNA is quite a surprise. It has been known that Puf6p acts as such a regulator (Gu et al., 2004). Consistent with its function, *puf6Δ* cells have an increased rate of Ash1p synthesis. But even surprising is the fact that translational control is affected when deleting *LOC1*. The increased rate of Ash1p synthesis was similar to that observed in *puf6Δ* cells. How Loc1p can mechanistically contribute to translation repression is not clear. Loc1p and Puf6p are both nucleolar proteins and copurify with pre-60S particles, and there is evidence that they interact physically (Stephan Jellbauer, personal communication). Loc1p could help to recruit Puf6p to the *ASH1* mRNA. Nevertheless, the mutual influence of these trans-acting factors regarding *ASH1*-binding is still elusive and needs to be determined in the future in order to understand the mechanistic detail of nuclear RNP assembly.

Translational control has a similar role in *oskar* mRNA localization. The translation of *oskar* during transport is repressed by the binding of the Bruno protein and HRP48 to three sites in its 3'-UTR (Gunkel et al., 1998; Yano et al., 2004). HRP48 binds to *oskar* mRNA very early, probably co-transcriptionally. Binding of HRP48 is required to recruit other factors to the 3' UTR such as Bruno. More recently, Bruno has been reported to bind CUP protein, which by its interaction with eIF4E inhibits translation. Mutants in *cup* therefore cause the premature translation of *oskar* mRNA, which disrupts also its localization because the passage of ribosome along the mRNA displaces the EJC (Nakamura et al., 2004; Wilhelm et al., 2003).

4 Materials

4.1 Consumables

Akku-Jet	Neolab (Heidelberg)
Analytical balance	Sartorius Universal
Autodiagraphy cassettes	Sigma, Deisenhofen
Fluorescence Microscope BX60	Olympus
Freezer -20°C	Liebherr
Freezer -80°C	New Brunswick
Fridge	Liebherr
Gel documentations system	Mitsubishi
Gel electrophoresis chamber	ZMBH (Heidelberg)
Light microscope ICS/KF 2	Zeiss
Magnetic Stirrer MR3000	Heidolph
Microwave oven	Bosch
PCR cycler Primus 96 plus	MWG-Biotech
PCR cycler PTC-200	MJ Research
Pipetman Gilson P10, P20, P200, P1000	Abimed
Rotor SLC6000	Kendro Sorvall
Rotors SS-34, GS-3, GSA	DuPont
Shaking incubator	New Brunswick
Sonifier 200	Branson
Stratalinker UV Crosslinker	Stratagene
Tabletop centrifuge Micro 2000	Hettich
Typhoon 9400 PhosporImager	Amersham Pharmacia
Ultracentrifuge	Beckmann
Vortexer Genie 2	Scientific Industries
Waterbath Shaker SW 2	Julabo

4.2 Commercially available kits

Access RT-PCR System	Promega
Nucleospin Miniprep Kit	Machery&Nagel
Nucleobond AX 100 Midiprep Kit	Machery&Nagel

QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Quick Ligation Kit	New England Biolabs (NEB)
QuikChange® Site-directed Mutagenesis	Stratagene
Prime-It®II Random Primer Labeling Kit	Stratagene
T7-Mega-Shortscript-Kit	Ambion
Topo TA Cloning®	Invitrogen

4.3 Enzymes

Alkaline Phosphatase	Roche, Mannheim
CIP (Calf intestinal phosphatase)	Roche, Mannheim
DNaseI (RNase-free)	Promega
Lysozyme	Biomol
Platinum-*Pfx*-Proofreading Polymerase	Invitrogen
Restriction enzymes	New England BioLabs (NEB)
RNase A	Roche, Mannheim
Quick-T4-DNA-Ligase	NEB
Taq-DNA-Polymerase	Axon
Vent-DNA-Polymerase	New England Biolabs (NEB)
Zymolyase 100T	ICN

4.4 Antibodies

3F10; rat-anti-HA	Roche
16B12; mouse anti-HA	Convance
9E10; mouse anti-Myc	(Evan et al., 1985)
anti-mouse-IgG-Horseradish Peroxidase	Dianova
anti-rabbit-IgG-Horseradish Peroxidase	Dianova
anti-rat-IgG-Horseradish Peroxidase	Dianova
Alexa®488 rabbit anti-mouse-IgG	MoBiTec
Alexa®488 goat anti-rabbit-IgG	MoBiTec
Alexa®594 goat anti-rabbit-IgG	MoBiTec
Alexa®488 goat anti-mouse-IgG	MoBiTec

Alexa®594 goat anti-mouse-IgG	MoBiTec
Alexa®594 rabbit anti-mouse-IgG	MoBiTec
Rabbit anti-She2 (323/4-E12)	This work, (Schmid et al., 2006)
MCA-38F3 mouse anti-Nop1	Encor BioTechnology
Rabbit anti-Nhp2	(Henras et al., 2004)
Rabbit anti-Rpa49	Gift from H. Tschochner
MAB1501 mouse anti-actin	Chemicon

4.5 Oligonucleotides

4.5.1 Primer for *she2::KANMX4* gene deletion

Name	Sequence (5'-3')
RJO 1813	CTTATAGAATGGTTCTTCGTGCATGCC
RJO 1964	CCTAAATTGGGGTCCCTCCCACATCAGAGG

4.5.2 Primer for *she2::HISMX6* gene deletion

Name	Sequence (5'-3')
RJO 2070	GAATTTGATGTTGTCGCTACTAAATGGCATGACAAATTTGGTA AATTGAAAAACcgtacgctgcaggtcgac
RJO 2071	CTATTAACTAGTGGTACTTATTTGCTCTTTTTGAGCTAAAAACT GAAGGCCatcgatgaattcgagctcg

4.5.3 Primer for *rrp6::natNT2* gene deletion

Name	Sequence (5'-3')
RJO 2390	ATAGGAACAACAAACAGCTTATAAGCACCCAATAAGTGCGTTA TGcgtacgctgcaggtcgac
RJO 2391	TACCATAATTTATAAATAAAAAAATACGCTTGTTTTACATAATCA atcgatgaattcgagctcg

4.5.4 Primer for Puf6p epitope tagging

Name	Sequence (5'-3')
RJO 2037	GATGAAAGTAACAAAGGCTCTCAGCTTTTGGCTAAATTGTTAA AACGTACGCTGCAGGTCGAC
RJO 2038	GTACAGATGCTTATATACCAAATATTGTGACTTTATCGTAGAAA ATATCGATGAATTCGAGCTCG

4.5.5 Primer for Khd1p epitope tagging

Name	Sequence (5'-3')
RJO 2115	AAGAAGAACCTCAAGAGAATCATGATAACAAAGAGGAGCAGTC GCGTACGCTGCAGGTCGAC
RJO 2116	TTTGTTTTGTCTGTGTGGGACGTGCGCACGCACACGTATATAA TCGATGAATTCGAGCTC

4.5.6 Primer for Loc1p epitope tagging

Name	Sequence (5'-3')
RJO 2039	GCTAGTGAAAGTAAAACTGAAGGAAGGAAGGTAAAAAAAGTCT CATTTGCTCAACGTACGCTGCAGGTCGAC
RJO 2040	GGATGTTATATATTATACAACAGACTTATCCGTATTTAGTTTAG TCAATCAAACTAATCGATGAATTCGAGCTCG

4.5.7 Primer for cloning of YCplac111-She2

Name	Sequence (5'-3')
RJO 1720	GGGCCAACCAAAGGATCCGAAATTCGAAGC
RJO 1721	TTTTGAATTCGGCAATTTTTCTTAGCGAAGTATACG
RJO 1722	TTTTGGTACCAGCAAAGACAAAGATATCAAAGTCACTCC
RJO 1723	TTTTGGTACCTTTTTCAATTTACCAAATTTGTCATGCC

4.5.8 Primer for cloning of YCplac111-GFP-She2

Name	Sequence (5'-3')
RJO 1728	TTTTGGTACCCCCCCCGGGCCCCCCTCAGCATGCAGTAAAGG AGAAGAACTTTTCACTGG
RJO 1729	TTTTGGTACCGCTTGGCTGCAGTTTGTATAGTTCATCCATGCC

4.5.9 Primer for cloning of YCplac111- She3N-She2

Name	Sequence (5'-3')
RJO 1932	TTTTTTGGTACCTCGGACCAGGATAATACCC
RJO 1933	TTTTTTGGTACCGGGGGGCCCGGGGGGATCTGAACCATAATT TAAATTTTG

4.5.10 Primer for cloning of p413-HA_6-She3C

Name	Sequence (5'-3')
RJO 2677	TTTTTGGATCCATGACTAGTTCAAATTCAGATATAG
RJO 2678	TTTTTGAATTCCTAGGATTGGGCCCCGTGAACAACC
RJO 2694	TTTTTACTAGTATGGTCGACTCCGGTTCTGCTGCTAG
RJO 2699	TTTTTACTAGTGCGGCCGCATAGGCCACTAGTGCT

4.5.11 Primer for *ASH1* RT-PCR

Name	Sequence (5'-3')
RJO 73	TACATGGATAACTGAATCTC
RJO 74	CAGGATGACCAATCTATTGC

4.5.12 Primer for *ASH1* probe (Northern blot)

Name	Sequence (5'-3')
RJO 176	CCAATAGAACCATGGAGCGC
RJO 217	GAAGATGCCGCGGCGTG

4.5.13 She2-N36S mutagenesis primer

Name	Sequence (5'-3')
RJO 1999	CTCATCTTATATTCACGTGCTGAgCAAGTTCATCAGTCATTTGCG
RJO 2000	CGCAAATGACTGATGAACTTGCTCAGcACGTGAATATAAGATGAGAGATACC

4.5.14 She2-R63K mutagenesis primer

Name	Sequence (5'-3')
RJO 2001	GATTAAATTTGTTAAGAAATTGAaATTTTACAACGATTGTGTGTTAAGC
RJO 2002	GCTTAACACACAATCGTTGTAAAATtTCAATTTCTTAACAAATTTAATC

4.5.15 She2-S120Y mutagenesis primer

Name	Sequence (5'-3')
RJO 2003	CTGAACTATTATCTAACGCAGTacTTACAAAAGGAAATTTTATCTAAAACTTTGAACG
RJO 2004	CGTTCAAAGTTTTAGATAAAATTTCCTTTTGTAAgtACTGCGTTAGATAATAGTTCAG

4.5.16 Sequencing primer

Name	Sequence (5'-3')
RJO 813	CTTATAGAATGGTTCTTCGTGCATGCC
RJO 1814	GCCATTAAATGCGCAGATGAGG
RJO 1816	CGCAAATGACTGATGAACTTGTTCAGC
RJO 1817	CCGTATGTTGCATCGCCTTCACCC
RJO 1818	GCCCTTTCGAAAGATCCCAACG
RJO 1964	CCTAAATTGGGGTCCCTCCCACATCAGAGG
RJO 2775	CCCAGGTGGAAAGCAACAACGC

4.6 Vectors and Plasmids

4.6.1 Vectors

pGEX4T-2 (Pharmacia)
p41x-*GAL1* series (Mumberg et al., 1995)
YCplac series (Gietz and Sugino, 1988)
YEplac series (Gietz and Sugino, 1988)
pYM series (Knop et al., 1999)

4.6.2 Plasmids

RJP 88	YEplac181-*ASH1* (C3319 in Long et al., 1997)
RJP 132	YEplac195-*ASH1*
RJP 309	pRS426-pGAL1-*ASH1*
RJP 392	YIplac211-pGAL1-10-*ASH1*
RJP 630	pGEX-TEV-*SHE2*
RJP 916	YCplac111-*SHE2* for N-terminal cloning
RJP 919	YCplac111-*GFP-SHE2*
RJP 920	YCplac111-*GFP-SHE2*
RJP 922	YCplac22-*GFP-SHE2*
RJP 932	pRS314-*yra1-1*
RJP 1081	pRS314-*sub2-85*
RJP 1082	pRS314-*SUB2*
RJP 1098	YCplac111-*SHE3N-SHE2*
RJP 1100	YCplac22-*SHE3N-SHE2*
RJP 1101	YCplac22-*SHE2*
RJP 1146	YCplac111-she2-N36S
RJP 1150	pRS313-she2-N36S-R63K
RJP 1292	YCplac111-*GFP-she2-N36S-R63K*
RJP 1461	pGal413-*HA6-SHE3C*

4.7 Bacterial strains

E.coli BL21 (Stratagene)
E.coli TOP10

RJB 20 pGEX-*TEV-SHE2* in BL21

4.8 Yeast strains

W303 *MAT a; ade2-1; trp1-1; can1-100; leu2-3; 112 his3-11; 15 ura3; GAL psi+*

Name	Genotype
RJY 126	*MAT α*, *HO-ADE2*, *HO-CAN1*, *she2::URA3*
RJY 280	*MAT* α, *ash1::URA3*, *TRP1::GAL1-ASH1-myc9* (2 copies)
RJY 358	*MAT a*
RJY 612	*MAT a*, *mex67:HIS3* (pUN100-*mex67-5*), Segref et al. (1997)
RJY 646	*MAT a*, *mex67:HIS3* (pUN100-*MEX67*)
RJY 915	*MAT α*, *LOC1-myc9::K.l.TRP1*
RJY 1121	*MAT a*, *HO-ADE2*, *she3::URA3*, *MYO4-myc3*, *she2::TRP*
RJY 1149	*MAT a*, *mex67:HIS3* (pUN100-*mex67-5*), pGAL1-*ASH1::URA3*
RJY 2182	*MAT α*, *HO-ADE2*, *HO-CAN1*, *she2::URA3*, p413-*GAL1-ASH1*, YCplac111-*GFP-SHE2*
RJY 2198	*MAT a*, *mex67:HIS3* (pUN100-*mex67-5*), pGAL1-*ASH1::URA3*, YCplac22-*GFP-SHE2*
RJY 2239	*MAT a*, *mex67:HIS3* (pUN100-*MEX67*), pGAL1-*ASH1::URA3*
RJY 2264	FY, *MAT α*, *ura3-52*, *his3Δ200*, leu2*Δ*1, trp1*Δ*63, *sub2::kanMX4*, pRS314-*sub2-85*, pGAL1-*ASH1::URA*
RJY 2273	RS453, *MAT a*, *ura3*, *ade2*, *his3*, *leu2*, *trp1*, *yra1::HIS3* pRS314-*yra1-1,* YCplac111-*GFP-SHE2*, pRS426-pGAL1-*ASH1*
RJY 2354	FY, *MAT α*, *ura3-52*, *his3Δ200*, leu2*Δ*1, trp1*Δ*63, *sub2::kanMX4* pGAL-*ASH1::URA*, YCplac111-*GFP-SHE2*
RJY 2414	BY4742; *Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; she2::kanMX4,* YCplac111-*SHE3N-SHE2*, YEplac195-*ASH1*

RJY 2415 *MAT a*, *mex67:HIS3* (pUN100-*mex67-5*), pGAL1-*ASH1::URA3, she2::KANMX4*

RJY 2416 *MAT a*, *mex67:HIS3* (pUN100-*MEX67*), pGAL1-*ASH1::URA3, she2::KANMX4*

RJY 2420 *MAT a*, *mex67:HIS3* (pUN100-*mex67-5*), pGAL1-*ASH1::URA3, she2::KANMX4,* YCplac22-*SHE3N-SHE2*

RJY 2421 *MAT a*, *mex67:HIS3* (pUN100-*mex67-5*), pGAL1-*ASH1::URA3*, *she2::KANMX4*, YCplac22-*SHE2*

RJY 2422 *MAT a*, *mex67:HIS3* (pUN100- *MEX67*), pGAL1-*ASH1::URA3, she2::KANMX4*, YCplac22-*SHE3N-SHE2*

RJY 2645 *MAT a*, *mex67:HIS3* (pUN100-*mex67-5*), pGAL1-*ASH1::URA*, *KHD1-HA6::K.l.TRP*

RJY 2646 *MAT a*, *mex67:HIS3* (pUN100-*MEX67*), pGAL1-*ASH1::URA*, *KHD1-HA6::K.l.TRP*

RJY 2663 *MAT a, PUF6-myc9::K.l.TRP*

RJY 2785 *MAT α*, *HO-ADE2*, *HO-CAN1*, *she2::URA3*, YCplac111-*GFP-she2-N36S-R63K*

RJY 2960 *MAT α*, *HO-ADE2*, *HO-CAN1*, *she2::URA3*, YCplac111-*she2-S120Y*

RJY 2997 BY4742; *Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;* YEplac195-*ASH1*

RJY 3162 *MAT* α, *ash1::URA3*, *TRP1::GAL1-ASH1-myc9* (2 copies), *loc1::HIS3MX6*

RJY 3163 *MAT* α, *ash1::URA3*, *TRP1::GAL1-ASH1-myc9* (2 copies), *puf6::HIS3MX6*

RJY 3164 *MAT* α, *ash1::URA3*, *TRP1::GAL1-ASH1-myc9* (2 copies), *she2::HIS3MX6*

RJY 3269 *MAT* α, *ash1::URA3*, *TRP1::GAL1-ASH1-myc9* (2 copies), *she2::HIS3MX6*, YCplac111-*SHE3N-SHE2*

RJY 3270 *MAT* a, *she3::URA3*, *MYO4-myc6* she2::TRP, YCplac111-*SHE3N-SHE2*

RJY 3271 *MAT* a, *she3::URA3*, *MYO4-myc6*, *she2::TRP*, YCplac111-*SHE3N-SHE2*, pGal413-*HA6-SHE3C*

5 Methods

5.1 Cell density of a yeast culture

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 Transformation of yeast cells

'One-step' transformation with plasmid DNA was performed according to Chen *et al.* (Chen et al., 1992). High-efficiency yeast transformations with linear DNA fragments and PCR products were performed as was described in Gietz *et al.* (Gietz and Schiestl, 1991).

5.3 Epitope-tagging of proteins

In yeast, tagging of genes by chromosomal integration of PCR-amplified cassettes is a standard method to label proteins *in vivo*. This 'one-step tagging' strategy directs the amplified tags to the desired chromosomal loci due to flanking homologous sequences provided by the PCR-primers (Wach et al., 1994). These tags are combined with different selectable marker genes, resulting in PCR amplificable modules (Janke et al., 2004; Knop et al., 1999). In this thesis, several proteins were tagged at the C-terminus using 9xmyc or 6xHA epitope modules. Primers used for amplification of these modules contain 50 bp 5'-overhangs, which are required to target the resulting PCR product directly to the 3'-end of a gene by homologous recombination. After transformation into yeast cells, the PCR-product is integrated into the genome. Cells, which have successfully integrated the epitope module, can be also selected for the marker gene using appropriate selective media plates. Candidate colonies were tested for a positive integration by PCR. Alternatively, the expression of the myc- or HA-tagged proteins can be checked in a western blot or also using indirect immunofluorescence.

5.4 Gene deletions

Genes were deleted with a similar PCR-based strategy using deletion cassettes (Knop *et al.,* 1999). The primers amplify only a selectable marker gene and contain overhangs of 50 bp, which direct the PCR-product to homologous sequences in 5'- and 3'-UTR of the target gene, respectively. After transformation into yeast cells, the endogenous target gene is replaced by the marker gene by homologous recombination. Transformed yeast cells were then selected for the marker gene using appropriate media plates, and the putative candidates were tested for the deleted gene using Colony-PCR.

5.5 Yeast Colony PCR

After transformation, colonies growing on selective media were directly subjected to colony PCR in order to test whether they have integrated the desired epitope-tag or a gene deletion. Colonies were resuspended in 100 µl 0.02 M NaOH. The same volume of glass beads was added and shaken for 5 min in a thermo mixer at 100°C in order to break the cells. After pelleting the beads and cell debris, the supernatant was transferred to a new tube. 5 µl was used as template for PCR.

5.6 Rapid Isolation of Yeast Chromosomal DNA (Ausubel et al., 1998)

Cells of a 10 ml overnight culture were harvested (3 min, 3000x g), and the pellet was resuspended in 200 µl of breaking buffer. 200µl of glass beads together with 200µl Phenol/Chloroform (50:50) were added. Cells were broken by heavily vortexing this mixture for 3 min at full speed, and 200µl TE buffer was added. After a spin at 13.000 rpm for 5 min, only the aqueous layer was transferred to a new tube. DNA was precipitated by the addition of 1 ml 100% ethanol and pelleted at 13.000 rpm for 3 min. The pellet was resuspended in 400µl TE buffer, and 30 µg of DNase-free RNaseA was added. Digestion of RNA occurred for 20 min at 37°C, and DNA was finally precipitated by the addition of 10 µl of 4 M ammonium acetate and 1 ml of 100% ethanol. After washing the pelleted DNA with 70% ethanol, it was spun again at full speed and resuspended in 100 µl TE buffer.

Breaking buffer:
2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA

TE buffer:
50 mM Tris-HCl pH 8, 1 mM EDTA pH 8

5.7 Isolation of RNA from yeast (Cross and Tinkelenberg, 1991)

In order to determine expression levels of *ASH1* mRNA, total RNA was extracted and analyzed in a Northern blot. Therefore, 15-20 ml of a logarithmically growing culture was harvested (3 min/3000x g). The pellet was resuspended in ice-cold TE buffer and transferred to a 2 ml eppendorf tube. After a short spin at 4°C, the supernatant was discarded, and 350 µl of Phenol/Chloroform/Isoamylalcohol (50:49:1, Roth), 450µl Cross-RNA 1 buffer and 200µl glass beads were added to the remaining pellet. This mixture was vortexed at 4°C for 10 min in order to break the cells and spun for 5 min at 13.000 rpm in a cooling centrifuge. 1 ml of pre-cooled (-20°C) ethanol was added to the supernatant, and after carefully mixing, RNA was precipitated for 10 min at -20°C. After centrifugation for 5 min at 4°C, the resulting pellet was resuspended in 200 µl Cross-RNA 2 buffer. RNA dissolved better when was incubated at 65°C for 10 min. RNA concentrations were measured in a UV spectrophotometer at 260 nm.

Cross RNA 1 buffer:
0,3 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2 % SDS
Cross RNA 2 buffer:
1x TE, 0.2 % SDS

5.8 Northern blot analysis

Total RNA was separated in a gel and transferred to a membrane. Expression level of a specific mRNA was detected by autoradiography using ^{32}P-labelled probes. These DNA probes were previously amplified by PCR from genomic DNA, purified and labelled with 'Prime-It II Random Primer Labelling Kit' (Stratagene). 25 ng of each probe was subjected to the labelling reaction, which was performed according to the manufacturer's manual. The radioactive probe was further purified using RNA

columns (BioRad). For the separation of RNA in a gel, 20-40 µg of total RNA was dissolved in 15 µl of DEPC-treated water and mixed with 5µl 10x MOPS, 9 µl formaldehyde (37%), 21 µl formamide and 10 µl of RNA loading buffer. The mixture was incubated for 15 min at 65°C. 20µl was loaded onto a 1.5% formaldehyde-agarose gel, and RNA was separated at a constant current of 6 V/cm. Before transfer of the RNA to the Hybond^{+} Nylon Membrane (Amersham Pharmacia), the gel was washed several times with DEPC water and equilibrated in 10x SSC. Transfer occurred overnight in 10x SSC taking advantage of capillary forces generated by several layers of Whatman®-paper and tissue paper above the gel and the membrane. After the transfer, the membrane was washed with 2x SSC, dried, and the RNA was UV cross-linked using the "UV Stratalinker" (Stratagene). To estimate the loaded amounts of RNA, the membrane was subjected to methylene blue staining (0.04% methylene blue in 0.5 M Na-acetate, pH 5.5). The membrane was pre-incubated in hybridization solution (11.5 ml H_2O, 115 µl Salmon-sperm DNA (10 mg/ml), 13.5 ml Scp/Sarc/DS) at 65°C for one hour. The ^{32}P-labelled probe was then added to the membrane for overnight hybridization at 65°C. The membrane was washed two times with 2x SSC/1% SDS and once with warm (55°C) 2x SSC/0.1% SDS. After a wash in 3 mM Tris-HCl the membrane was finally subjected to autoradiography at the phosphor-imager (Amersham Pharmacia)

RNA loading buffer:

1 mM EDTA, pH 8.0; 0.25% Bromophenol blue; 0.25% Xylene cyanol, 50% glycerol

10x MOPS:

48.1 g MOPS + 800 ml H2O; adjust pH 7.0 with NaOH.

16.6 ml 3 M sodium acetate and 20 ml 0.5 M EDTA pH 8.0 was added and filled up to 1l with H_2O.

Hybridization solution:

11.5 ml H_2O, 115 µl Salmon-sperm DNA (10 mg/ml), 13.5 ml Scp/Sarc/DS

20x Scp:

2M NaCl; 0,6M Na_2HPO_4; 0,02M EDTA; pH 6.2

Scp/Sarc/Ds-Mix:
20 g Dextransulfate dissolved in 60 ml 20x Scp, ad 101 ml H_2O.
Addition of 7 ml 30% N-Lauroylsarcosine (SLS).

20xSSC:
3M NaCl; 0,3M Na3-citrat

5.9 Yeast Whole Cell Extract

20 ml of an exponentially growing culture was harvested (3 min/3000x g), and the pellet resuspended in 400µl breaking buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 mM DTT, 1 mM PMSF, 1x Protease inhibitor mix). After addition of 400µl glass beads, yeast cells were broken using the IKA vibrax at 4°C. Cells were vortexed two times for 3 min with a pause of 3 min on ice in between. The supernatant was spun for 10 min at 13.000 rpm in a cooling centrifuge to get rid of remaining cell debris. Protein concentrations were estimated by measuring OD at 280 nm in the UV spectral photometer. 50µl of 4x Laemmli buffer was added to 150 µl of clear protein extract. The sample was cooked for 5 min at 95°C, and after a spin at 13.000 rpm for 5 min, 1 OD_{280}-unit was loaded on a SDS-polyacrylamide gel.

5.10 SDS-Polyacrylamide Gel electrophoresis (Laemmli, 1970)

Sodium dodecylsulfate (SDS) is an amphipathic detergent. In the presence of SDS, the intrinsic charge of a protein is masked. During SDS-PAGE, all proteins migrate toward the anode and the rate of migration of SDS-treated proteins is effectively determined by molecular weight. The samples were mixed with the same volume of 2x Laemmli buffer and incubated for 95°C to denature the proteins before loading on the SDS gel. Separation of proteins occurred at 25 mA for mini gel (Hoeffer) and 40 mA for a big SDS gel (ZMBH format). After electrophoresis, proteins either were transferred to a membrane (Western blot) or were directly stained with Coomassie.

2x Laemmli-buffer:
100 mM Tris-HCl pH 6.8, 4% SDS, 2% 2-Mercaptoethanol, 20% glycerol, 0,002% Bromophenol blue

10x SDS buffer (1 l):

30.2 g Tris, 144g glycine, 100 ml 10% SDS

12% separating gel (40 ml):

16 ml Acrylamide/Bisacrylamide (29:1), 8 ml 1,5M Tris-HCl, pH 8.8, 15.2 ml H_2O, 400 µl 10% SDS, 200 µl 10%APS, 18 µl TEMED

stacking gel (20 ml):

3.4 ml Acrylamide/Bisacrylamide (29:1), 4 ml 0,5M Tris-HCl, pH 6.8, 12.7 ml H_2O, 200 µl 10% SDS, 100 µl 10% APS, 15 µl TEMED

5.11 Western Blot

In order to detect a specific protein in a given sample of an extract, proteins were separated by SDS-PAGE and then transferred to a PVDF membrane Hybond P (Amersham Pharmacia), and probed using antibodies specific to the target protein. Protein transfer was performed using a semi-dry blotting apparatus (PeqLab) according to the manufacturer's manual. The PVDF membrane was previously activated and washed with 1x semi-dry buffer. A 'sandwich' consisting of 3 layers of wetted Whatman®-paper, PVDF membrane, polyacrylamide gel and another 3 Whatman®-papers on top was piled up for protein transfer, which occurred at 8 mA/cm^2 for 2h. To monitor effectiveness of transfer, blotted proteins were reversibly stained with Ponceau-S® dye. Blocking of non-specific binding is achieved by placing the membrane 3 times in 1x TBS, 0.1% Tween, 5% non-fat dry milk (each 15 min). After blocking, a dilute solution of primary antibody is incubated with the membrane under gentle agitation. Depending on the specificity of the antibody, incubation occurred either for 2 hours at room temperature or overnight at 4°C. After three rounds of washes with 1x TBS (15 mM NaCl, 0.1% Tween (10 min each) to remove unbound primary antibodies, a dilute solution of the secondary antibody linked to horseradish peroxidase (Dianova) was incubated with the membrane for one hour. After two additional washes with 1x TBS, 0.1% Tween, the membrane can be stored in 1x TBS. Detection of the secondary antibody was performed using the enhanced chemoluminescent kit (ECL, Pierce) according to the manufacturer's manual.

Signals were detected using ECL film (Amersham Pharmacia) or the LAS-3000 Image Reader (Fuji). Signal intensities were measured using Multigauge 3.0 software (Fuji).

1x TBS: 10 mM Tris/HCl pH 7.5, 150 mM NaCl.

10x Semi-dry-buffer:
30.3g Tris, 144.1g Glycine, 10 ml 20% SDS, ad 1l H2O

5.12 Isolation of nuclei (Hurt et al., 1988)

Isolation of yeast nuclei was performed according to Hurt *et al.* (1988). 500 ml of an exponentially growing culture (OD_{600}~1) was harvested in a SLC6000 rotor at (3000 rpm/5 min). After a wash with 500 ml H_2O, cells were resuspended in 25 ml 0.1 M Tris-HCl pH 9.4, 10 mM DTT and incubated at 25°C for 5 min while shaking. After washing cells with 25 ml spheroplasting buffer (1.2 M sorbitol, 20 mM potassium phosphate pH 7.4, the pellet was resuspended in spheroplasting buffer containing 5 mg Zymolyase 20T (ICN) per g of net wet weight. Cells were spheroplasted in a 50 ml falcon tube for 20 min at 25°C while gently shaking. Spheroplasted cells were then pelleted carefully for 5 min at 3500 rpm in SS34 rotor. After washing with 10 ml spheroplasting buffer, cells were resuspended in 50 ml osmotic lysis buffer and transferred into a 60 ml douncer with a loose pistil (Neolab). Cells were lysed by 3-4 strokes with the pistil. Cell debris and intact cells were pelleted at 5000 rpm in the SS34 rotor. The whole cell extract (WCE) was then further separated by a second centrifugation (15000 rpm/25 min in SS34 rotor) into an upper layer containing lipids and vacuoles, the post nuclear supernatant (PNS) and the crude nuclear pellet (CNP), which was enriched with nuclei and mitochondria. A 100µl aliquot of PNS and CNP was removed for Western blot analysis. The supernatant was removed, and the remaining CNP was resuspended in 2 ml nuclear resuspension buffer. The CNP was loaded onto a 5-step Ficoll/sucrose density gradient (2M, 1.8M, 1.5M, 1.3M, 1.2M sucrose), and centrifuged for 1 hour at 25000 rpm in a SW40 rotor (Beckmann). 200 µl of cellular material, which accumulated in interphase I-V were collected with a 1 ml syringe using a 21G needle. The collected fractions were finally analyzed in Western blot.

Osmotic lysis buffer:

18% Ficoll DL-400 (w/v); 0.5 mM $MgCl_2$; 20 mM K-phosphate pH 6.45

Nuclear pellet resuspension buffer:

0.3 M sucrose, 16.6% Ficoll; 0.5 mM $MgCl_2$; 20 mM K-phosphate pH 6.45

5-step Ficoll/sucrose gradient:

The 5 steps of the gradient contain 2, 1.8, 1.5, 1.3 und 1.2 M sucrose in 9 % Ficoll buffer (2 ml for each step), and are mixed by the combination of two buffers:

Buffer A: 9% Ficoll (w/v); 0.5 mM $MgCl_2$, 20 mM K-phosphate pH 6.45

Buffer B: 2 M sucrose, 9% Ficoll, 0.5 mM $MgCl_2$, 20 mM K-phosphate pH 6.45

	Mixing ratio A : B (v/v)
1.8 M step	1 : 9
1.5 M step	2.5 : 7.5
1.3 M step	3.5 : 6.5
1.2 M step	4 : 6

5.13 Purification of the She2-antigen

5.13.1 Recombinant expression in E. coli

GST-She2 was recombinantly expressed in *E. coli* BL21 strain RJB20 using a pGEX-GST-*TEV*-She2 plasmid (RJP630). 800 ml of LB medium containing ampicillin and chloramphenicol was inoculated with an overnight culture to an OD of 0.2 and grown at 37°C until the culture reaches OD 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 and stored at -20°C.

5.13.2 Lysis of cells

For the lysis, cells were resuspended in 15 ml lysis-buffer containing 100 mg/l Lysozyme and rotated in a 50 ml Falcon tube for 30 min at 30°C. After 3 rounds of freezing (liquid nitrogen) and thawing (37°C water bath), cells were subjected to sonification 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 in SDS-PAGE.

5.13.3 Affinity purification

The GST-She2 fusion protein was purified using 500 µl of Glutathione Fast Flow Slurry (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 and II, and equilibrated in 10 ml TEV-buffer using a Poly-Prep Chromatography Column (BioRad). The remaining slurry was then transferred to a mobil-col 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. She2 was eluted by centrifugation of the mobil-col column 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). Beads were washed 3 times with 10 ml TEV–buffer in a mobil-col column. The TEV-eluate was added to the beads and rotated for 2 hours at 4°C. Pure She2 antigen was eluted into a fresh eppendorf tube by a short spin for 1 min at 2000 rpm in a cooling centrifuge. Concentration of She2 antigen was further increased using vivaspin® concentrators. The concentration was determined using Bradford (Roth) according to the manufacturer's manual. 150 mM NaCl was added to concentrated She2 solution and stored at -20°C.

Lysis buffer:

25 mM Hepes-KOH pH 7.5, 0.1 mM EDTA pH 8, 1 M NaCl, 2 mM DTT, 1x protease inhibitors

Wash buffer I:

25 mM Hepes-KOH pH 7.,5, 0.1 mM EDTA pH 8, 12.5 mM $MgCl_2$, 1 M NaCl, 0.1% NP40, 2 mM DTT, 1x protease inhibitors,

Wash buffer II:

25 mM Hepes-KOH pH 7.5, 0.1 mM EDTA pH 8, 12.5 mM $MgCl_2$, 0.7 M NaCl, 0.1% NP40, 1 mM DTT

TEV buffer:

50 mM HEPES-KOH pH 7.5, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT (1M), 0.15% NP40, 1 mM PMSF (freshly added before use)

5.14 Generation of a polyclonal antibody

For the generation of a specific polyclonal antibody against She2p, purified antigenic material was injected into rabbits. For the first boost, 200 µg of the antigen (volume 400µl) was mixed with 400 µl Titermax™ (Sigma) and subcutaneously inoculated at the dorsal area. The rabbits were injected every four weeks with 150 µg antigen (300 µl) mixed with 300 µl Freund's Adjuvans™ incomplete (Sigma). 10-12 days after each injection 20-30 ml blood was removed from the ear vein. After coagulation, the blood was centrifuged 5 min at 3000x g and 15 min at 40000x g. The complement system of the serum was deactivated by incubation at 56 °C for 30 min. The yielded serum was stored at -20°C.

5.15 Affinity purification of polyclonal antibodies

In order to reduce unspecific cross reactivity in Western blot detection, the serum was further purified in an affinity step. Therefore, antigenic protein was coupled to sepharose column material.

5.15.1 Preparation of affinity chromatography columns

0.24 g of CNBr-activated sepharose (Amersham Pharmacia) was resuspended in 10 ml 1 mM HCl and rotated for 10 min at room temperature. After three rounds of washing with 10 ml 1 mM HCl, 1.5 mg of antigenic protein was dissolved in 3 ml 0.1 M $NaHCO_3$, 0.5 M NaCl, 1 mM PMSF, 1 mM EDTA pH 8.3 and added to the sepharose material. 10 µl was removed previously for analysis in SDS-PAGE. The suspension was rotated in Polyprep-chromatography columns (BioRad) for 1 ½ hours at room temperature. After binding, 10 µl of the eluate was analyzed in SDS-PAGE to determine coupling efficiency. The column was washed three times with 0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3. After another washing step with 5 ml 0.1 mM Tris-HCl pH 8.0, the column material was rotated in 10 ml 0.1 mM Tris-HCl pH 8.0 for 2 hours at room temperature (or overnight at 4°C) in order to block unspecific binding sites. After three rounds of washes with 5 ml 0.1 M Na-acetate, 0.5 M NaCl pH 4.0, the column was equilibrated with 5 ml 0.5 M NaCl pH 8.0 and stored in 0.1 M Tris-HCl pH 8.0, 10 mM NaN_3 at 4°C.

5.15.2 Affinity purification

Before antibody binding, the column was equilibrated by the flow-through of 10 ml 10 mM Tris-HCl pH 7.5, 10 ml 100 mM Glycine-HCl pH 2.5, 10 ml Tris-HCl pH 8.8, 10 ml 100 mM Na-phosphate pH 11.5 and 10 ml 10 mM Tris-HCl pH 7.5.
4 ml of serum was mixed with 16 ml 10 mM Tris-HCl pH 7.5, 100 µl 200 mM PMSF, 40 µl 0.5 M EDTA, 80 µl 0.5 M EGTA and loaded onto the affinity column to allow binding of the antibody by gravity flow. After four rounds of binding, the column material was washed with 20 ml Tris-HCl pH 7.5 and 20 ml Tris-HCl pH 7.5, 500 mM NaCl. The bound antibody was eluted using three different pH conditions:

1) 10 ml 100 mM Na_3-citrate pH 4.0
2) 10 ml 100 mM Glycine-HCl pH 2.5
3) 10 ml 100 mM Na-phosphate pH 11.5

Eluates were collected in fractions of 1 ml. In order to allow quick neutralization tubes for the citrate-eluates should already contain 160 µl 1 M Tris base. Glycine-fractions

were neutralized with 70 µl 1 M Tris Base, and phosphate-fractions with 300 µl 1 M Glycine-HCl pH 2.5. After washing with 30 ml 10 mM Tris-HCl pH 7.5, the affinity column was stored in 10 ml 10 mM Tris/HCl pH 7.5, 0.05% (w/v) NaN_3 at 4°C. One column can be used for up to 4 rounds of affinity purification. The elution fractions were tested for the antibody titer using Bradford (BioRad). In a microtiter plate, 200 µl of Bradford (dilution 1:5) was mixed with 20 µl of a fraction. Unspecific proteins and antibodies were washed off in the citrate-fraction. The highest titer of eluted antibody was indicated by a blue staining, usually in the first three Glycine-fractions. The antibody-containing fractions were stored in 100 µl aliquots at -20°C. Quality of the purified antibody was assayed in Western blot analysis or in indirect immunofluorescence.

5.16 Immunoprecipitation followed by RT-PCR (IP-RT)

5.16.1 Immunoprecipitation

Immunoprecipitation of She2 and of myc-tagged proteins was done using antibodies coupled to magnetic Protein G beads (Dynal). Coupling of the antibody was performed according to the manufacturer's guide. 25 µl of coupled beads were used for immunoprecipitation of a 50 OD culture. Beads were washed 2 times in breaking buffer and rotated overnight at 4°C in beads buffer to block unspecific binding sites. 50 OD units of an exponentially growing yeast culture were harvested (3 min /3.000x g). The pellet was transferred to a 2 ml eppendorf tube, and 500 µl breaking buffer, 500 µl glass beads, 10 µl RNasin (final conc. 0.8 u/µl, Promega) was added to the pellet. The cells were broken by heavily vortexing 4 times for 3 min with 3 min pause on ice using IKA vibrax at 4°C. Cell debris and beads were pelleted at 8.000 rpm in a cold centrifuge. 400 µl of the supernatant was incubated with 25 µl pre-washed (2x breaking buffer) uncoupled or non-specific magnetic beads for a pre-clear of the lysate. An aliquot 30µl (+10µl 4x Laemmli buffer) was removed as 'total input' (T) for Western blot analysis. An aliquot of 50µl (+50 µl breaking buffer) was taken when performing a subsequent RT-PCR reaction ('total' for RT-PCR). After incubation for 30 min at 4°C, the pre-clearing beads were pelleted in a cold centrifuge and the remaining supernatant was added to 25 µl of previously blocked beads and rotated for two hours at 4°C to allow binding. After binding, a 30 µl aliquot of the supernatant

(+10µl 4x Laemmli buffer) was removed for Western analysis (S). Beads were washed two times with wash buffer and resuspended in 300µl 1x PBS.

5.16.2 Elution of the immune pellet for Western blot analysis

100µl of the final bead suspension was pelleted with the MPC magnet and resuspended in 75 µl 100 mM Glycine-HCl pH 2.5. After incubation for 7 min at room temperature, beads were pelleted in the magnet, and the supernatant (Glycine elution, G E) was removed and mixed with 25 µl 4x Laemmli buffer. Beads were finally resuspended in 1x Laemmli buffer. All collected samples (T, S, G E, SDS E) were incubated at 95°C and analyzed in a Western blot.

5.16.3 Elution of the immune pellet for RT-PCR

After immunoprecipitation, 200 µl of the final bead suspension was subjected to RT-PC. For the elution, beads were pelleted in the magnet, resuspended in 100 µl 100 mM Glycine-HCl pH 2.5, and incubated for 7 min at room temperature. Beads were pelleted, and the supernatant was withdrawn (Glycine elution, G E). Finally, beads were resuspended in 100 µl TE buffer pH 8 (SDS E).

5.16.4 RNA extraction

For RT-PCR, all samples collected (T, G E, SDS E) were subjected to RNA extraction with phenol/chloroform (Rotiphenol, Roth). 5 µl 20% SDS, 10 µl 3 M Na-acetate pH 5.2 and 100 µl Rotiphenol was added to each sample and vortexed for 1 min. After centrifugation (5 min, 13000 rpm, 4°C), only the aqueous layer was taken, and the containing RNA was precipitated overnight with 180 µl ice-cold ethanol and 1 µl glycogen (Roche).

5.16.5 Treatment with DNaseI

Prior to RT-PCR, samples were treated with DNaseI to avoid unspecific signals caused by DNA. Samples were pelleted in a cold centrifuge at 13.000 rpm, washed with ice-cold 70% ethanol, and spun again for 15 min at 13.000 rpm. The pellet was

resuspended in 33 µl H_20. For DNA digestion 2 µl RNasin, 10 µl RQ1-DNaseI (Promega), 2 µl RNasin (Promega) and 5 µl 10x DNaseI buffer was added, and mixture was incubated for 25 min at 37°C. After DNaseI treatment, 50 µl Rotiphenol was added and centrifuged for 5 min at 13.000 rpm. The aqueous layer was removed and added to 100 µl ice-cold 100% ethanol, 5 µl 5 M Na-acetate pH 5.2 and 1 ml glycogen for RNA precipitation (2 hours at -20°C). Samples were pelleted for 30 min at 13000 rpm in a cold centrifuge. After a wash with 70% ice-cold ethanol, the pellet was finally resuspended in 15 µl RNase-free water.

5.16.6 RT-PCR

Extracted RNA of all samples (T, G E and SDS E) was analyzed for *ASH1* E3 element using primers RJO73/RJO74 and the Access RT-PCR System (Promega) according to the provided manual. 1 µl of each sample or of the corresponding dilution was used as template. For the control reaction without Reverse Transcriptase (-RT), 1 µl of the glycine elution (G E) was used.

5.17 Temperature-shift of mex67-5^{ts} cells

The shift of *mex67-5*^{ts} cells to non-permissive temperatures was performed as was previously described in Kruse *et al.*, 2002. Cells of *mex67-5*, which express *ASH1* under the control of the *GAL1* promoter (RJY1149, RJY2239, RJY2415, RJY2849 and RJY3002) were grown in Raffinose-containing media at 26°C. After two cell divisions (OD~0.8), 3% Galactose was added to induce *ASH1* expression for 1 hour. Cells were then shifted to restrictive temperature by placing the flasks into a 37°C shaking water bath for 30-120 min. After temperature shift, cells were fixed at 37°C for indirect immunofluorescence or *in situ* hybridisation using a final concentration 3.7% formaldehyde.

5.18 Indirect Immunofluorescence (Adams, 1997)

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

5.18.1 Preparation of cells

Cells of a logarithmically growing culture (10 ml) were fixed with formaldehyde in a final concentration of 3.7%. Fixation of cells 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), 0.5 M $MgCl_2$). Cells were spheroplasted in 500µl spheroplasting buffer containing 100 µg/ml of Zymolyase 100T (ICN) and 0.2% 2-Mercaptoethanol for 45 minutes at 30°C. Spheroplasted cells were pelleted at low speed (3000 rpm/1000x g) in a tabletop centrifuge for one minute. Cells were washed and finally resuspended in 200 µl spheroplasting buffer. The cell suspension can be stored in aliquots at -80°C or directly used for immunofluorescence.

5.18.2 Immunofluorescence

Multi-well slides (Neolab) used for immunofluorescence microscopy were coated with drops of 0.02% Poly-L-Lysine for 3 min and washed with distilled water. A drop of the cell suspension (~10 µl) was applied to binding onto each well for 5 min. Cells were blocked for 5 min with blocking solution (1x PBS, 1% BSA, 0.05% Na-azide). 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, 0.05% Na-azide), 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).

Primary antibodies

Name	Dilution in blocking solution
Mouse α-HA (16b12)	1:1000
Mouse α-myc (9E10)	1:1000
Mouse α-Nop1 (Aris & Blobel, 1988)	1:250
Rabbit α-Nhp2 (Henras *et al.*, 2001)	1:100
Rabbit α-She2 (this work, Schmid et al. 2006)	1:2000
Rat α-HA (3F10)	1:100

Secondary antibodies (Molecular Probes)

Name	Dilution in blocking solution
Alexa®488 goat anti rabbit	1:300
Alexa®488 goat anti mouse	1:300
Alexa®488 goat anti rat	1:100
Alexa®594 goat anti rabbit	1:300
Alexa®594 goat anti mouse	1:250

5.19 Fluorescent in situ hybridisation using oligonucleotides (FISH)

Cellular localization of *ASH1* mRNA and the *ITS2* of rRNA was determined in the microscope using fluorescently labelled anti-sense oligonucleotides.

5.19.1 Preparation of the probes

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.

ASH1 probe (1 ng/µl oligonucleotides, 1 mg/ml *E.coli* tRNA and 1 mg/ml salmon sperm DNA in DEPC water)

ITS2 probe (5 ng/µl oligonucleotides, 1 mg/ml *E.coli* tRNA and 1 mg/ml salmon sperm DNA in DEPC water)

5.19.2 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 or at 37°C in order to maintain non-permissive conditions. Cells were pelleted and washed three times with Buffer B (1.2 M sorbitol, 100 mM potassium phosphate pH 7.4). Cells were spheroplasted in 200µl spheroplasting buffer containing 100 µg/ml Oxalyticase (Enzogenetics) at 30°C. After spheroplasting, cells were pelleted carefully for one minute at low speed (3000 rpm/1000x g) and washed with buffer B. Cells finally were resuspended in 100 µl buffer B. Multi-well slides were coated with 0.02% Poly-L-Lysine and washed with DEPC water for 5 min. 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.

Spheroplasting buffer (1 ml)

720 µl	1.4 x Buffer B (1.7 M sorbitol, 140 mM K-phosphate 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	2-Mercaptoethanol
171.,5 µl	DEPC-water

5.19.3 Hybridisation

Multi-well slides were rehydrated in a jar with 2x SSC and 2x SSC/40% formamide for 5 min. A frozen aliquot of the *ASH1* or *ITS2* probe was resuspended in 15 µl of Solution 1 and incubated at 80°C for 3 min. Probes were mixed carefully with 15 µl of ice-cold Solution 2 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 two times with warm solution (37°C) of 2x SSC/40% formamide 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).

20x SSC: 3 M NaCl; 0,3 M Na_3-citrate

Solution1:
49,3 µl Formamide
0.63 µl 1M Na-phosphate pH 7
11.7 ul DEPC-water

Solution2:
12,3 µl BSA (20 mg/ml; Boeringer)
12,3 ul 20 x SSC
0,75 µl Rnasin (Promega)
36.2 µl DEPC-water

5.20 High efficiency transformation of DNA into Bacteria (Pope and Kent, 1996)

5.20.1 Generation of competent E. coli cells

TOP10 *E. coli* cells were grown at 37°C in 100 ml 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. After pelleting, cells were resuspended in half a volume of 0.1 M $CaCl_2$ and cooled on ice for 30 min. Cells were then pelleted and resuspended in 5 ml 0.1 M $CaCl_2$. Competent cells were stored in 10% glycerol at -80°C as aliquots of 100 µl.

5.20.2 Transformation

1-10 ng of Plasmid DNA or 5-10µl of a ligation mix was pre-cooled and incubated with 50 µl of thawed cell for 5 min on ice. Cells were directly streaked out on selective

LB plates, and incubated overnight at 37°C. Candidate colonies were picked to inoculate a 3 ml LB Medium containing 50 µg/ml ampicillin for plasmid preparation (Miniprep), and incubated in a 37°C-shaker over night.

5.21 Preparation of Plasmid-DNA

Isolation of pure plasmid DNA for restriction analysis and sequencing was performed with the Nucleospin Miniprep Kit (Machery&Nagel). Plasmid preparations in a larger scale were performed with the Nucleobond AX 100 Midiprep Kit (Machery&Nagel).

6 Summary

Messenger RNA localization occurs in the cytoplasm and allows temporal and spatial regulation of gene expression. In yeast, the localization of *ASH1* mRNA to the tip of budding cells allows the asymmetric sorting of Ash1 protein, which has a key function in the regulation of mating-type switching. After cell division, asymmetric distribution of Ash1p restricts mating type switching to only the mother cell. The cytoplasmic transport of *ASH1* mRNA to the bud tip depends on the myosin Myo4p, its adaptor She3p, and the specific RNA binding protein She2p. Three additional *trans*-acting factors Khd1p, Puf6p and Loc1p are involved in this process. All known RNA-binding proteins of *ASH1* mRNA have revealed a nuclear connection, when following their cellular distribution by indirect immunofluorescence. Thus, an early step in the localization pathway might be the early recruitment of specific *trans*-acting factors to the mRNA already in the nucleus. The aim of this thesis was to investigate how nuclear key events such as early binding to localized transcripts and the subsequent assembly into a nuclear RNP can account for effective RNA localization. Following the route of She2p, it was possible to show nucleo-cytoplasmic shuttling of this RNA binding protein. Moreover, *ASH1* mRNA and She2p were found accumulated within the nucleolus upon arrest of mRNA export. Interestingly, two additional *trans*-acting factors, Loc1 and Puf6p, both involved in *ASH1* mRNA localization are also nucleolar proteins. Moreover, She2's nuclear history seems to be important for an effective sorting of Ash1p. When restricting *ASH1*-She2p association to the cytoplasmic compartment artificially, the *ASH1* mRNA was still localized but was prematurely translated during its transport. This suggests that nuclear RNP assembly has an influence on the later stages of cytoplasmic translational control. The nucleolus might represent the appropriate cellular compartment to provide the spatial framework for the assembly of localization competent RNPs since many RNPs are assembled in this region.

7 References

Ainger, K., Avossa, D., Diana, A.S., Barry, C., Barbarese, E. and Carson, J.H. (1997) Transport and localization elements in myelin basic protein mRNA. *J Cell Biol*, **138**, 1077-1087.

Alavian, C.N., Politz, J.C., Lewandowski, L.B., Powers, C.M. and Pederson, T. (2004) Nuclear export of signal recognition particle RNA in mammalian cells. *Biochem Biophys Res Commun*, **313**, 351-355.

Aris, J.P. and Blobel, G. (1988) Identification and characterization of a yeast nucleolar protein that is similar to a rat liver nucleolar protein. *J Cell Biol*, **107**, 17-31.

Arn, E.A., Cha, B.J., Theurkauf, W.E. and Macdonald, P.M. (2003) Recognition of a bicoid mRNA localization signal by a protein complex containing Swallow, Nod, and RNA binding proteins. *Dev Cell*, **4**, 41-51.

Aronov, S., Gelin-Licht, R., Zipor, G., Haim, L., Safran, E. and Gerst, J.E. (2007) mRNAs encoding polarity and exocytosis factors are cotransported with the cortical endoplasmic reticulum to the incipient bud in Saccharomyces cerevisiae. *Mol Cell Biol*, **27**, 3441-3455.

Barbarese, E., Koppel, D.E., Deutscher, M.P., Smith, C.L., Ainger, K., Morgan, F. and Carson, J.H. (1995) Protein translation components are colocalized in granules in oligodendrocytes. *J Cell Sci*, **108 (Pt 8)**, 2781-2790.

Beach, D.L. and Bloom, K. (2001) ASH1 mRNA localization in three acts. *Mol Biol Cell*, **12**, 2567-2577.

Beach, D.L., Salmon, E.D. and Bloom, K. (1999) Localization and anchoring of mRNA in budding yeast. *Curr Biol*, **9**, 569-578.

Berteaux-Lecellier, V., Zickler, D., Debuchy, R., Panvier-Adoutte, A., Thompson-Coffe, C. and Picard, M. (1998) A homologue of the yeast SHE4 gene is essential for the transition between the syncytial and cellular stages during sexual reproduction of the fungus Podospora anserina. *Embo J*, **17**, 1248-1258.

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S.M., Singer, R.H. and Long, R.M. (1998) Localization of ASH1 mRNA particles in living yeast. *Mol Cell*, **2**, 437-445.

Bhoite, L.T., Yu, Y. and Stillman, D.J. (2001) The Swi5 activator recruits the Mediator complex to the HO promoter without RNA polymerase II. *Genes Dev*, **15**, 2457-2469.

Bobola, N., Jansen, R.P., Shin, T.H. and Nasmyth, K. (1996) Asymmetric accumulation of Ash1p in postanaphase nuclei depends on a myosin and restricts yeast mating-type switching to mother cells. *Cell*, **84**, 699-709.

Böhl, F., Kruse, C., Frank, A., Ferring, D. and Jansen, R.P. (2000) She2p, a novel RNA-binding protein tethers ASH1 mRNA to the Myo4p myosin motor via She3p. *Embo J*, **19**, 5514-5524.

Boisvert, F.M., van Koningsbruggen, S., Navascues, J. and Lamond, A.I. (2007) The multifunctional nucleolus. *Nat Rev Mol Cell Biol*, **8**, 574-585.

Broadus, J., Fuerstenberg, S. and Doe, C.Q. (1998) Staufen-dependent localization of prospero mRNA contributes to neuroblast daughter-cell fate. *Nature*, **391**, 792-795.

Brodsky, A.S. and Silver, P.A. (2000) Pre-mRNA processing factors are required for nuclear export. *Rna*, **6**, 1737-1749.

Brown, J.D., Hann, B.C., Medzihradszky, K.F., Niwa, M., Burlingame, A.L. and Walter, P. (1994) Subunits of the Saccharomyces cerevisiae signal recognition particle required for its functional expression. *Embo J*, **13**, 4390-4400.

Bruhn, L. and Sprague, G.F., Jr. (1994) MCM1 point mutants deficient in expression of alpha-specific genes: residues important for interaction with alpha 1. *Mol Cell Biol*, **14**, 2534-2544.

Carmo-Fonseca, M., Mendes-Soares, L. and Campos, I. (2000) To be or not to be in the nucleolus. *Nat Cell Biol*, **2**, E107-112.

Catlett, N.L., Duex, J.E., Tang, F. and Weisman, L.S. (2000) Two distinct regions in a yeast myosin-V tail domain are required for the movement of different cargoes. *J Cell Biol*, **150**, 513-526.

Catlett, N.L. and Weisman, L.S. (1998) The terminal tail region of a yeast myosin-V mediates its attachment to vacuole membranes and sites of polarized growth. *Proc Natl Acad Sci U S A*, **95**, 14799-14804.

Chandarlapaty, S. and Errede, B. (1998) Ash1, a daughter cell-specific protein, is required for pseudohyphal growth of Saccharomyces cerevisiae. *Mol Cell Biol*, **18**, 2884-2891.

Chang, F. and Drubin, D.G. (1996) Cell division: why daughters cannot be like their mothers. *Curr Biol*, **6**, 651-654.

Chartrand, P., Meng, X.H., Huttelmaier, S., Donato, D. and Singer, R.H. (2002) Asymmetric sorting of ash1p in yeast results from inhibition of translation by localization elements in the mRNA. *Mol Cell*, **10**, 1319-1330.

Chartrand, P., Meng, X.H., Singer, R.H. and Long, R.M. (1999) Structural elements required for the localization of ASH1 mRNA and of a green fluorescent protein reporter particle in vivo. *Curr Biol*, **9**, 333-336.

Chen, D.C., Yang, B.C. and Kuo, T.T. (1992) One-step transformation of yeast in stationary phase. *Curr Genet*, **21**, 83-84.

Ciufo, L.F. and Brown, J.D. (2000) Nuclear export of yeast signal recognition particle lacking Srp54p by the Xpo1p/Crm1p NES-dependent pathway. *Curr Biol*, **10**, 1256-1264.

Condeelis, J. and Singer, R.H. (2005) How and why does beta-actin mRNA target? *Biol Cell*, **97**, 97-110.

Corral-Debrinski, M. (2007) mRNA specific subcellular localization represents a crucial step for fine-tuning of gene expression in mammalian cells. *Biochim Biophys Acta*, **1773**, 473-475.

Corral-Debrinski, M., Blugeon, C. and Jacq, C. (2000) In yeast, the 3' untranslated region or the presequence of ATM1 is required for the exclusive localization of its mRNA to the vicinity of mitochondria. *Mol Cell Biol*, **20**, 7881-7892.

Cosma, M.P., Tanaka, T. and Nasmyth, K. (1999) Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. *Cell*, **97**, 299-311.

Cross, F.R. and Tinkelenberg, A.H. (1991) A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. *Cell*, **65**, 875-883.

Darzacq, X., Powrie, E., Gu, W., Singer, R.H. and Zenklusen, D. (2003) RNA asymmetric distribution and daughter/mother differentiation in yeast. *Curr Opin Microbiol*, **6**, 614-620.

Denisenko, O. and Bomsztyk, K. (2002) Yeast hnRNP K-like genes are involved in regulation of the telomeric position effect and telomere length. *Mol Cell Biol*, **22**, 286-297.

Du, T.G., Schmid, M. and Jansen, R.P. (2007) Why cells move messages: the biological functions of mRNA localization. *Semin Cell Dev Biol*, **18**, 171-177.

Entian, K.D., Schuster, T., Hegemann, J.H., Becher, D., Feldmann, H., Guldener, U., Gotz, R., Hansen, M., Hollenberg, C.P., Jansen, G., Kramer, W., Klein, S., Kotter, P., Kricke, J., Launhardt, H., Mannhaupt, G., Maierl, A., Meyer, P., Mewes, W., Munder, T., Niedenthal, R.K., Ramezani Rad, M., Rohmer, A., Romer, A., Hinnen, A. and et al. (1999) Functional analysis of 150 deletion mutants in Saccharomyces cerevisiae by a systematic approach. *Mol Gen Genet*, **262**, 683-702.

Ephrussi, A. and St Johnston, D. (2004) Seeing is believing: the bicoid morphogen gradient matures. *Cell*, **116**, 143-152.

Epstein, H.F., Waterston, R.H. and Brenner, S. (1974) A mutant affecting the heavy chain of myosin in Caenorhabditis elegans. *J Mol Biol*, **90**, 291-300.

Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol*, **5**, 3610-3616.

Evangelista, M., Blundell, K., Longtine, M.S., Chow, C.J., Adames, N., Pringle, J.R., Peter, M. and Boone, C. (1997) Bni1p, a yeast formin linking cdc42p and the actin cytoskeleton during polarized morphogenesis. *Science*, **276**, 118-122.

Farina, K.L. and Singer, R.H. (2002) The nuclear connection in RNA transport and localization. *Trends Cell Biol*, **12**, 466-472.

Gerber, A.P., Herschlag, D. and Brown, P.O. (2004) Extensive association of functionally and cytotopically related mRNAs with Puf family RNA-binding proteins in yeast. *PLoS Biol*, **2**, E79.

Gerbi, S.A., Borovjagin, A.V. and Lange, T.S. (2003) The nucleolus: a site of ribonucleoprotein maturation. *Curr Opin Cell Biol*, **15**, 318-325.

Gietz, R.D. and Schiestl, R.H. (1991) Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast*, **7**, 253-263.

Gietz, R.D. and Sugino, A. (1988) New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene*, **74**, 527-534.

Giorgi, C. and Moore, M.J. (2007) The nuclear nurture and cytoplasmic nature of localized mRNPs. *Semin Cell Dev Biol*, **18**, 186-193.

Gonsalvez, G.B., Lehmann, K.A., Ho, D.K., Stanitsa, E.S., Williamson, J.R. and Long, R.M. (2003) RNA-protein interactions promote asymmetric sorting of the ASH1 mRNA ribonucleoprotein complex. *Rna*, **9**, 1383-1399.

Gonsalvez, G.B., Little, J.L. and Long, R.M. (2004) ASH1 mRNA anchoring requires reorganization of the Myo4p-She3p-She2p transport complex. *J Biol Chem*, **279**, 46286-46294.

Gonzalez, I., Buonomo, S.B., Nasmyth, K. and von Ahsen, U. (1999) ASH1 mRNA localization in yeast involves multiple secondary structural elements and Ash1 protein translation. *Curr Biol*, **9**, 337-340.

Govindan, B., Bowser, R. and Novick, P. (1995) The role of Myo2, a yeast class V myosin, in vesicular transport. *J Cell Biol*, **128**, 1055-1068.

Grosshans, H., Deinert, K., Hurt, E. and Simos, G. (2001) Biogenesis of the signal recognition particle (SRP) involves import of SRP proteins into the nucleolus, assembly with the SRP-RNA, and Xpo1p-mediated export. *J Cell Biol*, **153**, 745-762.

Grunstein, M. (1998) Yeast heterochromatin: regulation of its assembly and inheritance by histones. *Cell*, **93**, 325-328.

Gu, W., Deng, Y., Zenklusen, D. and Singer, R.H. (2004) A new yeast PUF family protein, Puf6p, represses ASH1 mRNA translation and is required for its localization. *Genes Dev*, **18**, 1452-1465.

Gunkel, N., Yano, T., Markussen, F.H., Olsen, L.C. and Ephrussi, A. (1998) Localization-dependent translation requires a functional interaction between the 5' and 3' ends of oskar mRNA. *Genes Dev*, **12**, 1652-1664.

Haarer, B.K., Petzold, A., Lillie, S.H. and Brown, S.S. (1994) Identification of MYO4, a second class V myosin gene in yeast. *J Cell Sci*, **107 (Pt 4)**, 1055-1064.

Haber, J.E. (1998) Mating-type gene switching in Saccharomyces cerevisiae. *Annu Rev Genet*, **32**, 561-599.

Hachet, O. and Ephrussi, A. (2001) Drosophila Y14 shuttles to the posterior of the oocyte and is required for oskar mRNA transport. *Curr Biol*, **11**, 1666-1674.

Hann, B.C. and Walter, P. (1991) The signal recognition particle in S. cerevisiae. *Cell*, **67**, 131-144.

Harnpicharnchai, P., Jakovljevic, J., Horsey, E., Miles, T., Roman, J., Rout, M., Meagher, D., Imai, B., Guo, Y., Brame, C.J., Shabanowitz, J., Hunt, D.F. and

Woolford, J.L., Jr. (2001) Composition and functional characterization of yeast 66S ribosome assembly intermediates. *Mol Cell*, **8**, 505-515.

Henras, A.K., Capeyrou, R., Henry, Y. and Caizergues-Ferrer, M. (2004) Cbf5p, the putative pseudouridine synthase of H/ACA-type snoRNPs, can form a complex with Gar1p and Nop10p in absence of Nhp2p and box H/ACA snoRNAs. *Rna*, **10**, 1704-1712.

Herskowitz, I. (1988) Life cycle of the budding yeast Saccharomyces cerevisiae. *Microbiol Rev*, **52**, 536-553.

Herskowitz, I. (1992) Fungal physiology. Yeast branches out. *Nature*, **357**, 190-191.

Hicks, J. and Strathern, J.N. (1977) Interconversion of mating type in S. cerevisiae and the Cassette model for gene transfer. *Brookhaven Symp Biol*, 233-242.

Hilleren, P., McCarthy, T., Rosbash, M., Parker, R. and Jensen, T.H. (2001) Quality control of mRNA 3'-end processing is linked to the nuclear exosome. *Nature*, **413**, 538-542.

Hilleren, P. and Parker, R. (2001) Defects in the mRNA export factors Rat7p, Gle1p, Mex67p, and Rat8p cause hyperadenylation during 3'-end formation of nascent transcripts. *Rna*, **7**, 753-764.

Hiscox, J.A. (2002) The nucleolus--a gateway to viral infection? *Arch Virol*, **147**, 1077-1089.

Hoek, K.S., Kidd, G.J., Carson, J.H. and Smith, R. (1998) hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. *Biochemistry*, **37**, 7021-7029.

Hughes, J.R., Bullock, S.L. and Ish-Horowicz, D. (2004) Inscuteable mRNA localization is dynein-dependent and regulates apicobasal polarity and spindle length in Drosophila neuroblasts. *Curr Biol*, **14**, 1950-1956.

Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S. and O'Shea, E.K. (2003) Global analysis of protein localization in budding yeast. *Nature*, **425**, 686-691.

Hurt, E.C., McDowall, A. and Schimmang, T. (1988) Nucleolar and nuclear envelope proteins of the yeast Saccharomyces cerevisiae. *Eur J Cell Biol*, **46**, 554-563.

Hüttelmaier, S., Zenklusen, D., Lederer, M., Dictenberg, J., Lorenz, M., Meng, X., Bassell, G.J., Condeelis, J. and Singer, R.H. (2005) Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. *Nature*, **438**, 512-515.

Irie, K., Tadauchi, T., Takizawa, P.A., Vale, R.D., Matsumoto, K. and Herskowitz, I. (2002) The Khd1 protein, which has three KH RNA-binding motifs, is required for proper localization of ASH1 mRNA in yeast. *Embo J*, **21**, 1158-1167.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M. and Sakaki, Y. (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc Natl Acad Sci U S A*, **98**, 4569-4574.

Izaurralde, E., Kutay, U., von Kobbe, C., Mattaj, I.W. and Gorlich, D. (1997) The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *Embo J*, **16**, 6535-6547.

Jacobson, M.R. and Pederson, T. (1998) Localization of signal recognition particle RNA in the nucleolus of mammalian cells. *Proc Natl Acad Sci U S A*, **95**, 7981-7986.

Jambhekar, A., McDermott, K., Sorber, K., Shepard, K.A., Vale, R.D., Takizawa, P.A. and DeRisi, J.L. (2005) Unbiased selection of localization elements reveals cis-acting determinants of mRNA bud localization in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **102**, 18005-18010.

Janke, C., Magiera, M.M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E. and Knop, M. (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast*, **21**, 947-962.

Jansen, R.P., Dowzer, C., Michaelis, C., Galova, M. and Nasmyth, K. (1996) Mother cell-specific HO expression in budding yeast depends on the unconventional myosin myo4p and other cytoplasmic proteins. *Cell*, **84**, 687-697.

Jensen, T.H., Patricio, K., McCarthy, T. and Rosbash, M. (2001) A block to mRNA nuclear export in S. cerevisiae leads to hyperadenylation of transcripts that accumulate at the site of transcription. *Mol Cell*, **7**, 887-898.

Johnston, G.C., Prendergast, J.A. and Singer, R.A. (1991) The Saccharomyces cerevisiae MYO2 gene encodes an essential myosin for vectorial transport of vesicles. *J Cell Biol*, **113**, 539-551.

Kadowaki, T., Hitomi, M., Chen, S. and Tartakoff, A.M. (1994) Nuclear mRNA accumulation causes nucleolar fragmentation in yeast mtr2 mutant. *Mol Biol Cell*, **5**, 1253-1263.

Kadowaki, T., Schneiter, R., Hitomi, M. and Tartakoff, A.M. (1995) Mutations in nucleolar proteins lead to nucleolar accumulation of polyA+ RNA in Saccharomyces cerevisiae. *Mol Biol Cell*, **6**, 1103-1110.

Karpova, T.S., Reck-Peterson, S.L., Elkind, N.B., Mooseker, M.S., Novick, P.J. and Cooper, J.A. (2000) Role of actin and Myo2p in polarized secretion and growth of Saccharomyces cerevisiae. *Mol Biol Cell*, **11**, 1727-1737.

Kiebler, M.A., Jansen, R.P., Dahm, R. and Macchi, P. (2005) A putative nuclear function for mammalian Staufen. *Trends Biochem Sci*, **30**, 228-231.

Kim, M., Canio, W., Kessler, S. and Sinha, N. (2001) Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato. *Science*, **293**, 287-289.

Kim, S.H., Ryabov, E.V., Brown, J.W. and Taliansky, M. (2004) Involvement of the nucleolus in plant virus systemic infection. *Biochem Soc Trans*, **32**, 557-560.

Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K. and Schiebel, E. (1999) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast*, **15**, 963-972.

Krebs, J.E., Kuo, M.H., Allis, C.D. and Peterson, C.L. (1999) Cell cycle-regulated histone acetylation required for expression of the yeast HO gene. *Genes Dev*, **13**, 1412-1421.

Kress, T.L., Yoon, Y.J. and Mowry, K.L. (2004) Nuclear RNP complex assembly initiates cytoplasmic RNA localization. *J Cell Biol*, **165**, 203-211.

Krichevsky, A.M. and Kosik, K.S. (2001) Neuronal RNA granules: a link between RNA localization and stimulation-dependent translation. *Neuron*, **32**, 683-696.

Kruse, C., Jaedicke, A., Beaudouin, J., Bohl, F., Ferring, D., Guttler, T., Ellenberg, J. and Jansen, R.P. (2002) Ribonucleoprotein-dependent localization of the yeast class V myosin Myo4p. *J Cell Biol*, **159**, 971-982.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Levadoux, M., Mahon, C., Beattie, J.H., Wallace, H.M. and Hesketh, J.E. (1999) Nuclear import of metallothionein requires its mRNA to be associated with the perinuclear cytoskeleton. *J Biol Chem*, **274**, 34961-34966.

Li, T., Stark, M.R., Johnson, A.D. and Wolberger, C. (1995) Crystal structure of the MATa1/MAT alpha 2 homeodomain heterodimer bound to DNA. *Science*, **270**, 262-269.

Libri, D., Dower, K., Boulay, J., Thomsen, R., Rosbash, M. and Jensen, T.H. (2002) Interactions between mRNA export commitment, 3'-end quality control, and nuclear degradation. *Mol Cell Biol*, **22**, 8254-8266.

Lillie, S.H. and Brown, S.S. (1994) Immunofluorescence localization of the unconventional myosin, Myo2p, and the putative kinesin-related protein, Smy1p, to the same regions of polarized growth in Saccharomyces cerevisiae. *J Cell Biol*, **125**, 825-842.

Long, R.M., Gu, W., Lorimer, E., Singer, R.H. and Chartrand, P. (2000) She2p is a novel RNA-binding protein that recruits the Myo4p-She3p complex to ASH1 mRNA. *Embo J*, **19**, 6592-6601.

Long, R.M., Gu, W., Meng, X., Gonsalvez, G., Singer, R.H. and Chartrand, P. (2001) An exclusively nuclear RNA-binding protein affects asymmetric localization of ASH1 mRNA and Ash1p in yeast. *J Cell Biol*, **153**, 307-318.

Long, R.M., Singer, R.H., Meng, X., Gonzalez, I., Nasmyth, K. and Jansen, R.P. (1997) Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA. *Science*, **277**, 383-387.

Macchi, P., Brownawell, A.M., Grunewald, B., DesGroseillers, L., Macara, I.G. and Kiebler, M.A. (2004) The brain-specific double-stranded RNA-binding protein Staufen2: nucleolar accumulation and isoform-specific exportin-5-dependent export. *J Biol Chem*, **279**, 31440-31444.

Machesky, L.M. and Gould, K.L. (1999) The Arp2/3 complex: a multifunctional actin organizer. *Curr Opin Cell Biol*, **11**, 117-121.

Mahon, P., Partridge, K., Beattie, J.H., Glover, L.A. and Hesketh, J.E. (1997) The 3' untranslated region plays a role in the targeting of metallothionein-I mRNA to the perinuclear cytoplasm and cytoskeletal-bound polysomes. *Biochim Biophys Acta*, **1358**, 153-162.

Mahowald, A.P. (2001) Assembly of the Drosophila germ plasm. *Int Rev Cytol*, **203**, 187-213.

Margeot, A., Blugeon, C., Sylvestre, J., Vialette, S., Jacq, C. and Corral-Debrinski, M. (2002) In Saccharomyces cerevisiae, ATP2 mRNA sorting to the vicinity of mitochondria is essential for respiratory function. *Embo J*, **21**, 6893-6904.

Mason, N., Ciufo, L.F. and Brown, J.D. (2000) Elongation arrest is a physiologically important function of signal recognition particle. *Embo J*, **19**, 4164-4174.

Maxon, M.E. and Herskowitz, I. (2001) Ash1p is a site-specific DNA-binding protein that actively represses transcription. *Proc Natl Acad Sci U S A*, **98**, 1495-1500.

Mayford, M., Bach, M.E. and Kandel, E. (1996) CaMKII function in the nervous system explored from a genetic perspective. *Cold Spring Harb Symp Quant Biol*, **61**, 219-224.

Miki, T. and Yoneda, Y. (2004) Alternative splicing of Staufen2 creates the nuclear export signal for CRM1 (Exportin 1). *J Biol Chem*, **279**, 47473-47479.

Mingle, L.A., Okuhama, N.N., Shi, J., Singer, R.H., Condeelis, J. and Liu, G. (2005) Localization of all seven messenger RNAs for the actin-polymerization nucleator Arp2/3 complex in the protrusions of fibroblasts. *J Cell Sci*, **118**, 2425-2433.

Mohr, S.E., Dillon, S.T. and Boswell, R.E. (2001) The RNA-binding protein Tsunagi interacts with Mago Nashi to establish polarity and localize oskar mRNA during Drosophila oogenesis. *Genes Dev*, **15**, 2886-2899.

Mowry, K.L. (1996) Complex formation between stage-specific oocyte factors and a Xenopus mRNA localization element. *Proc Natl Acad Sci U S A*, **93**, 14608-14613.

Mumberg, D., Muller, R. and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*, **156**, 119-122.

Münchow, S., Sauter, C. and Jansen, R.P. (1999) Association of the class V myosin Myo4p with a localised messenger RNA in budding yeast depends on She proteins. *J Cell Sci*, **112 (Pt 10)**, 1511-1518.

Munro, T.P., Magee, R.J., Kidd, G.J., Carson, J.H., Barbarese, E., Smith, L.M. and Smith, R. (1999) Mutational analysis of a heterogeneous nuclear ribonucleoprotein A2 response element for RNA trafficking. *J Biol Chem*, **274**, 34389-34395.

Nakamura, A., Sato, K. and Hanyu-Nakamura, K. (2004) Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. *Dev Cell*, **6**, 69-78.

Nasmyth, K. (1993) Regulating the HO endonuclease in yeast. *Curr Opin Genet Dev*, **3**, 286-294.

Neville, M. and Rosbash, M. (1999) The NES-Crm1p export pathway is not a major mRNA export route in Saccharomyces cerevisiae. *Embo J*, **18**, 3746-3756.

Niessing, D., Huttelmaier, S., Zenklusen, D., Singer, R.H. and Burley, S.K. (2004) She2p is a novel RNA binding protein with a basic helical hairpin motif. *Cell*, **119**, 491-502.

Nissan, T.A., Bassler, J., Petfalski, E., Tollervey, D. and Hurt, E. (2002) 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. *Embo J*, **21**, 5539-5547.

Norvell, A., Kelley, R.L., Wehr, K. and Schupbach, T. (1999) Specific isoforms of squid, a Drosophila hnRNP, perform distinct roles in Gurken localization during oogenesis. *Genes Dev*, **13**, 864-876.

Oleynikov, Y. and Singer, R.H. (2003) Real-time visualization of ZBP1 association with beta-actin mRNA during transcription and localization. *Curr Biol*, **13**, 199-207.

Olivier, C., Poirier, G., Gendron, P., Boisgontier, A., Major, F. and Chartrand, P. (2005) Identification of a conserved RNA motif essential for She2p recognition and mRNA localization to the yeast bud. *Mol Cell Biol*, **25**, 4752-4766.

Palacios, I.M., Gatfield, D., St Johnston, D. and Izaurralde, E. (2004) An eIF4AIII-containing complex required for mRNA localization and nonsense-mediated mRNA decay. *Nature*, **427**, 753-757.

Paquin, N., Menade, M., Poirier, G., Donato, D., Drouet, E. and Chartrand, P. (2007) Local activation of yeast ASH1 mRNA translation through phosphorylation of Khd1p by the casein kinase Yck1p. *Mol Cell*, **26**, 795-809.

Politz, J.C., Lewandowski, L.B. and Pederson, T. (2002) Signal recognition particle RNA localization within the nucleolus differs from the classical sites of ribosome synthesis. *J Cell Biol*, **159**, 411-418.

Politz, J.C., Yarovoi, S., Kilroy, S.M., Gowda, K., Zwieb, C. and Pederson, T. (2000) Signal recognition particle components in the nucleolus. *Proc Natl Acad Sci U S A*, **97**, 55-60.

Pope, B. and Kent, H.M. (1996) High efficiency 5 min transformation of Escherichia coli. *Nucleic Acids Res*, **24**, 536-537.

Pruyne, D., Evangelista, M., Yang, C., Bi, E., Zigmond, S., Bretscher, A. and Boone, C. (2002) Role of formins in actin assembly: nucleation and barbed-end association. *Science*, **297**, 612-615.

Pruyne, D.W., Schott, D.H. and Bretscher, A. (1998) Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. *J Cell Biol*, **143**, 1931-1945.

Reck-Peterson, S.L., Provance, D.W., Jr., Mooseker, M.S. and Mercer, J.A. (2000) Class V myosins. *Biochim Biophys Acta*, **1496**, 36-51.

Reed, R. (2003) Coupling transcription, splicing and mRNA export. *Curr Opin Cell Biol*, **15**, 326-331.

Rook, M.S., Lu, M. and Kosik, K.S. (2000) CaMKIIalpha 3' untranslated region-directed mRNA translocation in living neurons: visualization by GFP linkage. *J Neurosci*, **20**, 6385-6393.

Ross, A.F., Oleynikov, Y., Kislauskis, E.H., Taneja, K.L. and Singer, R.H. (1997) Characterization of a beta-actin mRNA zipcode-binding protein. *Mol Cell Biol*, **17**, 2158-2165.

Rossanese, O.W., Reinke, C.A., Bevis, B.J., Hammond, A.T., Sears, I.B., O'Connor, J. and Glick, B.S. (2001) A role for actin, Cdc1p, and Myo2p in the inheritance of late Golgi elements in Saccharomyces cerevisiae. *J Cell Biol*, **153**, 47-62.

Ruiz-Medrano, R., Xoconostle-Cazares, B. and Lucas, W.J. (1999) Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development*, **126**, 4405-4419.

Ruiz-Medrano, R., Xoconostle-Cazares, B. and Lucas, W.J. (2001) The phloem as a conduit for inter-organ communication. *Curr Opin Plant Biol*, **4**, 202-209.

Schmid, M., Jaedicke, A., Du, T.G. and Jansen, R.P. (2006) Coordination of endoplasmic reticulum and mRNA localization to the yeast bud. *Curr Biol*, **16**, 1538-1543.

Schneiter, R., Kadowaki, T. and Tartakoff, A.M. (1995) mRNA transport in yeast: time to reinvestigate the functions of the nucleolus. *Mol Biol Cell*, **6**, 357-370.

Schott, D., Ho, J., Pruyne, D. and Bretscher, A. (1999) The COOH-terminal domain of Myo2p, a yeast myosin V, has a direct role in secretory vesicle targeting. *J Cell Biol*, **147**, 791-808.

Schuldt, A.J., Adams, J.H., Davidson, C.M., Micklem, D.R., Haseloff, J., St Johnston, D. and Brand, A.H. (1998) Miranda mediates asymmetric protein and RNA localization in the developing nervous system. *Genes Dev*, **12**, 1847-1857.

Segref, A., Sharma, K., Doye, V., Hellwig, A., Huber, J., Luhrmann, R. and Hurt, E. (1997) Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. *Embo J*, **16**, 3256-3271.

Shen, C.P., Knoblich, J.A., Chan, Y.M., Jiang, M.M., Jan, L.Y. and Jan, Y.N. (1998) Miranda as a multidomain adapter linking apically localized Inscuteable and basally localized Staufen and Prospero during asymmetric cell division in Drosophila. *Genes Dev*, **12**, 1837-1846.

Shepard, K.A., Gerber, A.P., Jambhekar, A., Takizawa, P.A., Brown, P.O., Herschlag, D., DeRisi, J.L. and Vale, R.D. (2003) Widespread cytoplasmic mRNA transport in yeast: identification of 22 bud-localized transcripts using DNA microarray analysis. *Proc Natl Acad Sci U S A*, **100**, 11429-11434.

Shestakova, E.A., Wyckoff, J., Jones, J., Singer, R.H. and Condeelis, J. (1999) Correlation of beta-actin messenger RNA localization with metastatic potential in rat adenocarcinoma cell lines. *Cancer Res*, **59**, 1202-1205.

Shore, P. and Sharrocks, A.D. (1995) The MADS-box family of transcription factors. *Eur J Biochem*, **229**, 1-13.

Sil, A. and Herskowitz, I. (1996) Identification of asymmetrically localized determinant, Ash1p, required for lineage-specific transcription of the yeast HO gene. *Cell*, **84**, 711-722.

Sobel, S.G. and Wolin, S.L. (1999) Two yeast La motif-containing proteins are RNA-binding proteins that associate with polyribosomes. *Mol Biol Cell*, **10**, 3849-3862.

Sommerville, J., Brumwell, C.L., Politz, J.C. and Pederson, T. (2005) Signal recognition particle assembly in relation to the function of amplified nucleoli of Xenopus oocytes. *J Cell Sci*, **118**, 1299-1307.

St Johnston, D. (2005) Moving messages: the intracellular localization of mRNAs. *Nat Rev Mol Cell Biol*, **6**, 363-375.

Strasser, K. and Hurt, E. (2000) Yra1p, a conserved nuclear RNA-binding protein, interacts directly with Mex67p and is required for mRNA export. *Embo J*, **19**, 410-420.

Strasser, K. and Hurt, E. (2001) Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. *Nature*, **413**, 648-652.

Strathern, J.N., Klar, A.J., Hicks, J.B., Abraham, J.A., Ivy, J.M., Nasmyth, K.A. and McGill, C. (1982) Homothallic switching of yeast mating type cassettes is initiated by a double-stranded cut in the MAT locus. *Cell*, **31**, 183-192.

Sylvestre, J., Margeot, A., Jacq, C., Dujardin, G. and Corral-Debrinski, M. (2003) The role of the 3' untranslated region in mRNA sorting to the vicinity of mitochondria is conserved from yeast to human cells. *Mol Biol Cell*, **14**, 3848-3856.

Takizawa, P.A., DeRisi, J.L., Wilhelm, J.E. and Vale, R.D. (2000) Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin diffusion barrier. *Science*, **290**, 341-344.

Takizawa, P.A., Sil, A., Swedlow, J.R., Herskowitz, I. and Vale, R.D. (1997) Actin-dependent localization of an RNA encoding a cell-fate determinant in yeast. *Nature*, **389**, 90-93.

Takizawa, P.A. and Vale, R.D. (2000) The myosin motor, Myo4p, binds Ash1 mRNA via the adapter protein, She3p. *Proc Natl Acad Sci U S A*, **97**, 5273-5278.

Thomsen, R., Libri, D., Boulay, J., Rosbash, M. and Jensen, T.H. (2003) Localization of nuclear retained mRNAs in Saccharomyces cerevisiae. *Rna*, **9**, 1049-1057.

Titus, M.A. (1997) Motor proteins: myosin V--the multi-purpose transport motor. *Curr Biol*, **7**, R301-304.

Toi, H., Fujimura-Kamada, K., Irie, K., Takai, Y., Todo, S. and Tanaka, K. (2003) She4p/Dim1p interacts with the motor domain of unconventional myosins in the budding yeast, Saccharomyces cerevisiae. *Mol Biol Cell*, **14**, 2237-2249.

Tong, A.H., Evangelista, M., Parsons, A.B., Xu, H., Bader, G.D., Page, N., Robinson, M., Raghibizadeh, S., Hogue, C.W., Bussey, H., Andrews, B., Tyers, M. and Boone, C. (2001) Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science*, **294**, 2364-2368.

Urbinati, C.R., Gonsalvez, G.B., Aris, J.P. and Long, R.M. (2006) Loc1p is required for efficient assembly and nuclear export of the 60S ribosomal subunit. *Mol Genet Genomics*, **276**, 369-377.

van Hoof, A., Lennertz, P. and Parker, R. (2000) Yeast exosome mutants accumulate 3'-extended polyadenylated forms of U4 small nuclear RNA and small nucleolar RNAs. *Mol Cell Biol*, **20**, 441-452.

van Hoof, A. and Parker, R. (1999) The exosome: a proteasome for RNA? *Cell*, **99**, 347-350.

Veyrune, J.L., Campbell, G.P., Wiseman, J., Blanchard, J.M. and Hesketh, J.E. (1996) A localisation signal in the 3' untranslated region of c-myc mRNA targets c-myc mRNA and beta-globin reporter sequences to the perinuclear cytoplasm and cytoskeletal-bound polysomes. *J Cell Sci*, **109 (Pt 6)**, 1185-1194.

Wach, A., Brachat, A., Pohlmann, R. and Philippsen, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. *Yeast*, **10**, 1793-1808.

Wang, X., McLachlan, J., Zamore, P.D. and Hall, T.M. (2002) Modular recognition of RNA by a human pumilio-homology domain. *Cell*, **110**, 501-512.

Wendland, B., McCaffery, J.M., Xiao, Q. and Emr, S.D. (1996) A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. *J Cell Biol*, **135**, 1485-1500.

Wesche, S., Arnold, M. and Jansen, R.P. (2003) The UCS domain protein She4p binds to myosin motor domains and is essential for class I and class V myosin function. *Curr Biol*, **13**, 715-724.

Wilhelm, J.E., Hilton, M., Amos, Q. and Henzel, W.J. (2003) Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. *J Cell Biol*, **163**, 1197-1204.

Wilkie, G.S. and Davis, I. (2001) Drosophila wingless and pair-rule transcripts localize apically by dynein-mediated transport of RNA particles. *Cell*, **105**, 209-219.

Wolberger, C. (1998) Combinatorial transcription factors. *Curr Opin Genet Dev*, **8**, 552-559.

Xoconostle-Cazares, B., Xiang, Y., Ruiz-Medrano, R., Wang, H.L., Monzer, J., Yoo, B.C., McFarland, K.C., Franceschi, V.R. and Lucas, W.J. (1999) Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem. *Science*, **283**, 94-98.

Yano, T., Lopez de Quinto, S., Matsui, Y., Shevchenko, A., Shevchenko, A. and Ephrussi, A. (2004) Hrp48, a Drosophila hnRNPA/B homolog, binds and regulates translation of oskar mRNA. *Dev Cell*, **6**, 637-648.

Yao, W., Roser, D., Kohler, A., Bradatsch, B., Bassler, J. and Hurt, E. (2007) Nuclear export of ribosomal 60S subunits by the general mRNA export receptor Mex67-Mtr2. *Mol Cell*, **26**, 51-62.

Yin, H., Pruyne, D., Huffaker, T.C. and Bretscher, A. (2000) Myosin V orientates the mitotic spindle in yeast. *Nature*, **406**, 1013-1015.

Zhang, H.L., Eom, T., Oleynikov, Y., Shenoy, S.M., Liebelt, D.A., Dictenberg, J.B., Singer, R.H. and Bassell, G.J. (2001) Neurotrophin-induced transport of a beta-actin mRNP complex increases beta-actin levels and stimulates growth cone motility. *Neuron*, **31**, 261-275.

8 Abbreviations

aa	Amino acid
ab	antibody
α	anti
Amp	Ampicillin
ASH	asymmetric synthesis of HO
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
°C	Grade Celsius
CEN	Centromer
CIP	Calf intestine phospatase
C-terminal	Carboxy terminal
Da	Dalton
DAPI	diamidino-2-phenylindol dihydrochloride
DEPC	Diethylpyrocarbonate
DIC	Differential interference contrast
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	Desoxyribonuclease
dNTPs	Dideoxynucleotides
DTT	Dithiotreitol
E. coli	*Escherichia coli*
E1-3	*ASH1* mRNA localization element
ECL	Enhanced chemoluminiscence
EDTA	Ethylenediaminotetraaceticacid
eIF	Eukaryotic (translation-) initiation factor
ER	endoplasmatic reticulum
et al.	*et alii* (from Latin, “and others”)
Fig.	Figure
FISH	Fluorescence *in situ* hybridization
x g	Gravitational acceleration
g	Grams

GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
h	hour
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNP	Heterogeneous nuclear ribonucleoprotein
HO endonuclease	Homothallic switching endonuclease
Hsp	Heat shock protein
Ig	Immunoglobulin
kb	Kilo bases
l	Litre
LB-Medium	Liquid-Broth-Medium
LE	Localization element
M	Molar
mA	Milliampere
MDa	Megadalton
mg	Milligramm
µg	Microgramm
µl	Mikroliter
min	Minute
ml	Millilitre
mM	Millimolar
MOPS	3-(N-Morpholino)-propanesulfonic acid
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NMD	Nonsense-mediated decay
NP-40	Nonidet P-40 (Igepal-CA-630)
nt	Nucleotide
OD	Optical density
oligo	Oligonucleotide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Potential of Hydrogen
poly(A)+	Polyadenylic acid
pre-mRNA p	Precursor messenger ribonucleic acid
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic acid
s	Second
S	Svedberg unit
S. cerevisiae	Saccharomyces cerevisiae
SDS	Sodium dodecyl sulfate
SHE	Swi5p-dependent HO expression
snoRNA	Small nucleolar ribonucleic acid
TAE	Tris-acetate-EDTA buffer
TAP	Tandem affinity purification
TBS	Tris-buffered saline
TEMED	N, N, N’, N’-Tetramethylethylenediamine
Tris	Trishydroxymethylaminomethane
tRNA	Transfer ribonucleic acid
UTR	Untranslated region
UV	"beyond violet" (from Latin *ultra*, "beyond")
V	Volt
wt	Wild type
YEP	Yeast Extract Peptone
YNB	Yeast Nitrogen Base

9 Publications

Du, T.G., Schmid, M. and Jansen, R.P. (2007) Why cells move messages: the biological functions of mRNA localization. *Semin Cell Dev Biol*, **18**, 171-177.

Heuck, A., **Du, T.G.**, Jellbauer, S., Richter, K., Kruse, C., Jaklin, S., Müller, M., Buchner, J., Jansen R.P. and Niessing, D. (2007) Monomeric Myosin V Uses two Binding Regions for the Assembly of Stable *Proc Natl Acad Sci U S A. 2007*, *in press*

Schmid, M., Jaedicke, A., **Du, T.G**. and Jansen, R.P. (2006) Coordination of endoplasmic reticulum and mRNA localization to the yeast bud. *Curr Biol*, **16**, 1538-1543.

Schmitz-Linneweber, C., Regel, R., **Du, T.G**., Hupfer, H., Herrmann, R.G. and Maier, R.M. (2002) The plastid chromosome of Atropa belladonna and its comparison with that of Nicotiana tabacum: the role of RNA editing in generating divergence in the process of plant speciation. *Mol Biol Evol*, **19**, 1602-1612.

10 Acknowledgement

Mein besonderer Dank gilt natürlich Prof. Dr. Ralf-Peter Jansen, der mir die Möglichkeit gegeben hat, dieses spannende Thema in seiner Arbeitsgruppe zu verfolgen. Ich bedanke mich für die wissenschaftliche Freiheit, die ich unter seiner Obhut genossen habe, für die vielen Anregungen und Diskussionen, aber auch für die nötige Portion Humor, die insgesamt sehr zum Gelingen dieser Arbeit beigetragen haben.

Herrn Prof. Dr. Roland Beckmann danke ich sehr herzlich für die Erstellung des Zweitgutachtens.

Für die gute Unterhaltung war stets gesorgt, ohne die unser Laboralltag wohl einiges trister wäre. Ich danke Maria, Gonçalo, Stephan, Heidrun, Susi und Birgit für die angenehme Atmosphäre und für das miteinander Leiden und Freuen während der Doktorarbeit. Bei Maria bedanke ich mich für ihr offenes Ohr beim Kaffeekränzchen auf dem Sonnendeck. Gonzo ‚danke' ich für die unzähligen Ohrwürmer in der „outstation", von denen ich heute immer noch nicht alle losgeworden bin.

Viele wertvolle Tipps und geistige Anregungen von „zu Hause" habe ich von Magali erhalten. Ich danke ihr sehr für ihre Geduld und sorry, für die ständigen Verspätungen, weil die Experimente schlecht geplant waren!

Ich möchte mich auch noch bei Florian Fuchs bedanken, der mir in vielen Fragestellungen und mit seinen „bunnies" (for antibodies) weitergeholfen hat.

Witzige und hitzige Gespräche gab es auch immer beim ‚Klinikum lunch club'. Danke an Sabrina, Sharea und Christine.

LEBENSLAUF

NAME: Tung-Gia Du

GEBURTSTAG: 7. Januar 1974

GEBURTSORT: Saigon/Vietnam

NATIONAILTÄT: deutsch

FAMILIENSTAND: ledig

SCHULBILDUNG

1980-1983	Grundschule Tirschenreuth
1983-1984	Grundschule St. Josef (Dingolfing)
1984-1993	Gymnasium Dingolfing (Allgemeine Hochschulreife)

AKADEMISCHE AUSBILDUNG

1993-1995	Grundstudium Diplom Chemie an der LMU München
1995-2001	Lehramt an Gymnasien (Biologie/Chemie) an der LMU München
Juli 2001	Erste Staatsprüfung
2001-2003	Hauptstudium Diplom Biologie an der LMU München
	Diplomarbeit im Labor von PD Rainer Maier/Prof. R.-G. Herrmann: Genexpression in Plastiden Höherer Pflanzen: Entwicklung einer Strategie zur Analyse differentieller NEP/PEP-Promotoraktivitäten *in vivo*"
Januar 2003	Diplom in Biologie
Mai-Okt. 2003	Labor von Prof. B. Westermann: Biogenese und Vererbung von Mitochondrien in *S. cerevisiae*
Oktober 2003	Beginn der vorliegenden Dissertation im Labor von Prof. Dr. R.-P. Jansen am Institut für Biochemie, Genzentrum LMU München