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Biochemical purification and functional characterization of the She RNP complex from *S. cerevisiae*

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I declare that the work presented here has been performed independently and without auxiliary resources except for those indicated.

Munich, April 2004

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Andreas Jaedicke

Summary

Asymmetric mRNA localization is a widely used mechanism to sort cell fate determinants in development. In the budding yeast *Saccharomyces cerevisae ASH1* (for 'asymmetric synthesis of HO') mRNA localization to the tip of the growing bud leads to targeting of Ash1p to the daughter cell nucleus prior to cytokinesis and is a prerequisite for proper mating type switching. In a genetic screen 5 *SHE* (for 'Swi5p-dependent *HO* expression') genes have been isolated, coding for proteins required for the RNA localization process. *SHE1* is equivalent to *MYO4*, a locus that encodes a member of the class V unconventional myosins. The finding that a motor protein is required for *ASH1* mRNA targeting suggested a cytoskeleton-based, active transport mechanism. Functional characterization of the *SHE* genes in turn established a working model for the 'core She machinery' which implies She1p / Myo4p as the ATP-dependent motor protein, She2p as the *ASH1* mRNA-binding protein, and She3p as adapter protein connecting She2p to She1p / Myo4p. She4p has been suggested to function in myosin assembly, whereas She5p is required for cell cycle regulated remodeling of the actin cytoskeleton. In a shared project with C. Kruse we could show that Myo4p trafficking is regulated by the formation of a robust She RNP, and relies on She2p and RNA association. In addition to the She proteins, accessory factors such as Loc1p or Khd1p have been suggested to function in *ASH1* mRNA localization though they have not been identified in the genetic screen. Thus, in order to allow a detailed characterization of the *ASH1*-She RNP ('ribo-nucleoprotein') complex, I initiated a biochemical purification. In order to enrich for the She RNP two myo4p mutants were generated in the myosin ATPase, a domain required for Myo4p force generation. Localization studies reveal that the mutants do not transport *ASH1* mRNA anymore to the bud tip but instead accumulate in an intermediate, 'frozen' state in the cytoplasm. Affinity purification was carried out based on the TAP ('tandem affinity purification') protocol, using two alternative bait proteins (She2-TAP or Myo4-TAP). In either case I could purify the core She machinery together with *ASH1* mRNA. Further analysis of the She RNP by gel filtration experiments revealed a peak fraction with a molecular weight of approximately 4.5 MDa. Within this fraction I could identify Myo4p, She2p and *ASH1* mRNA, arguing for the integrity of a single RNP. In addition

to She1-3p mass-spectrometric analysis identified a number of so far unknown proteins, including the kinase Gin4p and the translation inhibitor Eap1p. Eap1p has been of outstanding interest since a systematic RNA localization assay with *ash1* mutants that contained premature stop codons inserted at various positions within the coding sequence have revealed severe localization defects, indicating that translation (and translational regulation) is required for correct localization. Eap1p has been characterized as an inhibitor of translation initiation and belongs to the family of 'eukaryotic initiation factor 4E-binding proteins', eIF 4E-BPs. Members of this protein family share a common sequence motif which mediates association with eIF 4E, thereby blocking initiation of translation. Initial studies with *Δ eap1* yeast strains have revealed a defective partial accumulation of Ash1p in mother cell nuclei, whereas *ASH1* mRNA localization to the bud tip and total Ash1p levels remained unaffected. This observation prompted me to introduce a new model for *ASH1* mRNA localization, including the regulation of translation initiation during cytoplasmic She RNP trafficking to the bud.

Zusammenfassung

Die spezifische Lokalisierung von mRNAs sowie eine sich daraus ableitende räumlich begrenzte Protein-Expression stellt einen häufig genutzten Mechanismus zur Generierung von Zell-Asymmetrie dar. In sich teilenden Zellen der Sproß-Hefe *S. cerevisiae* wird in einem aktiven Transportmechanismus entlang von Zytoskelett-Strukturen *ASH1* ('asymmetric synthesis of *HO*') mRNA an die Spitze der wachsenden Knospe lokalisiert. Die dadurch auf die Tochterzelle begrenzte Expression von Ash1p, eines Transkriptions-Repressors, ermöglicht die Akkumulation des Repressors ausschliesslich im Tochter-Zellkern und dadurch eine differenzielle Genexpression von Mutter- und Tochterzelle. Ein genetischer 'screen' für Faktoren, die zur Anreicherung von Ash1p im Tochter-Zellkern benötigt werden, identifizierte 5 She ('Swi5p-dependent *HO* expression') Proteine, She1-5. Es stellte sich heraus, daß She1p identisch zu Myo4p ist, einem Klasse V Myosin Motor-Protein. Desweiteren konnte gezeigt werden, dass She2p direkt an *ASH1* mRNA bindet, und durch She3p mit dem Myo4p Motor-Protein verknüpft wird. Die Analyse des *ASH1* Lokalisations Mechanismus ergab, dass die mRNA als Teil des She RNP ('Ribo-Nukleoprotein') Komplexes und in Assoziation mit She1-3 an die Tochterzell-Spitze transportiert wird. In einem gemeinsamem Forschungs-Projekt mit C. Kruse zur Untersuchung der Regulation dieses Myo4p-abhängigen Prozesses konnte gezeigt werden, dass der She RNP Transport in Richtung Tochterzell-Spitze von der Assoziation mit einer RNA als Fracht abängig ist. Berichte über die Entdeckung zusätzlicher Faktoren, die ähnlich wie die She Proteine am *ASH1* mRNA Lokalisations-Prozeß beteiligt sein sollen (Khd1p, Loc1p), ließen darauf schließen, daß in dem ursprünglichen genetischen 'screen' nicht sämtliche Faktoren isoliert werden konnten. Die Zielsetzung meiner Doktorarbeit war somit die biochemische Aufreinigung des She RNPs, sowie die Charakterisierung möglicher neuer, She-ähnlicher Faktoren. Zur Isolierung des She RNP entwickelte ich ein auf dem Prinzip der TAP ('tandem affinity purification') basierendes Versuchs-Protokoll. Mit Hilfe von myo4p Mutanten, die den Transport, nicht jedoch die Formation des RNPs blockieren, konnte ich das Ausgangsmaterial für die Aufreinigung anreichern. Die Analyse des isolierten She RNP ergab die Identifizierung der She Proteine She2p, She3p, sowie Myo4p und *ASH1*

mRNA. Zusätzlich zu den She Proteinen wurden einige bisher unbekannte Faktoren gefunden, darunter der Translations Repressor Eap1p und die Protein Kinase Gin4p. Mittels Gelfiltration wurde die Größe des She RNP auf etwa 4.5 MDa bestimmt. Die weiterführende Analyse der Säulenfraktionen ergab den Nachweis der She Proteine sowie der *ASH1* mRNA in einem einzigen 'peak', ein Hinweis, daß die gefundenen Faktoren Komponenten eines einzigen, intakten Komplexes sind. Da frühere Experimente gezeigt hatten, daß die Insertion eines Translations Stop Codons in den codierenden Bereich der *ASH1* mRNA zu einer Reduzierung der Lokalisierungs-Effizienz führt, folgerte ich, daß die Lokalisation translations-abhängig ist. Aus diesem Grund wählte ich Eap1p zur einführenden Charakterisierung bezüglich einer möglichen Funktion als *ASH1* Translations Repressor. In *Δ eap1* Mutanten konnte gezeigt werden, daß Ash1p partiell in Mutter-Zellkernen akkumuliert, wohingegen die Anreicherung der RNA an der Tochterzell-Spitze sowie die zelluläre Ash1p Gesamt-Proteinmenge von der Mutation nicht betroffen sind. In einem Modell verknüpfe ich den zytoplasmatischen She RNP Transport mit einer postulierten Rolle von Eap1p als *ASH1* Translations Repressor.

Contents

1. Introduction

A central question in developmental biology is to explain the ability of a mother cell to divide into two daughter cells with different developmental fates, an essential phenomenon for the generation of multi-cellular organisms. For this to occur, the sister cells need to express different genetic programs. Differences in gene expression can occur by the asymmetric segregation of cell fate determinants. mRNA localization and subsequent asymmetric cell division is one mechanism by which cell fate determinants can be sorted between sister cells (reviewed by Bashirullah *et al*., 1998; Palacios and St. Johnston, 2001). In this case one important prerequisite is the precise timing of protein synthesis, ensuring that the transcript to be sorted is not translated until specifically localized. Asymmetric mRNA localization in turn is followed by de-repression of a translation block and of local translation initiation. Thus an *intra*cellular positioning of a specific mRNA can be ultimately linked via translation control mechanisms to the generation of *inter*cellular protein diversity.

For instance, mRNA localization plays a key-role in early development of the fruit fly *Drosophila melanogaster* (P. Lawrence, 'The making of a fly'). During oogenesis the future body axes of the *Drosophila* embryo become predetermined by the cytoplasmic localization and local translation of specific maternally derived mRNAs within the oocyte (Riechmann and Ephrussi, 2001).

Another model organism in molecular biology is the budding yeast *Saccharomyces cerevisiae*. The work presented here focuses on the connection between yeast *ASH1* mRNA localization and translational control. To allow a detailed discussion of this phenomenon I start with an overview of general principles of mRNA localization and the regulation of protein synthesis.

1.1 Mechanisms of mRNA localization

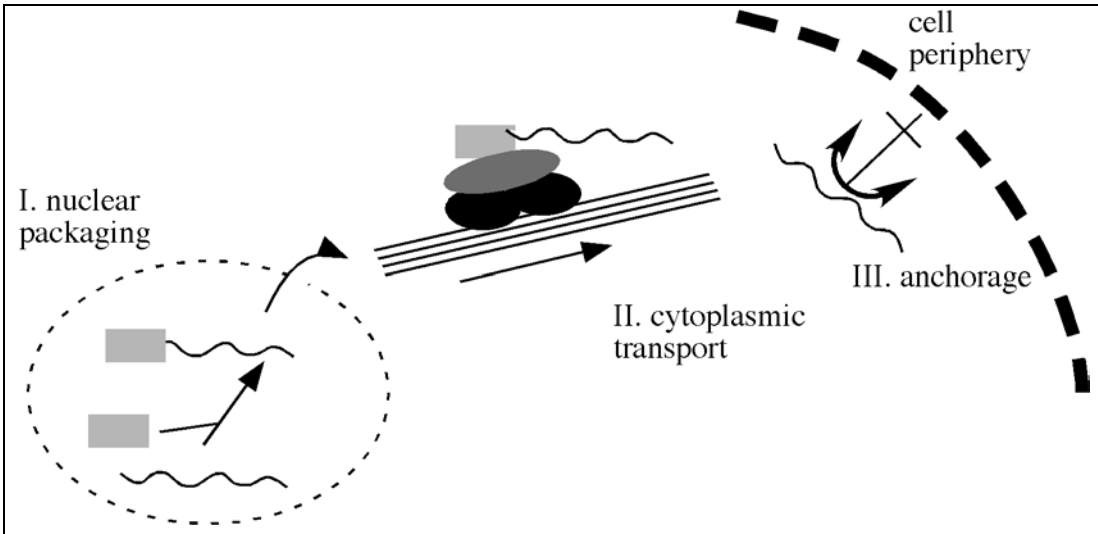
Intracellular mRNA localization is a widespread phenomenon among the animal kingdom, and has been described for more than 100 transcripts. Though the list is constantly growing, the localization mechanisms seem to be somewhat conserved, implying directional export of mRNA from the nucleus (Davis and Ish-Horowicz, 1991; Colon-Ramos *et al*., 2003), general degradation and selective stabilization at the site of localization (Bashirullah *et al*., 1999), diffusion coupled to specific retention (Glotzer *et al*, 1997; Forrest and Gavis, 2003) and motor-protein driven transport along cytoskeletal tracks (Yisraeli *et al*., 1990).

Highlighting only some examples, *hsp83* mRNA is specifically localized to the posterior pole of the *Drosophila* blastoderm embryo by degradation in all other parts and selective stabilization at the posterior (Bashirullah *et al*., 1999).

Most often mRNA localization depends on an intact cytoskeleton, suggesting structures for active transport or retention (reviewed by Jansen, 2001). For instance, *Vg1* mRNA targeting to the vegetal pole of *Xenopus laevis* oocytes depends on both actin and microtubules (Yisraeli *et al*., 1990), whereas *bicoid* mRNA localization to the anterior of the *Drosophila* oocyte is solely microtubule dependent (Pokrywka and Stephenson, 1991). Although the role of the cytoskeleton in anchoring has so far been only poorly characterized, several clear cases of transport along the cytoskeleton have emerged. It seems likely that active transport along the cytoskeleton represents the most common mechanism of mRNA localization (reviewed by Tekotte and Davis, 2002).

1.1.1 A 3-step working model for cytoplasmic mRNA localization by active transport

Early during transcription the pre-mRNA is processed and packed into an export competent ribo-nucleoprotein complex, RNP. Once released into the cytoplasm, the RNP is recognized by the transport machinery that mediates the delivery to a certain destination. Finally, having reached the cellular address, the mRNA becomes effectively retained and thereby concentrated against the cytoplasmic diffusion gradient.



Simplified 3-step-model for cytoplasmic mRNA localization by active transport (adapted from Wilhelm and Vale, 1993)

In the nucleus the RNA is processed and packed into an export-competent RNP. Upon export, the RNP is recognized by the cytoplasmic transport machinery, that delivers the RNA cargo to the site of destination (here the cell periphery). At the target membrane, an anchorage mechanism keeps the RNA from diffusion into the cytoplasm.

I. Nuclear RNP packaging

mRNA to be localized must be distinguished from bulk default cytoplasmic transcripts. The initial sorting event most likely takes place very early during the maturation process from a pre-mRNA to a translation-competent mRNA (Farina and Singer, 2002). During such maturation events the pre-mRNA becomes decorated with hnRNPs ('heterogeneous nuclear ribo-nucleoproteins'), forming an RNP whose composition is constantly changing. Some hnRNPs are restricted to the nucleus, whereas others escort the RNA during nuclear export and remain associated further on until correctly localized in the cytoplasm (reviewed by Jensen *et al*., 2003).

Thus, one function for hnRNPs is the deposition of a nuclear cue for RNAs destined to specific cytoplasmic locations. For instance, hnRNP A2 binds to 'myelin basic protein', *MBP* mRNA that is localized to the distal ends of dendrites in oligodendrocytes (Hoek *et al*., 1998).

5'-capping, splicing and 3'-end processing comprise a series of co-transcriptional maturation steps that render a transcript competent for export into the cytoplasm, where it potentially meets ribosomes and becomes translated into polypeptides. This processing of a pre-mRNA into a mature message results in the formation of an RNP that influences the fate of the mRNA (Daneholt, 2001). For example, the cap-binding complex binds to the 5' monomethylated cap structure, the exon-junction complex marks the (former) sites of splicing, and poly-(A) binding protein binds to the 3' tail.

Hence, similar to a quality-check in a man-made factory, having passed a cellular control checkpoint, the RNA becomes decorated with badges, monitoring the actual intermediate processing status. Falsely processed pre-mRNAs lack the corresponding batches. Those transcripts are blocked for nuclear export and become retained at the nuclear periphery (Galy *et al*., 2004), representing pivotal degradation substrates for the nuclear exosome (reviewed by Vasudevan and Peltz, 2003).

In order to assure an adequate hand-over of RNA intermediates, the cellular machinery that governs the nuclear RNA maturation events is not only functionally but most likely – at least temporarily - also physically linked. Several pieces of evidence meanwhile point into the direction that the main steps of RNA processing are carried out by large protein assemblies that are connected by a set of shuttling adapter proteins like Npl3p, Sub2p or Yra1p (reviewed by Reed and Hurt, 2002; Stutz and Izaurralde, 2003). The advantage is obvious: Coupling nuclear events ensures processivity, high turnover rates and accuracy, enabling cells to respond appropriately to changing environmental cues (reviewed by Maniatis and Reed, 2002).

Given that from their birth on, nascent precursor transcripts generated by RNA polymerase II in the nucleus are never 'naked' but initially become decorated with RNA-binding protein factors, the RNP composition is crucial for the determination of the RNA's cellular fate, including cytoplasmic mRNA localization.

II. Cytoplasmic transport events

Transcripts destined for specific sites within the cell are actively transported in form of large RNP granules along cytoplasmic routes. Upon nuclear export hnRNP's required for nuclear passage but primarily located in the nuclear compartment disassemble from the RNP and shuttle back. Strictly cytoplasmic factors in turn rally on and form a transport-competent cytoplasmic RNP. However, the mechanisms that govern loading and unloading of trans-acting factors are poorly understood.

Nevertheless, examples for strictly cytoplasmic components of an RNP transport complex are molecular motor proteins, physical linkers to the cytoskeletal surface supposed to form the track for the moving RNP. Similar to motors in automobiles, molecular motors generate their driving force through conversion of cellular fuel into mechanical energy. Repeated cycles of ATP hydrolysis within the motor protein's head domain allow movement with defined constant speed into a direction that is pre-determined by the orientation of the chosen track (Pruyne *et al*., 1998; Bertrand *et al*., 1998; Schott *et al*., 2002; Januschke *et al*., 2002). Concomitantly, mutants that disrupt the cytoskeletal organization also block mRNA localization. Thus, before the directed transport of the RNP can occur the cytoskeleton has to be coordinately rearranged and polarized.

Whether the cargo mRNA is translated during transport, or not before it has reached its destination is a matter of discussion, and probably both scenarios are exerted. During early *Drosophila* development *nanos* mRNA is translated into protein only upon anchorage at the posterior pole of the oocyte (Gavis and Lehmann, 1994). In yeast it has been suggested that *ASH1* mRNA transport and translation *ASH1* mRNA can occur simultaneously (Chartrand *et al*., 2002). This issue will be discussed in more detail below.

Taken together the general coordination of nuclear with cytoplasmic events in combination with the ability to rapidly answer to changing extra-cellular cues is crucial in order to assure correct targeting of RNPs.

III. Molecular anchorage

In order to facilitate the concentration of a specific transcript against the cytoplasmic diffusion gradient, it is generally assumed that an anchorage mechanism exists at the target site. However, the mechanisms that govern the retention of specific transcripts at a certain site within a cell are only poorly described. Once the RNP has reached the destination point it has to be ensured that the cargo mRNA is accurately handed over from the transport complex to the postulated molecular anchor, where it is effectively retained. After delivery, parts of the transport complex can be recycled back to the nuclear envelope, whereas others remain associated with the RNP.

During the docking step – maybe with the help of accessory factors like RNA helicases - the mRNA is thought to become unraveled from its tightly packed into a more accessible form, facilitating ribosome recruitment and the onset of protein synthesis. This mechanism is referred to as the idea of 'local translation', directly coupling mRNA localization with protein synthesis. It is likely that the translation process *per se* contributes to the anchorage of a localized mRNA. In yeast, anchorage of *ASH1* mRNA has been reported to require translation (Gonzalez *et al*., 1999). Similarly, in fruit flies *oskar* mRNA localization depends on local synthesis of Oskar protein (Rongo *et al*., 1995).

In addition to polyribosome-mediated retention, mRNAs can be anchored by other RNAs through complementary base-pairing or by specific RNA-binding proteins. In *Xenopus laevis* oocytes it has been shown that *Xlsirts* RNA, short non-protein-coding RNAs, are required for *Vg1* mRNA anchorage at the vegetal cortex (Kloc and Etkin, 1994). In *Drosophila* oocytes the double-stranded RNA-binding protein Staufen anchors *oskar* mRNA to the posterior (Ephrussi *et al*., 1991; St Johnston *et al*., 1992; Rongo *et al*., 1995) and *bicoid* mRNA to the anterior pole (St Johnston *et al*., 1992).

As mentioned earlier, for some transcripts the cytoskeleton has been imposed not only to take part in the transport-, but also in the anchorage process. Probably the most illustrating example is again the localization procedure of *Vg1* mRNA. While the cytoplasmic transport process requires microtubules, the cortical anchorage seems to depend on a meshwork of actin filaments and cytokeratin (Yisraeli *et al*., 1990; Alarcon and Elinson, 2001).

1.1.2 Cis- and trans-acting requirements for mRNA localization by active transport along the cytoskeleton

Directed mRNA transport within the cell cytoplasm is generally mediated through the interaction of trans-acting factors, the localization machinery, with one or several cis-acting

localization element(s), or "ZIPcodes", present within the mRNA sequence (Kislauskis and Singer, 1992). In most cases the localization elements are located within the 3'-untranslated region, 3'-UTR, of the mRNA (for review see Bashirullah *et al*., 1998). Most ZIPcodes comprise regions with extended secondary structure elements like stems, bulges, loops and hairpins, suggesting that it is rather the secondary structure than the primary sequence that confers RNA-protein specificity. The primary assembly of RNA-binding proteins and ZIPcodes is loaded onto the motor protein with the help of adapter proteins. Thus, the core localization machinery is thought to comprise three proteins, namely the RNA-binding-, the adapter- and the motor-protein.

In *Drosophila* it has been suggested that *bicoid* mRNA is linked to cytoplasmic dynein via the ZIPcode-binding protein Swallow, and dynein light chain as adapter protein (Schnörrer *et al*., 2000).

1.1.3 Tracks and motors

Cytoplasmic mRNA transport has been observed in a range of organisms, but traveling distances vary from extremely long in oocytes and neurons to relatively short in fibroblasts. Since the cytoskeletal requirements for mRNA localization can be easily analyzed using drugs that either specifically de-polymerize microfilaments or microtubules, it has been suggested that long-range transport depends on microtubules whereas shorter distances can be mediated by microfilaments (Nasmyth and Jansen, 1997).

For example, in oligodendrocytes the localization of *MBP* mRNA relies on an intact microtubule cytoskeleton (Carson *et al*., 1997), whereas targeting of *β-actin* mRNA to the leading edge in chicken fibroblasts requires microfilaments (Sundell and Singer, 1991). However, the choice for the track ultimately implies a decision for the kind of molecular motor, namely cytoplasmic dynein or kinesin in the case of microtubules (depending on the desired travelling direction), or an unconventional myosin for actin-based transport. In oligodendrocytes it was shown that localization of *MBP* mRNA depends on kinesin, a plus-end-directed microtubule-associated motor protein (Carson *et al*., 1997). Similarly, Kinesin 1 is required for *oskar* mRNA localization at the posterior pole of *Drosophila* oocytes (Brendza *et al*., 2000), whereas *bicoid* mRNA transport to the anterior pole is driven by cytoplasmic dynein (Schnörrer *et al*., 2000).

1.1.4 Cargo-binding to the motor involves distinct adapter proteins

RNA is only one of several goods that need to be distributed by active transport. In order to enable a motor protein to carry a set of different loads, cargo differentiation on the molecular level is specified by adapter proteins (reviewed by Bretscher, 2003).

For example in the yeast *S. cerevisiae*, Myo2p has been implicated in numerous bud-directed targeting processes, including organelle inheritance, vesicle transport (Govindan *et al*., 1995), and the orientation of the mitotic spindle (Yin *et al*., 2000). Thus cargo differentiation is crucial in order to sort each load accordingly. For Myo2p this goal has been accomplished by using a unique adapter for each kind of cargo, which in turn becomes tethered to separable cargo-binding regions within the Myo2p globular tail. Vacuole sorting involves the adapter proteins Vac17p / Vac8p (Catlett *et al*., 2000; Tang *et al*., 2003; Ishikawa *et al*., 2003), transport of secretory vesicles Sec4p (Pruyne *et al*., 1998; Schott *et al*., 1999), and segregation of mitochondria Ypt11p (Itoh *et al*., 2002). Sec4p and Ypt11p are organelle-specific Rab proteins with intrinsic GTPase activity that are located on the cargo membrane. Rab proteins have already earlier been shown to be implicated in endocytic trafficking pathways, and therefore match perfectly as organelle specific adapter proteins for active transport of membranous cargo (Munro, 2002). Another example for an adapter protein is She3p that links the *ASH1* mRNA-She2p assembly to the Myo4p motor protein, discussed in more detail in chapter 1.4.3.2.

1.1.5 Regulation of motor based movement

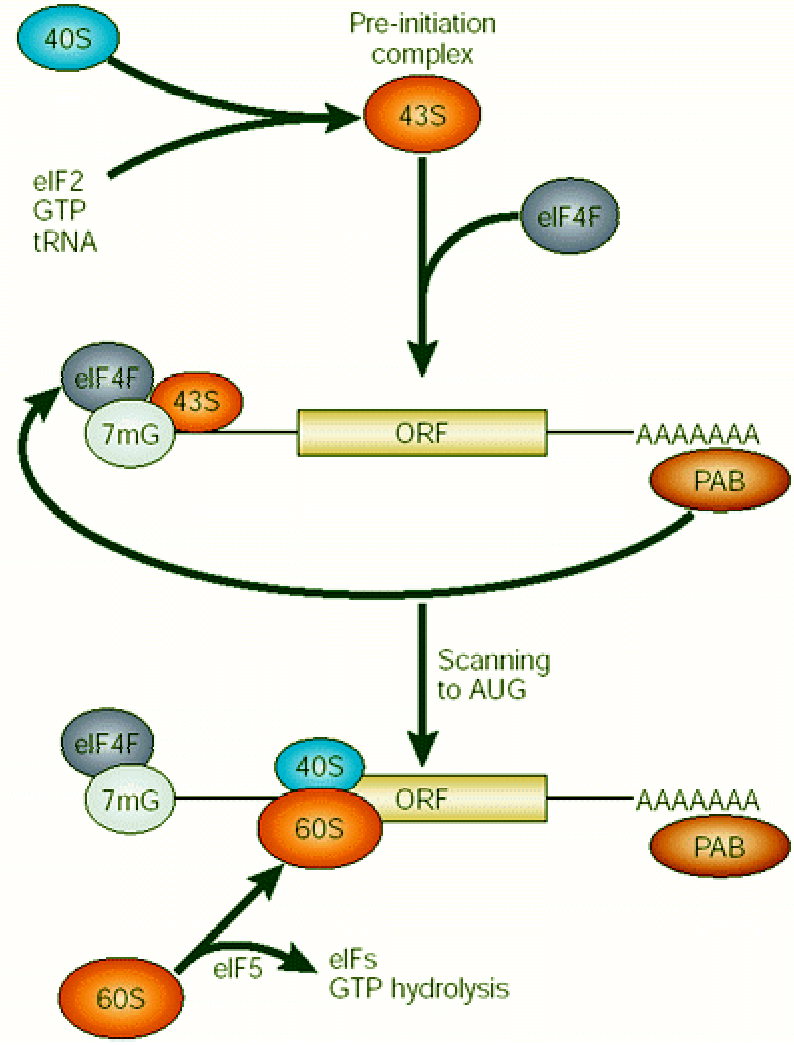
It is assumed that the activity of a given motor is regulated by the interaction with the cargo and cell-cycle-dependent post-translational modifications (reviewed by Karcher *et al*., 2002). For example in *Xenopus* melanophores, Myo5a mediated melanosome transport to the cell-periphery during interphase is ceased before mitosis (Rogers *et al*., 1999). Cell-cycle regulated phosphorylation of a conserved Ser residue causes dissociation of the motor from the cargo organelle (Karcher *et al*., 2001). The diffusive intracellular distribution of melanosomes is thought to ensure an adequate inheritance to the progeny cells during mitosis. In yeast, cell cycle dependent degradation of the vacuole adapter Vac17p is required for the release of the organelle from Myo2p upon bud delivery (Tang *et al*., 2003). Similarly transport of membranous cargo by microtubule-based kinesin motors has been shown to be regulated by cargo binding (Friedman and Vale, 1999).

1.2 A connection between mRNA localization and translation in development

During early development of multi-cellular organisms gene expression is controlled mainly on the translational level. For example, during *Xenopus laevis* oocyte maturation and early embryogenesis transcription is completely shut down, while proteins are synthesized from maternally derived mRNAs. The precise translational onset of such mRNAs, some of them encoding developmental key players, is a matter of elaborate molecular control mechanisms that become initialized by the hormonal trigger progesterone (reviewed by Mendez and Richter, 2001).

1.2.1 Translation initiation

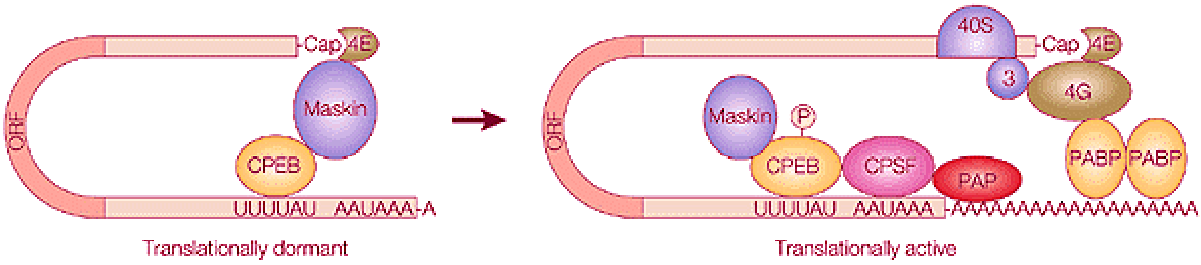
Generally speaking, translational control most often occurs during protein synthesis initiation, when binding of the 40S ribosomal subunit to the mRNA cap-complex is rate limiting.



Eukaryotic translation initiation (Kuersten and Goodwin, 2003)
In a simplified view of eukaryotic translation initiation, the 40S ribosomal subunit assembles with the ternary complex, composed of the eukaryotic initiation factor 2 (eIF2) bound to GTP and the initiator methionyl tRNA, to form the 43S pre-initiation complex. The pre-initiation complex in turn docks to the cap-binding complex eIF4F, composed of the RNA helicase eIF4A, the cap-binding protein eIF4E and the 40S subunit-binding scaffold protein eIF4G. Poly-(A)-binding protein (PAB) bound to the poly(A) tail facilitates translation initiation by communicating with the 5' end of the mRNA through its interaction with eIF4G, effectively joining the 5' and 3' ends and circularizing the transcript (Tarun and Sachs, 1996; Preiss and Hentze, 1998). The 43S complex in turn scans the mRNA until the AUG codon is recognized by the anticodon of the initiator tRNA. This triggers eIF5 to hydrolyze GTP bound to eIF2, the eIF's dissociate and the 60S ribosomal subunit joins, resulting in a fully functional ribosome that is competent for elongation.

1.2.2 Mechanisms of translational control by eIF 4E-binding proteins

Usually the 3'-UTR contains several regulatory elements that govern the spatial and temporal expression of an mRNA (reviewed by Kuersten and Goodwin, 2003).
During *Xenopus laevis* oogenesis, for example, dormant cytoplasmic mRNAs contain short poly-(A) tails, and it is only when these tails are elongated that translation can occur. Polyadenylation requires two elements located within the 3'-UTR, namely the hexanucleotide AAUAAA and the 'cytoplasmic polyadenylation element', CPE (Mendez and Richter, 2001). In the repressed state the CPE is bound by the 'CPE-binding protein', CPEB (Hake and Richter, 1994). During progesterone stimulated oocyte maturation CPEB becomes phosphorylated (Mendez *et al*., 2000a,b), an event that increases the affinity for the 'cleavage and polyadenylation specificity factor', CPSF (Dickson *et al*., 1999). In turn, CPSF binds to the AAUAAA sequence near the CPE and recruits poly-(A) polymerase to the mRNA. Because CPE-containing mRNAs are inactive in oocytes, it is likely that CPEB is involved in translational repression (masking) as well as polyadenylation, depending on the phosphorylation status. However, the masking function of CPEB has been shown to be only indirect, since another inhibitory protein has been found, maskin, that simultaneously interacts with CPEB and eIF4E (Stebbins-Boaz *et al*., 1999). Maskin thus can be counted to the family of 'eIF4E-binding proteins', eIF4E-BPs, with members in a wide range of organisms (reviewed by Gingras *et al*., 2000), including *Drosophila* and *S. cerevisiae* (see part 5.4.3.3). The interaction between maskin and eIF4E is mediated by a binding motif present in all eIF4E-BPs as well as eIF4G. Because of this motif, eIF4E-BPs and eIF4G compete for binding to the same region of eIF4E (Mader *et al*., 1995). Consequently a competition between maskin and eIF4G for occupancy of eIF4E determines the state of translation; when maskin is bound to eIF4E translation initiation is repressed (Cao and Richter, 2002).



Translational repression by maskin, an eIF4E binding protein (from Mendez and Richter, 2001). CPE-containing mRNAs are translationally dormant and reside in a complex with CPEB, maskin and eIF4E. Upon egg maturation CPEB becomes phosphorylated, followed by recruitment of CPSF and poly-(A) polymerase (PAP). Poly-(A) tail elongation in turn coincides with maskin dissociation from eIF4E, a prerequisite for joining of eIF4G, circularization and translation initiation to occur.

Translational repression by eIF4E-BPs is a widely used mechanism that allows the timing of protein synthesis. However, other important mechanisms of translation regulation are repression by deadenylation (Wreden *et al*., 1997) or occur at the ribosomal 60S subunit joining step (Ostareck *et al*., 2001).

1.3 Example: *Drosophila oskar* mRNA localization and translation are linked

During early development of the fruit fly *Drosophila melanogaster* the mechanisms of translational control and RNA localization co-operate in the spatial distribution of specific proteins essential for embryonic axis determination (reviewed by Johnstone and Lasko, 2001; Riechmann and Ephrussi, 2001). As an example, I have chosen *oskar* mRNA localization to illustrate the high complexity but also similarity to a central issue of this work, namely the cooperation between processes of mRNA localization and translation in yeast.

oskar mRNA is produced in the nurse cells adjacent to the egg chamber, transported in a translationally repressed state into the oocyte, and subsequently delivered to the posterior pole. Only there localized *oskar* mRNA is translationally activated, both *oskar* RNA and protein are anchored (Vanzo and Ephrussi, 2002), and Oskar protein stabilized (Riechmann *et al*., 2002). It has been suggested that the polarized microtubule network between the oocyte and the posterior follicle cells mediates transport of the mRNA (Brendza *et al*., 2000). *oskar* mRNA transport depends on cis-acting elements in its 3'-UTR (Kim-Ha *et al*.,1993) , on the RNA-binding protein Staufen (St Johnston *et al*., 1991), on components of the exon-junction complex, such as Y14 (Hachet and Ephrussi, 2001) and Mago nashi (Micklem *et al*., 1997), and on Barentsz, a protein enriched around the nurse cell nuclei and at the posterior pole (van Eeden *et al*., 2001). Sequence elements in the *oskar* 3'-UTR are required for translational repression of the mRNA during transport, and specific trans-acting factors such as Bruno and Cup mediate this process (Kim-Ha *et al*.,1995; Webster *et al*., 1997; Wilhelm *et al*., 2003; Nakamura *et al*., 2004). Upon localization, translational silencing is de-repressed. It has been shown that derepression requires cis-acting elements in the *oskar* 5'-UTR (Gunkel *et al*., 1998), Staufen protein (Micklem *et al*., 2000) and cAMP-dependent protein kinase R1 (Yoshida *et al*., 2003). In addition, *oskar* mRNA is subject to cytoplasmic polyadenylation, which is mediated by Orb, the *Drosophila* CPEB homolog (Christerson and McKearin, 1994; Chang *et al*., 1999).

In summary, *oskar* translational regulation involves a mixture of repressive and activating control mechanisms. The decisive switch between the translational off and on status seems to be triggered by the anchorage of the mRNA to the posterior pole of the oocyte.

1.4 *ASH1* mRNA localization in *S. cerevisiae*

1.4.1 Mating-type switching and the connection to mRNA localization

The budding yeast *S. cerevisiae* alternates between a diploid growing form and, under conditions of nutrient deprivation, a haploid growing state. The return to a diploid form is accomplished through the mating of two haploid cells of the opposite mating-type (a or α). Mother cells are capable of switching mating-type whereas daughter cells are not, ensuring that an isolated haploid spore will be able to form diploid cells through mating between its progeny. Mating-type switching is regulated by HO endonuclease (Nasmyth, 1993), which is expressed in mother cells but not in daughter cells. HO initiates a genomic rearrangement of the *MAT* locus, resulting in the conversion of an 'a' cell to an 'α" or vice-versa. In daughter cells the expression of *HO* is repressed by Ash1p ('asymmetric synthesis of HO'), which is asymmetrically distributed to daughter cell nuclei (Bobola *et al*., 1996; Sil and Herskowitz, 1996; Cosma *et al*., 1999; Maxon and Herskowitz, 2001). The asymmetric sorting of Ash1p to the daughter cell nucleus correlates with the localization of *ASH1* mRNA to the distal tip of daughter cells during anaphase of the cell cycle (Long *et al*., 1997; Takizawa *et al*., 1997). Taken together, Ash1p serves as a model system for studying the asymmetric segregation of cell-fate determinants through mRNA localization (reviewed by Darzacq *et al*., 2003).

1.4.2 Original *SHE*-screen

Five *SHE* genes ('Swi5p-dependent *HO* expression') were originally discovered in a screen for factors that are required for *HO* expression in mother cells (Jansen *et al*., 1996). Accordingly it could be shown that the *SHE* genes are necessary to restrict Ash1p to daughter cell nuclei (Bobola *et al*., 1996). The coincidence that the most often found open reading frame in the *SHE* screen, designated *SHE1*, encodes an unconventional non-muscle class V myosin, Myo4p (Jansen *et al*., 1996), and that asymmetric Ash1p distribution to daughter nuclei is achieved by *SHE*-dependent localization of *ASH1* mRNA to the tip of growing daughter cells (Long *et al*., 1997; Takizawa *et al*., 1997) raised the idea that the She proteins mediate this mRNA localization process.

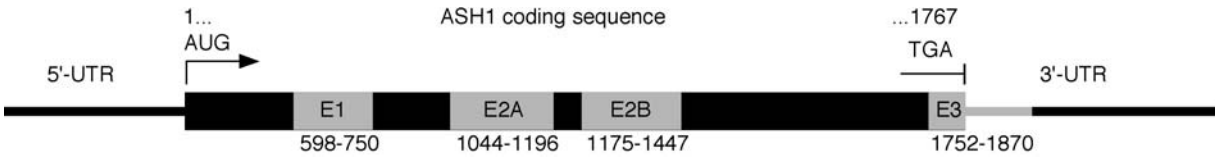
A few years later the function of each of the *SHE*-encoded factors could be unraveled, and today the cellular mechanism of how *ASH1* mRNA is actively transported to the yeast bud tip is regarded as a bonafide example for mRNA localization in students' textbooks.

Before the function of each She protein will be summarized in detail, I start with the cargo, namely *ASH1* mRNA.

1.4.3 The She-machinery

1.4.3.1 The cargo

I. *ASH1* mRNA



***ASH1* mRNA localization elements.**
Numbers indicate the position of the ZIPcodes relative to the *ASH1* open reading frame.

Four *ASH1* localization elements have been mapped as being essential for tight anchorage at the bud tip cortex, namely E1, E2a, E2b and E3 (Chartrand *et al*., 1999; Chartrand *et al*., 2002) or elements U1, U2 and U3 (Gonzalez *et al*., 1999). Of the elements, three (E1, E2A and E2B) are located within the coding region, while the remaining element (E3) spans from the end of the open reading frame into the 3'-UTR. RNA secondary structure prediction suggests that all *ASH1* mRNA ZIPcodes form extensive stem-loops and bulges (Chartrand *et al*., 1999; Gonzalez *et al*., 1999; Chartrand *et al*., 2002). Their disruption destroys the ability of these elements to direct RNA localization. Moreover, the integrity of the stem-loop structure of the element E3 is essential for the binding of She2p (Böhl *et al*., 2000; Long *et al*., 2000). Initial work has shown that only one element alone is sufficient to target a reporter RNA into the bud (Long *et al*., 1997; Takizawa *et al*., 1997; Bertrand *et al*., 1998; Beach *et al*., 1998), but all four elements are required for tight anchorage (Chartrand *et al*., 2002). In addition to serving as binding sites for trans-acting factors such as She2p and Khd1p (see below), the secondary structure elements within the *ASH1* ZIPcodes seem to act as molecular obstacles, slowing down the protein synthesis rate (Chartrand *et al*., 2002). It has been proposed that this molecular translation retardation mechanism contributes to the establishment of Ash1p asymmetry in yeast.

ASH1 is transcribed in anaphase (Bobola *et al*., 1996; Spellman *et al*., 1998), Besides recruitment to the *HO* promoter Ash1p also interacts with the promoters of *SGA1* and *PCL1*, two loci implicated in sporulation control and cell cycle control, respectively (Lee *et al*., 2002). Interestingly, Pcl1p is one of the cyclins associated with the Pho85 kinase complex, which has been demonstrated to phosphorylate Ash1p, thereby regulating its stability (McBride *et al*., 2001). In a *Δ pho85* strain, Ash1p is stabilized so that its activity persists in

the daughter cell nucleus during the cell cycle. This aberrantly represses mating type switching when the cell enters the next bud cycle. Besides its role in mating-type switching Ash1p asymmetry might also be crucial for the switch from vegetative to pseudohyphal growth phase (Chandarlapaty and Errede, 1998).

II. Other localized mRNAs in yeast

In yeast *ASH1* mRNA was the first example for a transcript that is specifically localized. Using biochemical precipitation and DNA microarray analysis, a second mRNA to be localized by the She machinery joined the field, *IST2* (Takizawa *et al*., 2000), coding for a putative ion transporter (Entian *et al*., 1999). The septin ring located at the mother-bud junction has been implicated to function as a diffusion barrier for membrane proteins such as Ist2p and thereby compartmentalize the yeast plasma membrane. Based on the work of Takizawa and co-workers (2000) a set of 22 mRNAs has been discovered, all localized in a *SHE*-dependent manner to the tip of growing buds (Shepard *et al*., 2003), speaking for a widely used transport mechanism in *S. cerevisiae.* However, the biological relevance remains to be uncovered since only in the case of *ASH1*, transcript asymmetry clearly correlates with protein asymmetry. In addition, and together with earlier results (Long *et al*., 1997), this finding suggested that the She machinery-mediated mRNA transport can take place independently of the stage of the cell cycle.

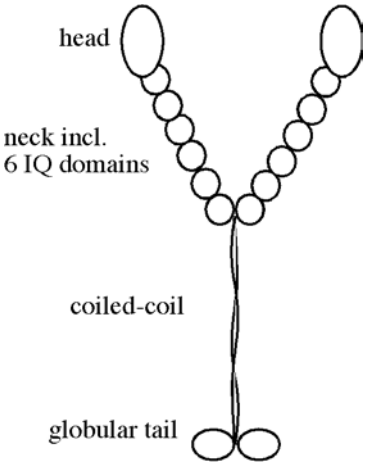
However, besides this essential function of the She machinery in cytoplasmic mRNA localization there is at least one example for *SHE*-independent mRNA targeting in yeast. The sorting of mRNAs encoding a subset of mitochondrial proteins to polysomes located directly at the organelle surface has been shown to be unaffected in *Δ she* mutants (Corral-Debrinski *et al*., 2000; Marc *et al*., 2002 and A. Margeot, personal communication). The cellular mechanism for how those transcripts become specifically targeted to mitochondria remains to be uncovered.

1.4.3.2 The localization machinery

I. The motor protein Myo4p

The molecular motor protein that mediates the active transport of the She mRNP is Myo4p (Jansen *et al*., 1996; Bertrand *et al*., 1998; Münchow *et al*., 1999). Myo4p is a non-muscle myosin that belongs to the family of unconventional class V myosins with the only other member Myo2p being 57% identical in amino acid sequence (Haarer *et al*., 1994). Myo2p is

essential for growth (Johnston *et al*., 1991), but *Δ myo4* strains do not display growth defects (Haarer *et al*., 1994). Both Myo2p and Myo4p localize to the bud tip during bud formation (Lillie and Brown, 1994; Schott *et al*., 1999; Karpova *et al*., 2000). Myo2p mediates numerous cytoplasmic transport processes as well as vacuole and spindle positioning (reviewed by Bretscher, 2003). Myo4p was thought to be solely implicated in mRNA localization (Long *et al*., 1997; Takizawa *et al*., 1997; Shepard *et al*., 2003), but a recent publication describes Myo4p to additionally function in cortical ER inheritance (Estrada *et al*., 2003), an issue that will be discussed in chapter 5.3.1.



Myo4p, a class V myosin. Characteristic structural motifs of class V myosins are a head, a neck, coiled-coil and globular tail domain, highly conserved in animals, fungi, plants, and slime mold (reviewed by Reck-Peterson *et al*., 2000).

The head contains the force-generating ATPase and directly contacts the actin-filaments, the trail for directed movement. It has been shown that She4p binds to the motor domain of both Myo2p and Myo4p (Wesche *et al*., 2003). While in *Δ she4* cells Myo4p is delocalized to cytoplasmic background, Myo2p distribution remains unaffected. More information on She4p is given below.

In both Myo2p and Myo4p the neck harbors six tandem IQ repeats (Johnston *et al*., 1991; Haarer *et al*., 1994), sequences found in proteins containing calcium-insensitive calmodulin-binding sites (Cheney and Mooseker, 1992). Two target proteins could be identified, Mlc1p and Rho3p, both binding to the IQ repeats within Myo2p. Mlc1p has been shown to act both as stabilizing (Stevens and Davis, 1998) as well as regulating factor for septum closure during yeast cell division (Wagner *et al*., 2002). Rho3p is a small GTPase that appears to influence cell growth by regulating polarization of the actin cytoskeleton (Robinson *et al*., 1999). Besides representing the docking sites for trans-acting factors, the number of IQ repeats within the Myo2p neck has been shown to determine the secretory vesicle velocity (Schott *et al*., 2002). However, by now there is no protein known that binds to the IQ repeats within Myo4p.

The primary sequence of Myo4p contains a stretch of 30 amino acids located within the first part of the tail, which is predicted to be capable of coiled-coil formation. Based on this short motif, it cannot necessarily be concluded that Myo4p functions as a di- or oligomeric protein complex. However, *in vitro* pulldown assays have shown that Myo4p can dimerize (C. Kruse, unpublished), suggesting that several myosins in concert might participate in moving one RNP.

In yeast class V myosins the COOH-terminal globular tail represents a major part of the protein. Primary sequence alignment of Myo2p versus Myo4p has revealed that the tail regions are the most divergent parts (Haarer *et al*., 1994; Schott *et al*., 1999). The differences have been explained by phylogenetic analysis of motor protein sequences, pointing out that both sequences stem from a relatively recent gene duplication event. It is believed that following this event the Myo4p tail has lost features involved in membrane transport which are conserved in Myo2p and animal myosin Vs (Schott *et al*., 1999).

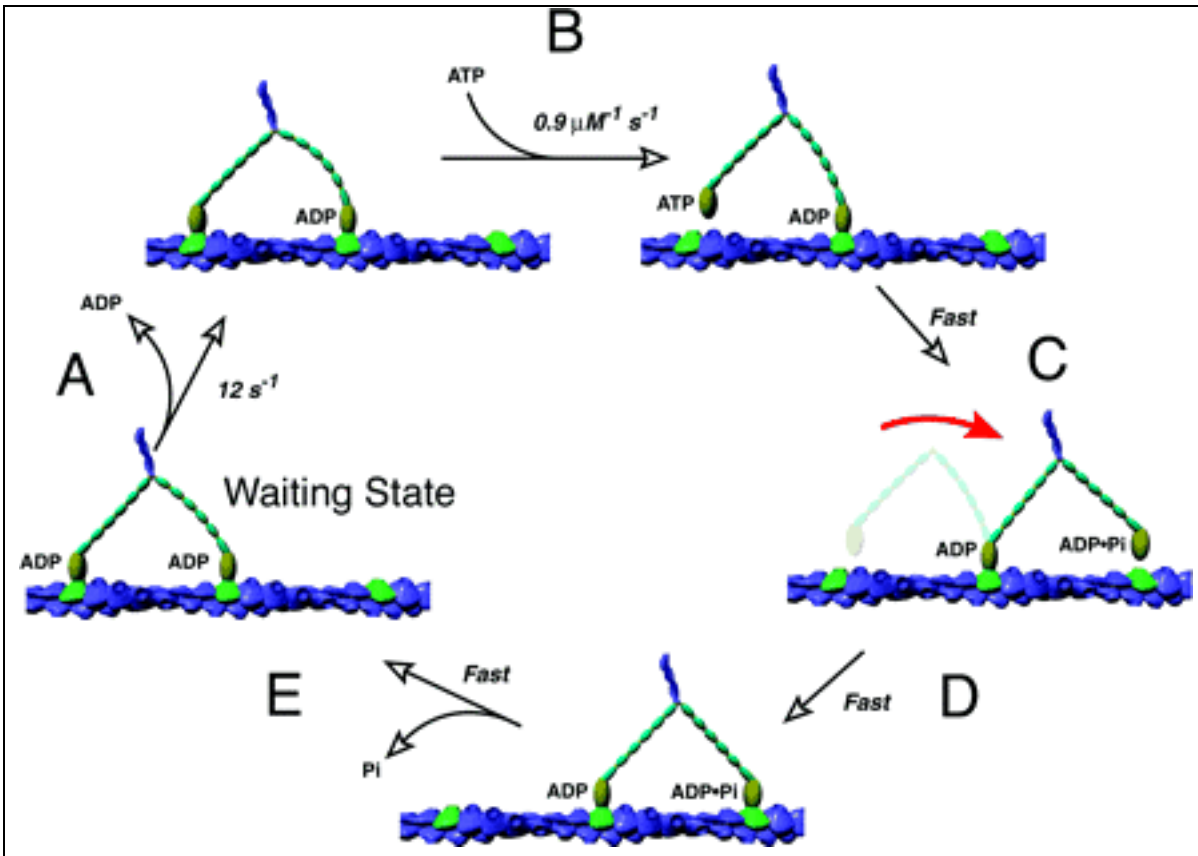
The Myo2p globular tail tethers cargo to the motor, whereas the NH2-terminal actin-binding domain targets the complex to growth sites (Catlett and Weisman, 1998; Schott *et al*., 1999). Moreover, the Myo2p tail contains at least two sub-domains with separable cargo-binding regions for secretory vesicles and the vacuole (Catlett *et al*., 2000; Ishikawa *et al*., 2003), indicating that different parts are responsible for binding to different cargoes, and that the movement of each cargo must be regulated uniquely. This finding suggests that, similar to Myo2p, also the Myo4p tail harbors regions that mediate a connection to cargo-specifying adapter proteins (see below).

Another interesting feature of the Myo2p globular tail is the observation that the rear end of Myo2p can localize independently from the motor domain and actin to growing bud tips (Ayscough *et al*., 1997; Reck-Peterson *et al*., 1999). In addition, overexpression of the tail in a wt background resulted in mislocalization of full-length endogenous Myo2p to cytoplasmic background. This phenomenon has been interpreted to mean that the tail competes out a factor necessary for Myo2p localization to the bud tip, and that it is the tail, not the motor, that directs Myo2p to growth sites.

In addition to published data on Myo4p I briefly summarize a few unpublished results obtained in collaboration with coworkers in the lab. Similar to Myo2p, the Myo4p tail is required for correct myosin localization, and overexpression of the tail alone affects the distribution of endogenous Myo4p and *ASH1* mRNA (C. Kruse, unpublished). Interestingly, Myo4p was found in a screen for protein-lipid interactions. It was shown that the motor specifically binds to phosphoinositide-3,4-bisphosphate, a component of yeast membranes

(Zhu *et al*., 2001 and references therein). In order to test for the possibility that the globular tail might mediate this interaction, COOH-terminal Myo4p truncation mutants have been generated in our lab, leaving Myo4p with reduced length of the globular tail (T. Güttler, C. Kruse and A. Jaedicke, unpublished). Quantification of the localization pattern against full-length Myo4p could show that the rate of correct Myo4p localization to the tip of growing buds significantly dropped with the truncation length. This observation prompted us to speculate that the Myo4p tail at least partly functions in tethering the myosin to the bud cortex.

The high degree of structural conservation and sequence homology within class V myosins of eukaryotic organisms ranging from slime molds to vertebrates offers an important tool to gain more insight into how those motor proteins produce force.



Force generation by class V myosins (Vale, 2003).
A current model for how class V myosins convert cellular energy in form of ATP into motion combines insights from electron microscopic images of the two-headed motor protein bound to actin in various conformational states with measurements of single molecule stepping kinetics. Based on a strictly ordered series of structural changes induced by ATP hydrolysis, the motor walks in a hand-over-hand mechanism (A-E).

In order to assure that at least one myosin head always remains attached to actin while the other arm swings and contacts to actin one position further, both motor heads by some means need to communicate and collectively organize movement. The swinging lever arm model predicts that the light chain-binding region within the neck of the myosin heavy chain, forms a rigid rod, or lever arm, that amplifies small movements of the actin-binding globular head (Uyeda *et al*., 1996).

In vitro experiments have allowed to determine the maximal velocity of Myo2p and Myo4p with 4.5μm / s and 1.1μm / s, respectively (Reck-Peterson *et al*., 2001). On the other hand, in vivo video microscopy demonstrated that Myo4p moved *ASH1* mRNA containing particles to the bud tip at 200-440nm/s (Bertrand *et al*., 1998). The discrepancy between the *in vitro* and *in vivo* rates might be explained by additional factors associated in vivo that have been missing during the *in vitro* measurements, or simply by differences in the detection limit of the chosen experimental setup.

II. The adapter protein She3p

An interaction partner of Myo4p is She3p, acting as adapter protein between the mRNA binding protein She2p and the motor (Takizawa and Vale, 2000). In more detail, 2-hybrid (Böhl *et al*., 2000) as well as 3-hybrid analysis (Long *et al*., 2000) has shown that Myo4p interacts with the NH2-terminus of She3p. The connection could be confirmed *in vitro* by GST pulldown assays using full-length She3p as bait against the Myo4p globular tail (including the predicted coiled-coil region), indicating a direct physical link (Böhl *et al*., 2000). Moreover, sucrose gradient centrifugation demonstrated a co-fractionation of Myo4p / She3p, suggesting a tight and permanent association of both proteins (Böhl *et al*., 2000). Those data fitted well to the earlier observed co-localization of Myo4p / She3p at the tip of growing buds (Münchow *et al*., 1999).

In addition, She3p influences on the *ASH1* mRNA binding ability of She2p. Gel-shift assays demonstrated that She2p-binding to an *ASH1* ZIPcode element is specific and effectively enhanced by She3p (Böhl *et al*., 2000).

III. The RNA-binding protein She2p

She2p acts as a specific mRNA binding protein, that bridges *ASH1* mRNA to the She3p COOH-terminus (Böhl *et al*., 2000; Long *et al*., 2000; reviewed by Kwon and Schnapp, 2001). However, the She2p primary sequence does not contain a canonical RNA-binding motif, and the region within She2p that contacts the RNA still remains to be uncovered.

Although all four localization elements within *ASH1* mRNA are contacted and needed for efficient localization (Böhl *et al*., 2000; Chartrand *et al*., 2002), She2p might have a somewhat higher affinity to elements E1 and E3 compared to E2A and E2B (Long *et al*., 2000). Nevertheless, elegant ZIPcode-swapping experiments combined with site-directed mutagenesis have nicely demonstrated that all four *ASH1* localization elements are redundant in function in respect to tight mRNA localization (Chartrand *et al*., 2002).
Under wt conditions She2p is distributed uniformly throughout the cytoplasm (Jansen *et al*., 1996), but upon *ASH1* overexpression, She2p co-localizes together with *ASH1* mRNA at the tip of growing buds (Böhl *et al*., 2000).

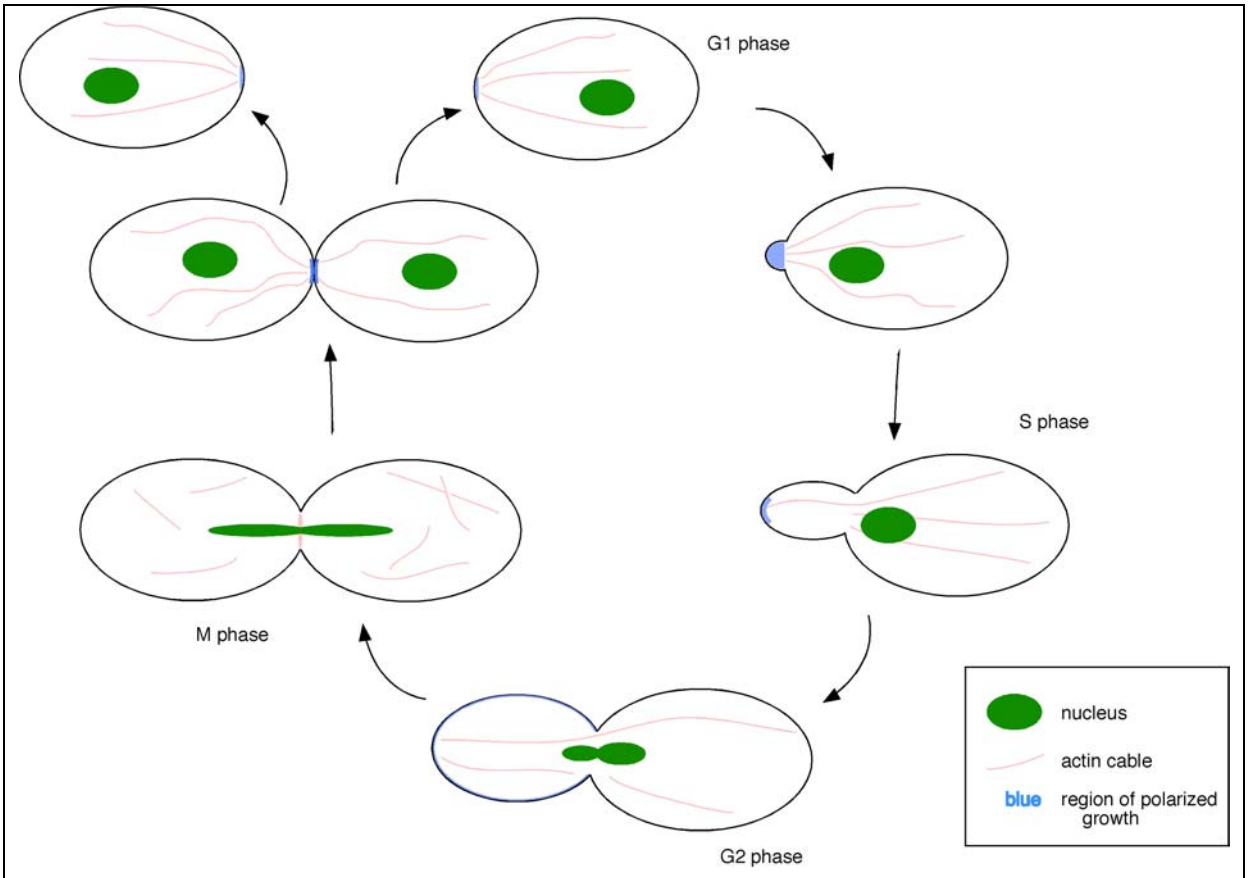
IV. She4p, a putative myosin assemblase

Only one mutant could be isolated in the original *SHE* screen that was defective in the *SHE4* open reading frame, indicating a rather modest influence on *ASH1* mRNA localization (Jansen *et al.*, 1996). She4p was initially characterized as protein involved in receptor-mediated endocytosis, organization of the cortical actin cytoskeleton and growth at elevated temperatures (Wendland *et al*., 1996). By sequence comparison it was found that *SHE4* shares a domain together with *Caenorhabditis elegans UNC45* (Epstein and Thomsen, 1974), and with *Podospora anserina CRO1* (Berteuaux-Leceillier *et al*., 1998), the 'UCS domain' (Barral *et al*., 1998; reviewed by Hutagalung *et al*., 2002).
In the nematode, Unc45p is implicated in correct folding and assembly of conventional thick muscle class II myosin (Barral *et al*., 2002), indicating a myosin-specific chaperone activity of the protein. This analogy prompted members of our group to investigate if She4p exerts a similar function on Myo4p. In turn, She4p was found to specifically interact via the UCS domain with the motor domain of class I and class V myosins, thereby promoting actin-binding of the myosin (Wesche *et al*., 2003; Toi *et al*., 2003). In the case of Myo4p this interaction is required for correct myosin localization, whereas the intracellular distribution of Myo2p in *Δ she4* cells remains unaffected. Importantly, the interaction with myosins has been characterized as very weak, explaining why She4p could not be detected in traveling *ASH1* mRNA granules using the 'green RNA' system, described in part 4.2 (Bertrand *et al*., 1998; Takizawa *et al*., 2000).

V. A key regulator of the actin cytoskeleton, Bni1p

SHE5 has been found to be equivalent to *BNI1*, a gene involved in the organization of the actin cytoskeleton.

Actin filaments constitute the polarized tracks for bud–directed cytoplasmic transport of various cargoes, including *ASH1* mRNA (Long *et al*., 1997; Takizawa *et al*., 1997; Karpova *et al*., 2000; Schott *et al*., 2002a). During the yeast cell cycle the actin cytoskeleton is subject of constant remodeling (reviewed by Schott *et al*., 2002b), a process that is initially regulated by Rho GTPase signalling (Dong *et al*., 2003).



Morphological stages during the yeast cell cycle (adapted from Schott *et al*., 2002).
Schematic drawing of yeast cells with varying bud size and the corresponding stage of the cell cycle. Nuclei are depicted in green, actin filaments in red and regions of polarized growth in blue. Note the distribution of actin filaments towards the regions of polarized growth at any given stage of the cell cycle.

Bni1p is a member of the formin protein family and has been shown to directly link Cdc42p and the actin cytoskeleton (Evangelista *et al*., 1997). For this to occur, Bni1p continually co-localizes with regions of cell-growth, namely at the bud tip and neck (Ozaki-Kuroda *et al*., 2001). Bni1p is required for actin filament assembly and maintenance (Evangelista *et al*., 2002; Sagot *et al*., 2002a), and purified protein can nucleate actin polymerization *in vitro*

(Pruyne *et al*., 2002; Sagot *et al*., 2002b). Thereby Bni1p binds the barbed end of actin filaments, suggesting that besides their function in microfilament nucleation, formins might directly anchor the growing end to sites of polarized growth (Pruyne *et al*., 2002). Importantly the barbed ends need to be oriented towards the growth sites because yeast class V myosins travel in a pointed-to-barbed-end direction.
In support of this view, *Δ bni1* cells fail to correctly localize *ASH1* mRNA to the bud tip (Long *et al*., 1997; Takizawa *et al*., 1997), but instead accumulate *ASH1* mRNA at the mother-bud junction. This discrete mislocalization phenotype is in agreement with a defect in promoting nucleation and polymerization of actin cables at the bud tip (Evangelista *et al*., 1997). However, Bni1p together with Bud6p has also been suggested to constitute a multi-purpose cortical scaffold, serving as docking platform and anchorage site for *ASH1* mRNA (Beach *et al*., 1999).
Taken together, the data implicate Bni1p as key protein in the cell cycle coordinated assembly and polarization of microfilaments, and thus as rather indirect factor in *ASH1* mRNA localization. Consistently, Bni1p could not be found to co-localize with *ASH1* containing cytoplasmic granules in the 'green RNA' approach, described in part 4.2 (Bertrand *et al*., 1998; Takizawa and Vale, 2000)

1.4.3.3 Accessory factors: Khd1p, Scp160p and Puf5p

In addition to the She machinery several other factors required for asymmetric distribution of *ASH1* mRNA have been discovered: *KHD1, SCP160, PUF5* and *LOC1*. Deletions of these genes do not display a delocalization phenotype as severe as observed in *she1-3* mutants. Instead they have been proposed to rather act as assistant factors for the basic transport machinery, with mutants that show only a relatively mild phenotype on *ASH1* mRNA or protein localization. Like She4p and Bni1p, the new factors are thought to play accessory roles in the trafficking process. Their deletion seems to affect general steps in cell metabolism, such as cell polarization (e.g. *BNI1* or *BUD6*), motor-based motility (*SHE4*), or translation (*KHD1, SCP160*).
Based on sequence homology to canonical RNA-binding motifs, three additional factors could be identified affecting *ASH1* mRNA localization as observed by FISH, namely Khd1p, Scp160p and Puf5p (Irie *et al*., 2002). Using the 'green RNA' approach in combination with indirect immunofluorescence it has been shown that Khd1p co-localizes with cytoplasmic RNA granules whereas Scp160p and Puf5p do not.

I. Khd1p

The KH-domain protein 1, Khd1p, has been reported to bind to the *ASH1* N-element (Irie *et al*., 2002), the region spanning the first 800 nucleotides of the coding sequence. The N-element was shown previously to be sufficient for targeting a reporter RNA to the bud (Gonzalez *et al*., 1999). Surprisingly, a deletion within *KHD1* had only little effect on *HO* expression, explaining why this factor was not identified in the original *SHE* screen. Moreover, no phenotype on the frequency in mating-type switching could be observed in *Δ khd1* mutants. Nevertheless, *KHD1* genetically interacts with a weak mutation in *MYO4*, *myo4-910*, but a physical interaction with the She proteins has not been shown.
Most remarkably, Irie *et al*., (2002) observed that *KHD1* overexpression resulted on one hand in a decrease of *ASH1* mRNA localization efficiency, and on the other hand in reduced Ash1 protein levels. The authors suggested that the anchorage deficiency might be a consequence of the inhibition of *ASH1* translation. Translation dependent *ASH1* mRNA anchorage to the bud tip has been observed earlier (Gonzalez *et al*., 1999), fitting nicely to the model that Khd1p might be involved rather in Ash1p translation control than in the *SHE*-mediated cytoplasmic transport process (Irie *et al*., 2002).

II. Scp160p

Scp160p has been described as a multi KH-domain protein and belongs to the family of vigilin-like proteins (Wintersberger *et al*., 1995). KH-domains are known to function as RNA-binding motif, and Scp160p has been shown to interact with membrane-bound polysomes (Frey *et al*., 2001), and to be a component of RNPs (Lang and Fridovich-Keil, 2000). The role of Scp160p on *ASH1* mRNA could be more general, as this protein was recently shown to interact with 69 mRNAs with diverse functions, among which *ASH1* mRNA was not found (Li *et al*., 2003). A discrete function of Scp160p in RNP targeting, maturation or stability has not been revealed so far, and - to make things even more difficult - the phenotypic characterization of *Δ scp160* is doubtful because mutants display severe chromosomal instability defects (Wintersberger *et al*., 1995).
Interestingly, Scp160p has recently been revealed to directly interact with Gpa1p, the α subunit of the G-protein complex involved in mating signal transduction (Guo *et al*., 2003).

III. Loc1p

The strictly nuclear protein Loc1p was isolated by 3-hybrid screening due to its ability to bind to the *ASH1* 3'-UTR (Long *et al*., 2001). In *Δ loc1* cells, asymmetric distribution of both

ASH1 mRNA and Ash1p is affected. However, *LOC1* has also been identified as part of the 66S pre-rRNA complex and has been shown to be involved in 60S rRNA processing (Harnpicharnchai *et al*., 2001). Likewise, Loc1p is localized strictly to nuclei, and binds rather unspecifically to double-stranded RNA. Moreover, *Δ loc1* strains display a severe slow growth phenotype at 30°C standard cultivation conditions and abnormal cell morphology (Long *et al*., 2001), typical for genes involved in ribosome synthesis pathways. Hence, the connection of Loc1p to the *ASH1* mRNA localization machinery is likely to be of indirect nature, via a general influence on protein synthesis.

1.4.3.4 Myo4p, She3p and She2p together with cargo RNA constitute the core She RNP

Since Myo4p, She3p and She2p all co-localize with trafficking *ASH1* mRNA containing granules (Takizawa and Vale, 2000; Irie *et al*., 2002), and co-precipitate with *ASH1* mRNA (Münchow *et al*., 1999; Takizawa and Vale, 2000; Irie *et al*., 2002), the three proteins are thought to constitute the minimal She RNP (Darzacq *et al*., 2003). Moreover, She3p and Myo4p have been shown to co-localize with *ASH1* mRNA in the cytoplasm on string-like filamentous structures (Münchow *et al*., 1999), strongly resembling transport intermediates located on actin filaments. Other factors might be associated only transiently (She4p), or are associated but not absolutely required for transport (Khd1p). In a combination of the 'green RNA approach' (chapter 4.2) with indirect immunofluorescence it has been demonstrated that She4p does not co-stain with *ASH1* mRNA granules (Takizawa and Vale, 2000). However, recently it has been shown that She4p and Myo4p co-localize under wt conditions at the bud tip (Wesche *et al*., 2003), suggesting a transient interaction only at the site of destination but not during transport.

1.5 Aim of this work

In a shared project with C. Kruse we have investigated the principles in the regulation of cytoplasmic She RNP trafficking. Nevertheless, the aim of the work presented here has been the biochemical purification of the intact cytoplasmic She RNP complex, including the identification of so far unknown associated factors and their initial functional characterization in respect to *ASH1* mRNA targeting.

2. Material

2.1 Plasmids

E.coli - S. cerevisiae shuttle vectors have been described in Sikorski and Hieter, 1989, and in Gietz and Sugino, 1988.

pRJ 46	YCplac111-ASH1-myc9, Long et al., 1997
pRJ 88	YEplac181-ASH1, equals to plasmid C3319 in Long et al., 1997
pRJ 124	pUC19-myc9-K.lactis TRP1, Zachariae et al., 1998
pRJ 135	pFA6a-HIS3MX6, Wach et al., 1997
pRJ 151	pTS15, EcoR1-pep4-URA3-pep4-Xho1, for pep4::URA3 disruption
pRJ 256	pBluescript II KS-ASH1, full length plus 1kb up- and 0.5kb down-stream
pRJ 272	pBluescript II KS-ash1-stop-E2
pRJ 273	YEplac181-ash1-stop-E3
pRJ 274	YEplac181-ash1-stop-E2
pRJ 276	pYM2, Knop et al., 1999
pRJ 277	pYM3, Knop et al., 1999
pRJ 279	pYM5, Knop et al., 1999
pRJ 280	pYM6, Knop et al., 1999
pRJ 287	YEplac181-ash1-stop-E1
pRJ 292	pBS1479, Rigaut et al., 1999
pRJ 298	pRS315-ASH1, SacI / SalI fragment from pRJ 88
pRJ 299	pRS315-ash1-stop-E1
pRJ 300	pRS315-ash1-stop-E2
pRJ 301	pRS315-ash1-stop-E3
pRJ 309	pRS426-pGAL1-ASH1, pGAL1-ASH1 plus 1kb downstream, XhoI / SacI
pRJ 318	pRS315-ash1-E2B-stop
pRJ 319	YEplac181-ash1-E2B-stop
pRJ 329	YEplac112-ASH1, harbors Sac1 / Sal I full length ASH1 from pRJ 88
pRJ 332	pSL1180-ash1 COOH-terminus, harbors a 1kb NcoI / SacII fragment
pRJ 334	YEplac181-ash1-E2A-stop
pRJ 365	pRS426-HO, EcoRI / SacI fragment contains HO endonuclease
pRJ 384	YEplac181-SHE3-MS2, pRL141, Long et al., 2000
pRJ 385	YEplac195-lacZ-ADH II (3'UTR), Chartrand et al., 1999
pRJ 386	YEplac 195 lacZ-MS2-ADHII (3'UTR), Bertrand et al., 1998
pRJ 392	YIplac211-pGAL1-10-ASH1, digest AvrII to integrate into ASH1 locus
pRJ 510	pRS316-pADH1-ASH1, full length ASH1 plus 1kb down-stream
pRJ 543	pWZ-2xGFP, Kruse et al., 2002
pRJ 602	pCORE, Storici et al., 2001
pRJ 627	YEplac181-SHE2-myc3, Kruse et al., 2002
pRJ 629	YEplac181-she2ΔN70-myc3, Kruse et al., 2002

2.2 Oligonucleotides

RJO 61	ATACATATATACATATATGGGCGTATATTTACTTTGTTCTTA CCTCGAGGCCGAAGACT
RJO 62	TTAGCTACTGTCAGTAAAATTATAAAATTAGACAGAAAATC CGGTTCTGCTGCTAGT
RJO 142	AAGCGGATCCATGTCAAGCTTATAC
RJO 211	GTAGAGGCCTCTTTTTTCTAGAGGAAATCCAGC
RJO 213	CTCCATGCATTGTTGG
RJO 214	GGGGGAGAGTCGAGAGC
RJO 215	CATCAAAACGTACATCTATCTAGAAAACTGATCTTACCC
RJO 217	GAAGATGCCGCGGCGTG
RJO 218	CCGTTGCTTATTTTGTATCTAGATAACTGAGACAG
RJO 268	GTTGTCGCTACTAAATGGCATGACAAATTTGGTAAATTGA AAAACCGTACGCTGCAGGTCGAC
RJO 269	ACTAGTGGTACTTATTTGCTCTTTTTGAGCTAAAAACTGAAG GCCATCGATGAATTCGAGCTCG
RJO 367	CTGTAATACGACTCACTATAGGAATGGAACTTGGACGACC
RJO 368	CGAATTTAGGTGACACTATAGTTTCCCATGCCAAATGCCAG
RJO 369	GCAATTTAGGTGACACTATAGCTGATCTTACCCATTGGTGT
RJO 370	CGTTAATACGACTCACTATACACTGGACCTTCTACTTCCC
RJO 371	GCAAATTAGGTGACACTATAGCATAACTGAGACAGTAGAGA
RJO 372	CGTTAATACGACTCACTATAATGCCGCGGCGTGTCGAATG
RJO 373	CGTTAATACGACTCACTATAGTGTTACCGAAAACATTTG
RJO 374	GCAATTTAGGTGACACTATAGGCAAGCATCAAATGTTTTCG
RJO 375	GCAATTTAGGTGACACTATAGGGGAAGTAGAAGGTCCAGTG
RJO 376	CGTTAATACGACTCACTATATCTCTACTGTCTCAGTTATGT
RJO 420	TCAAAGCAAGCATCAAATGTTTTCTAGAACACTGCAAGACA ATTGGT
RJO 465	ACGTTGTAAAACGACGGCC
RJO 466	CACAGGAAACAGCTATG
RJO 467	ACCAATTGTCTTGCAGTGTTCTAGAAAACATTTGATGCTT GCTTTGA
RJO 547	TATGTATATATACATATATACATATATGGGCGTATATTTACTTT GTTCATCGATGAATTCGAGCTCC
RJO 548	ACAGAGGGCTTAGCTACTGTCAGTAAAATTATAAAATTAGA CAGAAAACGTACGCTGCAGGTCGAC
RJO 553	GTTGTCGCTACTAAATGGCATGACAAATTTGGTAAATTGAAA AACTCCATGGAAAAGAGAAG
RJO 554	GCTATTCATGTATATATATATGTTCTATTAACTAGTGGTACTT ATTACGACTCACTATAGGG
RJO 558	GGGAGCTTCCATATGTAGTGTAGGATATATGTATATATA CATATATACGACTCACTATAGGG
RJO 559	GAGGGCTTAGCTACTGTCAGTAAAATTATAAAATTAGACAG AAAATCCATGGAAAAGAGAAG

RJO 1020	CAGTGGTGAATCCGGTGCTGGTACCACCGTCTCTGCTAAATA CATTATGAGATAC
RJO 1021	GTATCTCATAATGTATTTAGCAGAGACGGTGGTACCAGCACCG GATTCACCACTG
RJO 1022	CGGGGAATTGATGGCCATCATAAATTCTACTATAAAACCAAAT TCTGAAAAAAAGCC
RJO 1023	GGCTTTTTTTCAGAATTTGGTTTTATAGTAGAATTTATGATGG CCATCAATTCCCCG
RJO 1036	GATCCCAGCAAGATTAAG
RJO 1037	CCAGCACACCACAAGCTC
RJO 1038	CATGAATGGACAGATATATAC
RJO 1292	CGGGTAACTTAGAGACAGCATTAGTATATATACCAGCCC AGCTGAAGCTTCGTACGC
RJO 1293	GTTTTGTCTGTGTGGGACGTGCGCACGCACACGTATATA GCATAGGCCACTAGTGGATCTG

2.3 Yeast strains

- all strains are W303 background except if indicated
- strains #2018, #2022 and #2049 have been obtained from the Euroscarf collection, Frankfurt, Germany.

RJY 358	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+
RJY 359	Mat α, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+
RJY 375	Mat a, pep4::URA3, CSE1-myc9::HIS3, YEplac 181-ASH1, plasmid #88
RJY 449	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, MYO4-Myc6, pep4::URA3
RJY 585	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, ash1::S.pombe HIS3
RJY 586	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, ash1::S.pombe HIS3, YEplac181-ash1-stop-E3, plasmid #273
RJY 587	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, ash1::S.pombe HIS3, YEplac181-ash1-stop-E2, plasmid #274
RJY 588	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, ash1::S.pombe HIS3, YEplac181-ASH1, plasmid #88
RJY 602	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, ash1::S.pombe HIS3, YEplac181-ash1-stop-E1, plasmid #287
RJY 612	Mat a, ade2, his3, leu2, trp1, ura3, mex67:HIS3 (pUN100-LEU2-mex67-5) Segref et al. (1997) EMBO J. Vol 16 pp3256 RS453 background
RJY 617	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, MYO4-Myc6, pep4::URA3, she3::HIS3
RJY 620	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, ash1::S.pombe HIS3, pRS315-ASH1, plasmid #298

RJY 621	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, ash1::S.pombe HIS3, pRS315-ash1-stop-E1, plasmid #299
RJY 622	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, ash1::S.pombe HIS3, pRS315-ash1-stop-E2, plasmid #300
RJY 623	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, ash1::S.pombe HIS3, pRS315-ash1-stop-E3, plasmid #301
RJY 646	Mat a, ade2, his3, leu2, trp1, ura3, mex67:HIS3 (pUN100-LEU2-MEX67) RS453 background, Segref *et al*. (1997) EMBO J. Vol 16 pp3256
RJY 666	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1. ash1::S.pombe HIS3, YEplac181-ASH1-postE2 STOP, plasmid #319
RJY 676	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1 ash1::S.p.HIS3, she2::URA3
RJY 751	Mat a, ade2, his3, leu2, trp1, ura3, mex67:HIS3 (pUN100-LEU2-mex67-5), SHE3-MYC9::TRP
RJY 836	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1. ash1::S.pombe HIS3, YEplac181-ASH1-E2aSTOPE2b, plasmid #334
RJY 939	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1 ash1::S.p.HIS3, she2::URA3, pRS315
RJY 940	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1 ash1::S.p.HIS3, she2::URA3, pRS315-ASH1, plasmid #298
RJY 941	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1 ash1::S.p.HIS3, she2::URA3, pRS315-ASH1-STOP-E1, plasmid #299
RJY 942	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1 ash1::S.p.HIS3, she2::URA3, pRS315-ASH1-STOP-E2, plasmid #300
RJY 943	Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1 ash1::S.p.HIS3, she2::URA3, pRS315-ASH1-STOP-E3, plasmid #301
RJY 953	Mat a, ade2, his3, leu2, trp1, ura3, mex67:HIS3 (pUN100-LEU2-MEX67) SHE2-TAP::TRP1, pep4::URA3
RJY 968	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, MYO4-Myc6 she2::HIS3
RJY 983	Mat a, ade2, his3, leu2, trp1, ura3, mex67:HIS3 (pUN100-LEU2-mex67-5), SHE2-TAP::TRP1 pGAL1-10-ASH1::URA3
RJY 1004	Mat α, his3, leu2, ade2, trp1, ura3, HO-ADE2, HO-CAN1 SHE3-MYC6, ist2::HIS3MX6, ash1::TRP1
RJY 1082	Mat α, ade2-1; his3-11,15; ura3-52; leu2-3,112; trp1-1; can1-100; GAL+ RS 453 background
RJY 1083	Mat a, ade2-1; his3-11,15; ura3-52; leu2-3,112; trp1-1; can1-100; GAL+ RS 453 background
RJY 1164	Mat a, ade2-1; his3-11, 15; ura3-52; leu2-3, 112; trp1-1; can1-100; GAL+ mex67::HIS3; pUN100-mex67-5, SHE2-Myc9::K.l.TRP1 RS453 background
RJY 1193	Mat a, ade2, his3, leu2, trp1, ura3, mex67:HIS3 (pUN100-LEU2-mex67-5), SHE2-myc9::K.l. TRP1 RS 453 background

RJY 1303	Mat a, ade2-1; his3-11, 15; ura3-52; leu2-3, 112; trp1-1; can1-100; GAL+ mex67::HIS3; pUN100-mex67-5, MYO4-2xGFP::K.l.TRP1 RS453 background
RJY 1304	Mat a, his3, leu2, ade2, trp1, ura3, can1-100, she3::URA3 MYO4-2xGFP::K.l. TRP1
RJY 1310	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ MYO4-2xGFP::K.l. TRP1
RJY 1343	Mat a, ade2-1; his3-11, 15; ura3-52; leu2-3, 112; trp1-1; can1-100; GAL+ mex67::HIS3; pUN100-MEX67, MYO4-2xGFP::K.l.TRP1 RS453 background
RJY 1405	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ MYO4-2xGFP::K.l. TRP1, she2:: URA3
RJY 1420	Mat a, his3, leu2, MYO4-TAP::TRP1, pep4::URA3, SHE2myc3
RJY 1526	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ MYO4-2xGFP::K.l. TRP1, she2::HIS YEplac181-SHE2-myc3, plasmid #627
RJY 1528	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ MYO4-2xGFG::K.l. TRP1, she2::HIS YEplac181-she2ΔN70-myc3, plasmid #629
RJY 1529	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ myo4-2xGFP::klTRP1::HIS3, ATPase P-loop mutant
RJY 1531	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ myo4-2xGFP::klTRP1::HIS3, actin-binding mutant
RJY 1578	Mat α, ade2-1; his3-11, 15; ura3-52; leu2-3, 112; trp1-1; can1-100; GAL+ SHE2-Myc3::HIS3MX6, MYO4-HA6::K.l.TRP1 RS453 background
RJY 1581	Mat a; ade2-1; trp1-1; can1-100; leu2-3, 112; his3-11, 15, MYO4-HA6 pRS316-pADH1-ASH1, plasmid #510
RJY 1599	Mat α, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, she2::URA3 YEplac112-ASH1 (plasmid #329), YEplac181-SHE2myc3 (plasmid #627)
RJY 1600	Mat α, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, she2::URA3, YEplac112-ASH1 (plasmid #329) YEplac181-she2ΔN70-myc3 (plasmid #629)
RJY 1613	Mat α, ade2-1; his3-11, 15; ura3-52; leu2-3, 112; trp1-1; can1-100; GAL+ mex67::HIS3; pUN100-mex67-5, SHE2-Myc9::K.L.TRP1 MYO4-HA6::K.l.TRP1, pRS426-pGAL1-ASH1, plasmid #309 RS453 background
RJY 1616	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ MYO4-2xGFP::klTRP1, YEplac181-ASH1, plasmid #88
RJY 1624	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ MYO4-2xGFP::K.l. TRP1, ash1::K.l. TRP1, ist2::HIS3MX6
RJY 1629	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ Myo4-2xGFP::klTRP1, ash1::klTRP1, ist2::HIS3MX6, wsc2::URA3

RJY 1640	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ MYO4-2xGFP::K.l. TRP1, she2::HIS YEplac181-SHE3-MS2, plasmid #384 YEplac195-lacZ-ADH II, plasmid #385
RJY 1641	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ MYO4-2xGFP::K.l. TRP1, she2::HIS YEplac181-SHE3-MS2, plasmid #384 YEplac 195 lacZ-MS2-ADHII (3'UTR), plasmid #386
RJY 1933	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ MYO4-2xGFP::klTRP1, khd1::HIS3MX6
RJY 1938	Mat α, his3, ura3, trp1, leu2, SHE2-TAP::K.l.TRP1 myo4-2xGFP::K.l.TRP1::HIS3, actin-binding mutant
RJY 1939	Mat α, his3, ura3, trp1, leu2, SHE2-TAP::K.l.TRP1 myo4-2xGFP::K.l.TRP1::HIS3, P-loop mutant
RJY 1940	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ myo4-2xGFP::K.l.TRP1::HIS3, P-loop mutant YEplac181-ASH1, plasmid #88
RJY 1941	Mat a, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ myo4-2xGFP::K.l.TRP1::HIS3, actin-binding mutant YEplac181-ASH1, plasmid #88
RJY 2010	Mat α, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, SHE2-myc3, GIN4-HA6::K.lactis TRP1
RJY 2018	Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, gin4::kanMX4 BY4741 background
RJY 2022	Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, eap1::kanMX4 BY4741 background
RJY 2042	Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, gin4::kanMX4 YEplac181-ASH1, plasmid #88 BY4741 background
RJY 2046	Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, eap1::kanMX4 YEplac181-ASH1, plasmid #88 BY4741 background
RJY 2049	Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 BY4741 background
RYJ 2068	Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, eap1::kanMX4 YCplac111-ASH1-myc9, plasmid #46 BY4741 background
RJY 2069	Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 YCplac111-ASH1-myc9, plasmid #46 BY4741 background
RJY 2070	Mat a; his3D1; leu2D0; lys2D0; ura3D0; she2::kanMX4 YCplac111-ASH1-myc9, plasmid #46 BY 4741 background

2.4 Lab equipment and disposables

Standard lab equipment and disposals have been used; exceptions are listed below. All solutions have been autoclaved or sterilized by filtration.

Standards

chemicals	MERCK, FLUKA, J.T. BAKER, SERVA, SIGMA
enzymes	NEB, ROCHE, BIOMOL, ICN PROMEGA, MBI FERMENTAS
oligos	MWG, ARK SCIENTIFIC

Kits

Plasmid Miniprep	MACHEREY NAGEL, PEQLAB
Plasmid Midiprep	MACHEREY NAGEL
Gel Extraction/PCR Purification	QIAGEN
Massspec Compatible Silver Stain	INVITROGEN
Colloidal Blue Stain	INVITROGEN
QuikChange Site Directed Mutagenesis	STRATAGENE
Prime-It II Random Primer Labeling	STRATAGENE
T7-Mega-Shortscript	AMBION
ECL Detection	PHARMACIA
Rnase Away	MOLECULAR BIOPRODUCTS
Protein Assay	BIO-RAD
Quick Ligation Kit	NEB

Special equipment

I. Hardware

motor grinder RM 100	RETSCH
Criterion Electrophoresis System	BIO-RAD
IKA vibrax VXR	NEOLAB

II. Disposables

glass beads, 0.45-0.5mm	NEOLAB
PositiveTM Membrane	QBIOGENE
Hybond-P	PHARMACIA
Hyperfilm ECL	PHARMACIA
NytranN	SCHLEICHER&SCHÜLL
GB 003/GB 004 Gel-Blotting Paper	SCHLEICHER&SCHÜLL
Micro Bio-Spin 30 Chromatography Columns	BIO-RAD
Mini Quick Spin RNA Columns	ROCHE
Mobicol, 35µm pore filters	MOBITEC
Glas Econo columns	BIO-RAD

III. Reagents

Denhardt's	SIGMA
Poly-L-lysine	SIGMA
Triton-X-100	GERBU
Tween 20	SIGMA
phalloidin, rhodamine conjugated	MOLECULAR PROBES
IGEPAL CA-630, equals to NP 40	ICN
Trichloracetic acid, TCA	SIGMA
Deoxycholic acid, sodium salt	SERVA
CHAPS	GERBU
Salmon sperm DNA	ROCHE
Rnasin,	PROMEGA
RQ1 Dnase	PROMEGA
oxalyticase	ENZOGENETICS
Platinum Pfx DNA polymerase	INVITROGEN
CL-4B sepharose	PHARMACIA
IgG sepharose	PHARMACIA
ProG sepharose	PHARMACIA
calmodulin Affinity Resin	STRATAGENE
Complete EDTA-free Protease Inhibitor Cocktail	ROCHE
aminoethyl-p-benzenesulfonylfluoride, AEBSF	GERBU
ribonucleoside-vanadyl-complex, VRC	NEB
heparin	PHARMACIA
diethylpyrocarbonate, DEPC	ROTH
tRNA	ROCHE
diamidino-2-phenylindol dihydochloride, DAPI	ROTH
bovine serum albumine, BSA	ROCHE
digoxigenin-11-uridin-5'-triphosphate, DIG-UTP	ROCHE
zymolase 20T / 100T	ICN

Antibodies:

Primaries

rabbit anti protein A	SIGMA
Peroxidase anti peroxidase, PAP	SIGMA
12CA5, mouse anti HA	ROCHE
3F10, rat anti HA	ROCHE
9E10, mouse anti MYC	hybridoma supernatant
rabbit anti Scp160p	M.Seedorf, ZMBH, Heidelberg
rabbit anti Asc1p	M.Seedorf, ZMBH, Heidelberg
B34, mouse anti GFP	COVANCE
Mab414, mouse anti actin	CHEMICON
rabbit anti Myo2p tail	S.Reck-Peterson, Yale, New Haven
rabbit anti She2p	Gonsalvez et al., 2003

Secondaries

sheep anti mouse, peroxidase-conjugated	DIANOVA
rabbit anti rat, peroxidase-conjugated	DIANOVA
donkey anti rabbit, peroxidase-conjugated	DIANOVA
mouse anti DIG	ROCHE
rabbit anti mouse, Alexa 488	MOLECULAR PROBES
goat anti rabbit, Alexa 488	MOLECULAR PROBES
goat anti rabbit, Alexa 594	MOLECULAR PROBES
goat anti mouse, Alexa 488	MOLECULAR PROBES
goat anti rat, Alexa 488	MOLECULAR PROBES
rabbit anti mouse, Alexa 594	MOLECULAR PROBES

3. Methods

General methods to culture and manipulate yeast strains were used as described by Adams *et al*., 1997. Epitope-tagging was performed as described in Knop *et al*., 1999. Techniques in molecular biology have been adapted from Ausubel *et al*., 1999.

In order to minimize RNA degradation DEPC treated solutions, plasticware and gloves have been used.

3.1 Yeast plasmid transformation

modified from Chen et al., *Curr. Genet*. 21:83-84, 1992

You should have a culture of at least 10^8 cells/ ml. You need 1ml per transformation. They can be stationary phase cells from a YEPD culture or scraped off a fresh plate.

- Thaw salmon sperm DNA (2mg/ml), heat 5-10min at 95°C, then chill on ice.
- Pellet 1ml of culture by centrifugation in Eppendorf centrifuge, discard supernatant.
- Resuspend cells in 100µl of 'one-step' buffer, vortex heavily.
- Add 20µg ssDNA (10µl of 2mg/ml) + 100ng - 500ng plasmid DNA to be transformed, vortex, incubate at 45 ºC for 30 min.
- Add 1 ml YEPD (or YEPGal), mix and spin 10s full speed. Discard supernatant.
- Resuspend cell pellet in 1000µl YEPD (or YEPGal) and plate 100µl directly on appropriate selective plates. Colonies appear after 2 days.

'one-step' buffer 0.2 M LiAc, 40% PEG 3350,100 mM DTT.

3.2 Quick plasmid recovery from yeast

adapted from Robzyk and Kassir,1992

- harvest 1.5 ml yeast grown O/N or longer under selective condition with a 10s spin
- resuspend in 0.1ml of STET buffer
- add same amount of glass beads (0.45 mm) and vortex for 5min
- add again 0.1ml of STET buffer, vortex briefly and boil for 3min
- cool briefly on ice and spin for 10min at 4°C in a microfuge (full speed)
- transfer 0.1ml of the sup to a new tube containing 50µl of 7.5 M ammonium acetate
- incubate at -20°C for 30min and spin for 10min at 4°C (full speed)
- add 0.1ml of the supernatant to 0.2ml precooled EtOH
- spin for 10min in a microfuge and wash the pellet with 70% EtOH
- resuspend the pellet in 0.02ml water or TE
- use 1µl to transform DH5 by electroporation
  (or 10µl to transform DH5/TOP10 by $CaCl_2$-method)

STET buffer: 8% sucrose, 50mM EDTA, 50mM Tris-HCl pH8.0, 5% Triton X-100

3.3 High efficiency yeast transformation
adapted from Gietz and Schiestl, 1991

- inoculate 50 ml YEPD medium with an over-night culture so that the OD_{600} = 0.3
- harvest cells at OD_{600} = 0.8 in a Falcon tube at 2500 rpm, 5 min
- resuspend cells in 25 ml sterile water, centrifuge again
- resuspend cells in 1 ml 100mM LiAc and transfer the suspension to an Eppi
- pellet cells at top speed for 15 sec and remove the sup
- resuspend cells to a final volume of 500 µl - about 400 µl of 100 mM LiAc+pellet
- boil salmon sperm DNA 2mg/ml for 5 min, then quickly chill on ice
- vortex the cell suspension and pipette 50µl samples into labeled Eppis
- pellet cells and remove the LiAc
- add the transformation mix in the following order:

240µl PEG (50%w/v)
36 µl 1M LiAc
25 µl salmon sperm DNA (2mg/ml)
50 µl water and plasmid DNA (0,1-10 µg)

- vortex vigorously and incubate 30min 30°C
- heat shock 30min 42°

pellet cells at 6000-8000 rpm for 15 sec and remove the transformation mix
- resuspend in 700µl YEPD and plate 100µl cell suspension

3.4 Yeast RNA preparation
modified from Cross and Tinkelenberg,1991

Start with 15ml of YEPD cultures OD_{600} = 0.7 - 0.8.
Spin cells down at 4°C, 2000 rpm, 2min, discard supernatant.
Resuspend pellets into 1ml of ice-cold TE, transfer into 2ml safelock Eppendorf tubes.
Spin for 10s in the cold and discard supernatant.

To the pellet add:
1. 200µl of glass beads
2. 400µl of 50:49:1 mixture of phenol: chloroform: isoamylalkohol, equilibrated with TE
3. 500µl of RNA buffer 1

Shake vigorously on the Ika-Vibrax-VXR mixer in the coldroom for 10 min.
Spin for 5min in an angle microfuge and transfer the upper phase into 1ml of precooled -20°C ethanol in 1.5ml Eppendorf tubes. Mix and leave at -20°C for 10min.
Spin in the angle microfuge in the coldroom for 5 min full speed. Dissolve sediment in 30-100µl RNA buffer 2 by 10 min incubation at 65°C. Measure RNA concentration.
Store in -20 freezer.

RNA buffer1: 0.3 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.2 % SDS
→ shake the buffer well before use and try to get as homogenous suspension as possible

RNA buffer 2: 1x TE, 0.2 % SDS → DEPC-treated water recommendable.

3.5 Northern blot

→ for dot blot follow the instructions of the manufacturer and directly spot your sample onto the membrane hybridize as described below

→ specific probes for hybridization have been generated by PCR, and fragments have been labeled by nick-translation with P^{32} using the 'Prime It kit'.

Low-formaldehyde RNA gels

For 1.2% agarose gels:

small gels= 50ml (big gel = 150 ml - see values in brackets).

Boil 0.6g (1.8g) high quality agarose in 43.5ml (131.5ml) sterile H_20. Let it cool to 60°C in water-bath.

Add 5ml (15ml) 10x MOPS and 1.5ml (3.5ml) 37% formaldehyde (pH of the formaldehyde should be above 4), mix well and pour the gels usual way. The sample wells should be big enough for >20µl.

Put the gels into gel-box, fill box with 1xMOPS, and load samples prepared as follows . For each sample mix in an Eppendorf tube on ice:

- 5µl 10x MOPS
- RNA and H_2O up to 15µl
- 9µl 37% formaldehyde
- 21µl formamide

Vortex, spin to the bottom and heat for 15min at 65°C. Add 10µl loading buffer, vortex, respin and load 20-30µl.

Run the gels at 6V/cm

Transfer

Rinse gel several times in Millipore water and once in 10x SSC.

Trim the gel and set the blot in 10x SSC. Fold 2 sheets of Whatman wetted with 10xSSC around a glass plate and put this over a shallow rectangular plastic dish containing 10xSSC so that the ends of paper lay in the buffer. Place your gel upside down on the filters, surround it with parafilm to prevent buffer short-circuits, put a Hybond N+ nylon membrane pre-wetted in 10xSSC on the gel and 2-3 pieces of Whatman (prewetted in 10xSSC) on the membrane. Then put on the top a 5-10 cm stack of paper towels, second glass plate and a ca 0.5kg weight and leave to blot overnight.

Rinse the membrane briefly (< 1min) in 2xSSC, blot dry and fix the RNA by UV.

To check loading, stain the membrane in a solution of 0.04% methylene blue in 0.5M NaOAc pH 5.5, destain in water.

Hybridization
use 8.7ml of hybridization solution per 100cm2 of filter

1.4ml H_2O (0.46 vols.)
14µl SpermDNA (10mg/ml) (0.0046vols.)
heat at 95°C for 7min
rapidly chill on ice
add 1.6ml Scp/Sarc/DS Mix (0.54vols.)

Add the solution to filter in hybridisation-tube and incubate at 65°C for at least 30 min. Before adding the probe to the prehybridisation-solution, heat your probe at 95°C for 5min and chill on ice. Hybridize for at least 5 hrs.

Washing and autoradiography
After removing the probe wash the filter 2x briefly with 2xSSC, 0.1% SDS at RT and then 2x 20min in 0.5xSSC, 0.1% SDS at 43°C. Rinse in 3mM Tris-HCl pH 8 at RT, place your filter between 2 overhead-foils and autoradiograph with intensifying screen

10xMOPS: to 800ml of DEPC-treated water add 48.1 g MOPS, adjust pH to 7 with NaOH, add 16.6 ml of DEPC-treated 3M Na acetate and 20.0 ml of DEPC-treated 0.5 M EDTA pH 8, adjust volume to 1l with DEPC-treated water and filter. Keep at RT in a bottle wrapped in aluminium foil.

loading buffer: 1mM EDTA pH8, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol.

20x Scp: 2M NaCl, 0.6M Na_2HPO_4, 0.02M EDTA ph 6.2 (with HCl), autclave

Scp/Sarc/DS: Dissolve 20g of Dextran sulfate (500,000) in 60ml of 20x Scp, making up to 101 ml with H_2O. Then make up to a final vol of 108ml with 7ml of 30% SLS.

3.6 Northern dot blot
→ for dot blotting follow the instructions of the manufacturer

Sample preparation
- dissolve reasonable amount of RNA (10µg) in 10µl H_2O
- add 7µl 37% formaldehyde
  20µl 100% formamide
  2µl 20xSSC
- heat 15 min 68°C, cool on ice
- add 2 volumes (=78µl) of 20xSSC

Membrane preparation
- wet precut S+S Nytran for 10min in H_2O, then transfer for 10min to 6xSSC

Blot assembly
- place 1 sheet of GB003 blotting paper prewet in 6xSSC on filter support plate
- cover with equilibrated membrane and carefully assemble the clamps in anti-parallel direction

Sample application
- connect blotter with water pump and apply vacuum to a flow rate of approximately 1ml per minute per well
- wash wells 2x 500µl 6xSSC
- apply sample
- wash 1x500µl 6xSSC
- carefully open clamps in antiparallel direction and remove the membrane
- dry and crosslink with UV
→ hybridize as described in 3.5

3.7 Indirect immunofluorescence
adapted from Adams *et al*., 1997

- dilute overnight culture to OD_{600} = 0,2 and grow to 0.8
- to 4.5ml of your culture add 500µl 37% formaldehyde, and fix cells 1hr 30°C, shaking
- harvest 3min 2.5 krpm, resuspend in 1ml of spheroblasting premix and transfer to an Eppi, repeat 2 times
- resuspend cells in 500µl spheroblasting solution and incubate 45min in 30°C waterbath
- spin down cells for 3min at 3krpm, carefully resuspend in 1ml spheroblasting premix
- spin again 3min 3krpm, then carefully resuspend cells in 200µl spheroblasting premix
- coat a 10well slide with 0.02% polylysine, pipette 5µl onto the wells and wash off with distilled water under tap
- dry slide, then add 10µl of cell suspension
- after 4min suck off with water pump
- block with a drop of 1xPBS-1%BSA-0,05%NaAzid, aspirate off after 5 min
- add 1st antibody diluted in BSA-PBS-NaAzid, put slide on wet Kleenex on bench and cover with coated petri-dish top. Keeps it moist. Leave 2hrs RT.
- wash off antibody three times with15µl BSA-PBS-NaAzid-0.1% TritonX100, leave on well each time for about a minute or so
- add 2nd antibody diluted in BSA-PBS-NaAzid, cover Petri-dish with foil and keep it dark and moist for 1h at RT.
- wash three times in BSA-PBS-NaAzid-TritonX100.
- add1x PBS containing 10 µg/ml DAPI and incubate 5-10 min at RT. Wash once more with 1xPBS without DAPI.
- mount cells with 3µl 1xPBS/80% glycerol per well
- seal cover-slip with nailpolish

Solutions:
spheroblasting premix: 1.2 M sorbitol, 0.1 M Kphos pH 7.4, 0.5 mM MgCl2

spheroblasting solution: 1ml spheroblasting premix
+ 2 µl mercaptoethanol / ml.
+ 100 µg zymolase 100T, freeze-dried

3.8 'Fluorescence *in situ* hybridization', FISH

I. Hybridization with Cy3-labeled oligos
-all solutions must be DEPC-treated / RNase-free

Coating of multi-well-slides:
- wash slides with water, dry
- add 5µl of 0,02% poly-L-lysine on each well, let stand for 3 min
- aspirate off, dry
- wash slides in a jar, 3 x 10 min with DEPC-water
- airdry, store 4°C

Oxalyticase preparation:
- make a 2mg/ml solution of oxalyticase in 20mM Kphos pH 7.5.
- aliquot 50µl into microfuge tubes and dry in speedvac, store pellets at -20°C.

Oligo aliquots:
1.0µg ml probe
1.0mg/ml *E.coli* tRNA
1.0mg/ml salmon sperm DNA

→ aliquot 10µl into microfuge tubes, dry in speedvac and store -80°C

Solutions:
BufferB: 1,2M sorbitol,100mM Kphosphate pH7,4

spheroblasting buffer: 720µl 1,4xbuffer B, 3.5µl AEBSF, 100µl VRC, 3µl Rnasin, 2µl β-mercaptoethanol, 171.5µl water
→ add 1ml of the spheroblasting buffer to oxalyticase tube, incubate for 1min at RT and vortex before adding to the cells

hybridization solution:
prepare freshly and keep solution 2 on ice
1) 49,3µl formamide (mol. boil. grade)
0,63µl Naphos 1M pH 7
11,7µl water
2) 12,3µl BSA (20 mg/ml)
36,2µl water
12,3µl 20xSSC
0,75µl RNasin

- get oligo aliquot from –80°C, keep on ice and dark
- add 15µl solution 1 to one oligo, incubate 3min 80°C
- add 15µl solution 2 and resuspend carefully
- centrifuge15 min full speed

Prepare cells:
- dilute over-night culture to OD_{600} = 0.3 and grow until 0.8
- fix 9ml with 1ml 37% formaldehyde, shake 1h 30°C
- harvest 2min, 3000rpm and resuspend in 3ml cold BufferB, repeat 2 times
- harvest again, then resuspend in 1ml BufferB and transfer to Eppi
- harvest 30seconds full speed table-top centrifuge
- resuspend cells in 200µl sphaeroblasting buffer and incubate10min in 30°C waterbath
- spin down cells (1min, 3000rpm), wash cells one more time with 1ml BufferB
- spin down and resuspend the pellet in 150µl BufferB
- per well add 5µl cell suspension and keep 30min 4°C
- aspirate off the cells and wash once with BufferB
- aspirate off buffer and de-hydrate in a jar of 70% EtOH -20°C for at least 20min

In situ hybridization:
- prepare hybridization solution
- re-hydrate in a jar of 2x SSC, 5min RT
- pour off and add 2 x SSC/40% formamide, freshly prepared, 5min RT
- pour off and transfer slide into dark and moist chamber (large petri-dish covered with aluminium foil)
- add 5µl hybridization solution per well and cover with large cover-slip
- hybridize for at least 6hrs 37°C

Washing and DAPI stain:
- wash two times 15min with 37°C prewarmed 2xSSC/40% formamide, freshly prepared at 37°C
- wash one time with 2xSSC/0,1% triton X-100, 15 min RT , shaking
- wash two times with 1xSSC15min RT, shaking
- stain with 1xPBS/100ng/ml DAPI, 10µl on each well,10 min RT
- wash 10 min with 1x PBS RT
- mount cells in 3µl mounting solution per well, put a large cover-slip on the slide and seal with nailpolish

II. Hybridization with DIG-labeled probe

Probe preparation:
PCR 4-5 elements of +-250bp length, flanked by a suitable promoter for *in vitro* transcription, within the mRNA you want to detect
→ for ASH1 use template #88, and primer-pairs #369/367, #368/373, #374/370, #375/376, #372/371

→ use PCR products as templates for *in vitro* transcription (T7-Mega-Shortscript-kit)

mix 2µl10x transcription buffer
1,5µl ATP (75mM)
1,5µl CTP (75mM)
1,5µl GTP (75mM)
1,2µl UTP (75mM)
2µl DIG-UTP (10mM)
2µl enzyme Mix
50-200ng/µl template DNA
→ to 20µl with nuclease-free water

- incubate for 2hrs 37°C
- add 1µl DNase I (2U/µl)
- incubate for 15min, 37°C
- check the reaction on a gel
- purify the reaction using the 'Mini Quick Spin RNA Columns'
- phenol-chloroform-extract and EtOH precipitate overnight
-resuspend the pellet in 15µl water, mix 1µl of each element, estimate your amount of RNA on a gel, store the RNA at -80°C

Prepare the cells as described in part A

Hybridization:
-dilute your RNA-probe to a concentration of 100ng/µl
-for hybridization you need an end-concentration of 5ng/µl of your probe - dilute with the HybMIX to this concentration
-prepare the HybMIX (40ml for one jar)
20ml formamide100%
10ml 20xSSC
400µl 500mM EDTA;pH8
400µl 10% Tween
800µl 50x Denhardt's
400µl 10% CHAPS
400µl tRNA, 10mg/ml
400µl H.sperm, 10mg/ml
400µl Heparin, 10mg/ml
6,8ml water

-re-hydrate the slides in 5x SSC for 5min
-pre-hybridize in the HybMIX for 1h RT
-put your diluted probe on the well, put a coverslip on the slide and place it in a wet chamber
-hybridize ON, 37°C

Washes and detection:
-wash 15min 37°C with 2xSSC/40%formamide (pre-warmed)
-wash two times 15 minutes RT with 1xPBS/10%FCS/0,1%Triton
(this solution is also needed for the dilutions of the antibodies)
-add 1st antibody mouse-anti-DIG 1:250, incubate 2hrs RT
-wash 3 times with 1xPBS/0,1%BSA (leave on well for 1 minute, then aspirate off)
-add 2nd antibody rabbit-anti-mouse Alexa 488 1:2000, incubate 1hr RT
-wash 3 times with 1xPBS/0,1%BSA (leave on well for 1 minute, then aspirate off)
-optional: add 3rd antibody goat-anti-rabbit Alexa 488 1:10000, incubate
-wash 2 times with 1xPBS/0,1%BSA (leave on well for 1 minute, then aspirate off)
-stain with1xPBS/100ng/ml DAPI 10min RT
-wash 1 time with 1xPBS/0,1%BSA (leave on well for 1 minute, then aspirate off)
-mount the slide with 1xPBS/80%glycerol
-seal with nail-polish

III. Combined FISH and indirect immunofluorescence
- prepare cells and hybridize as described in part I
- after hybridization keep the slide moist and dark

Washes and immuno-fluorescence:
- two times with prewarmed 2xSSC/40% formamide, 37°C
- one time 2xSSC/ 0,1% triton, 15min, RT , shaking
- one time1x SSC, 15 min
- one time PBS, 15 min
- add first antibody: dilute with 1xPBS/1%BSA/0,05%NaAzid, incubate 2hrs, RT
- wash 2 times BSA(1%)-PBST(0,1%Triton), each for 5min
- wash 5min with 1xPBS
- add second antibody: dilute with 1xPBS/1%BSA/0,05%NaAzid, incubate 1h, RT
- wash three times with 1x PBS
- stain with1xPBS/100ng/ml DAPI,10min, RT
- wash 10min with1x PBS in jar
- dry, mount and seal as described

3.9 Yeast genomic DNA prep

- start with 5ml of YEPD stationary phase cells
- spin cells down 2-3min in benchtop 2-3000rpm
- add 1ml water to cell pellet, resuspend by vortexing and transfer to a 1.5ml Eppi
- spin 20sec in microfuge, suck off water
- resuspend cells in 0.2ml of SCE/zymolase/2-ME mix, incubate 37ºC with occasional shaking for 30-60min
- add 0.2ml of SDS solution, mix and vortex
- heat at 65ºC for 5min
- add 0.2ml 5M-KOAc, again mix by vortexing
- leave at 0ºC for 20min
- spin at high speed for 5min in microfuge, transfer 0.4-0.5ml supernatant to a fresh tube
- add 0.5 ml ethanol, mix well and spin at low speed for 15-30sec in an angle microfuge
- pour off supernatant, remove remaining sup by aspiration.
- completely dissolve pellet in 270µl TE, then + 1µl RNase A (1mg/ml), leave slowly shaking at 37ºC for 30min.
- add 30µl 3M-NaOAc (pH 5.2), mix and then add 300µl phenol
- spin 5min full speed and transfer upper phase into fresh tube
- add 0.6ml ethanol, mix and centrifuge 1min full speed
- rinse pellets in 70% EtOH, remove drops of 70% EtOH by a low speed spin followed by aspiration and dissolve pellets in 30µl of TE, store at +4ºC

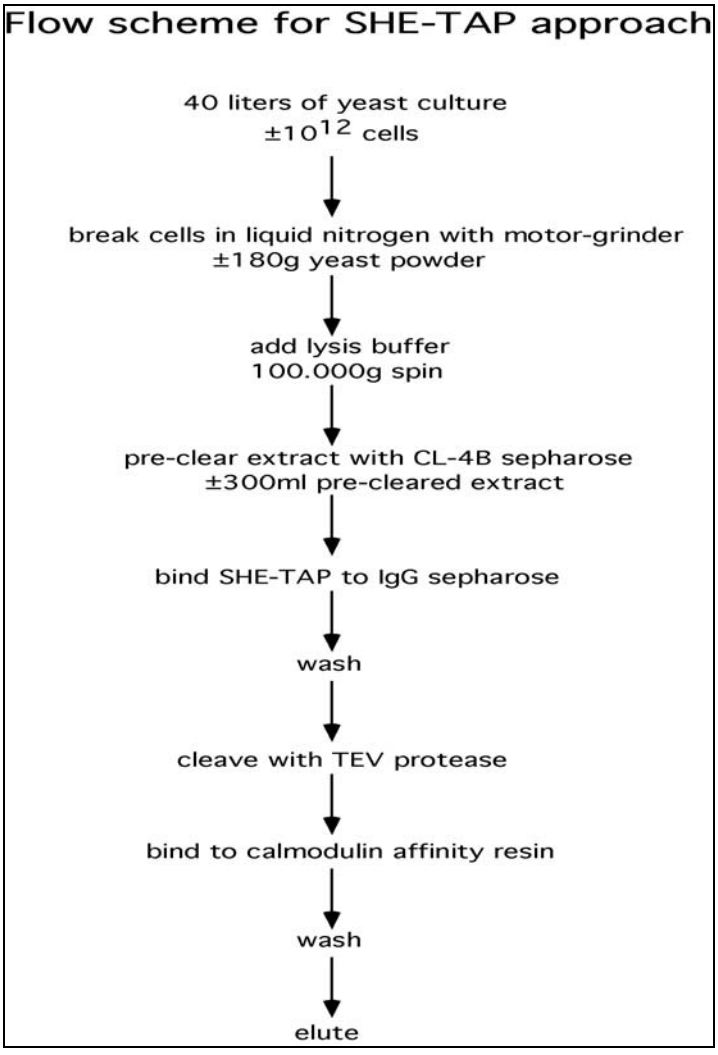
Solutions:

SCE:	1M-sorbitol, 0.1M-Na citrate, 0.06M-EDTA pH7.0
SCE/zymolase/2-ME mix:	to each ml of SCE add : 1mg zymolase 20000 and 8µl ß-mercaptoethanol
SDS solution:	2% SDS, 0.1M-Tris/Cl (pH 8.0), 0.05M-EDTA, 5M-KOAc

3.10 Large scale She 'tandem affinity purification', TAP

- you need 200g of yeast powder to get nice bands on a colloidal coomassie stained gel, corresponds to +- 50 liter culture

BREAKAGE	PRE-CLEAR	CALM.BEAD EQUI.
20mM HEPES, pH 8,0	20mM HEPES, pH 8,0	20mMHEPES,pH8,0
100mM KCl	100mM KCl	100mM KCl
0,15% NP40	0,1% NP40	0,1% NP40
1,5mM MgCl2	1,5mM MgCl2	1,5mM MgCl2
1mM DTT		0,5mM DTT
0,1mg/ml tRNA		2mM CaCl2
0,1mg/ml heparin		
1x protease inhibitor cocktail	ELUTION BUFFER	
	20mM HEPES, pH 8,0	
	5mM EGTA	



Starting material
- grow cells to OD_{600} = 3,5 and harvest by 10min spin 5krpm in GS3 rotor
- wash cells with cold water, spin as above
- transfer to in smallest water volume possible to open SS34 tubes and pack cells 10min 6,7 krpm in SS34 rotor
- remove all water
- use spoon to transfer cellmass into 50ml syringe
- press cells through syringe directly into grinder filled with liquid nitrogen
- manually pre-grind cells, thereby smash largest pieces, store -80°C

Breakage
- pre-cool motor grinder 2x with liquid nitrogen
- set grinder to position 7, add +-20g frozen yeast (1 filled 50ml Falcon) and grind for 12min, continually add smashed dry ice
- transfer yeast powder back into 50ml Falcon

Pre-clear extract
- weigh powder and prepare amount of "breakage buffer": use 1,3ml buffer per 1g powder
- place sterile 2 liter glas beaker into hot water bath and dissolve powder in buffer
- after powder is dissolved completely transfer suspension to GS3 bucket, cool down on ice
- spin 10min 5krpm in GS3 rotor and transfer sup to pre-cooled Ti45 ultracentrifuge tubes
- spin 1hr 30krpm (100.000g)
- meanwhile equilibrate 12ml CL4B sepharose in "pre-clear buffer"
- remove fatty upper phase and carefully transfer extract to a fresh GS3 bucket
- add equilibrated CL4B sepharose and rotate 1hr 4°C
- in the cold room load over large yellow BIURAD column to remove beads and collect extract
- add 5% glycerol, final, distribute to 50ml Falcons, snap-freeze in liquid nitrogen and store -80°C

Affinity purification
- equlibrate 0,5ml IgG sepharose in "pre-clear buffer"
- defreeze extracts in 50°C waterbath, then cool on ice
- pool extracts in GS3 bucket, add equilibrated IgG sepharose and rotate 2hrs 4°C
- spin 3,5krpm, mark position of bead pellet and carefully remove extract with a 25ml pipet
--> you may save the extract and store -80°C
- transfer last +- 40ml extract to 50ml Falcon, wash bucket with 10ml "pre-clear buffer+0,5mM DTT" and add to Falcon
- spin 5 min 4°C 2000krpm and carefully remove extract
- transfer beads to MOBICOL (small inserted filter, 35microns pore size) and empty by gravity flow (subsequently always empty by gravity flow)
- screw 20ml luer-lock syringe onto the MOBICOL and wash with 10ml "pre-clear buffer+0,5mM DTT"

- close MOBICOL bottom, add 150ul "pre-clear buffer+0,5mM DTT", 4ul TEV protease and close top with srew cap
- rotate 1hr 19°C, use hybridization oven placed into cold-room
- meanwhile equlibrate 600ul calmodulin affinity resin in "calm. bead equi. buffer", transfer to fresh MOBICOL, empty by gravity flow and close bottom
- carefully open TEV-MOBICOL, place into 2ml eppi without cap and collect TEV eluate by 15 seconds spin 2krpm 4°C
- determine volume of TEV eluate, add 2mM CaCl2, final, and load onto calm bead MOBICOL
- close top with screw cap and rotate 1hr 4°C
- elute by gravity flow, screw on 20ml syringe and wash with 5ml "calm. bead equi. buffer"
- close bottom, add 400ul "elution buffer", close top and warm tube 10min in hand
- place into 2ml eppi without cap and collect eluate by 15 seconds spin 2krpm 4°C
- TCA precipitate and analyze

3.11 Site-directed mutagenesis of the *ASH1* coding sequence

- *ash1* non sense mutations have been created by a 2 step PCR-based 'mega-primer' protocol, in any case using template pBluescript II KS-ASH1 (pRJ 256)
- the first PCR product containing the desired mutation has served as 'mega-primer' for a subsequent second PCR
- the product of the second PCR has been subcloned into pRJ 256, sequenced and then cloned into yeast/E. coli shuttle vectors

ash1-stop E1 construct:
1st PCR primer pair RJO 213 / RJO 215
2nd PCR primer pair product of PCR1 / RJO 142

ash1-stop E2 construct:
1st PCR primer pair RJO 214 / RJO 211
2nd PCR primer pair product of PCR1 / RJO 142

ash1-stop E3 construct:
1st PCR primer pair RJO 217 / RJO 218
2nd PCR primer pair product of PCR1 / RJO 142

ash1-E2B-stop construct:
1st PCR primer pair RJO 290 / RJO 217
2nd PCR primer pair product of PCR 1 / RJO 142

YEplac181-ash1-E2A-stop (pRJ 334) has been created as follows:
PCR A) primer pair RJO 466 / RJO 467, template pRJ 332
PCR B) primer pair RJO 465 / RJO 420, template pRJ 332
→ PCR product A was digested NcoI / XbaI, PCR product B was digested SacII / XbaI and both fragments were ligated SacII / XbaI in a triple ligation into pRJ 88

3.12 Live cell imaging of cells expressing Myo4-2xGFP
as described in Kruse *et al*., 2002

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 3.01 software (Improvision).

- in brief, for widefield fluorescence microscopy cells were grown with vigorous shaking in selective media to cell densities below 10^7cells/ml
- 3µl cell suspension were dropped onto a microscope slide that has been coated with a pad of the respective media containing 2% agarose, trimmed to a square of approximately 15x15mm
- for temperature shift experiments, agarose pads were equilibrated to the respective temperature before adding cells, images were acquired directly after adding cells

Time-lapse imaging of MYO4–2xGFP strains RJY1310 (wt) or RJY1405 *(Δ she2*) at 30°C was performed using a PerkinElmer Ultraview confocal microscope equipped with a Nikon 100x NA 1.3 oil objective (PerkinElmer). Cells were attached to coverslip chambers (MatTek) coated with 5 mg/ml ConA and directly observed in SC-trp medium. Due to the weak signal of Myo4–GFP, only five focal planes (0.5-µm distance in Z) per stack were acquired with a 450-ms exposure time at 2 x 2 binning, resulting in a total acquisition time of 2.5 s per stack. A total of 50 stacks were acquired per experiment (125 s total). Focal planes of each stack were projected by maximum intensity projection using the ImageJ software package (NIH). Before exporting as Quicktime movies, the resulting images were filtered to remove noise. Single frames from representative movies were exported into Adobe Photoshop 6® and assembled.

FLIP was performed on an LSM 510 confocal microscope essentially as previously described (Ellenberg *et al*., 1997; Daigle *et al*., 2001). The LSM 510 was fitted with selected PMTs (Carl Zeiss MicroImaging, Inc) and custom dichroics and emission filters (Chroma Technology Corp.) for fluorescent protein imaging using a PlanApochromat 63x NA 1.4 oil DIC objective (Carl Zeiss MicroImaging, Inc) and an optical section of 2.5 µm (FWHM). Circular bleach regions were defined to cover 50% of the mother cell, taking care not to directly bleach the bud itself (Fig. 7). Low intensity scanning to monitor intensity changes after bleaching did not significantly reduce fluorescence over the time of the experiment, as verified in control cells in the same field (not depicted). Bleaching was performed at an intensity 200 times higher than scanning.

3.13 Temperature shift experiments with *mex67-5* cells
as described in Kruse *et al*., 2002

- cells were grown in selective media to cell densities below 10^7cells/ml at 26°C
- harvest by low speed spin in equilibrated centrifuge buckets at RT and resuspend in the same volume of 36°C prewarmed medium
- for transcription induction from the GAL promoter, cells were grown overnight in selective medium containing 2% raffinose / 0.02% glucose, diluted the next morning to OD_{600} = 0.3 in selective medium 2% raffinose only and grown until OD_{600} = 0.6, then 2% galactose, final, was added and yeast were cultivated for another 3hrs
- for simultaneous detection of Myo4-HA and She2-myc in mex67-5 cells, yeast were grown overnight in selective medium containing 2% raffinose / 0.02% glucose at 26°C
- next morning cells were diluted in pre-equilibrated selective medium containing 2% raffinose only to OD_{600} = 0.3 and grown at 26°C until OD_{600} = 0.6
- galactose was added to a final concentration of 2%, and cells were grown another 3hrs at 26°C
- cells were harvested by low speed spin in equilibrated centrifuge buckets at RT and resuspended in 37°C prewarmed selective medium containing 2% galactose only
- aliquots were taken at indicated timepoints and processed for indirect immunofluorescence as described above

3.14 TCA protein precipitation
- add 2% deoxycholate Na salt, DOC to 0.02% final (for 100µl sample, add 1µl 2% DOC)
- incubate RT for 15min
- add 24% TCA to 6% final (for 100µl sample, add 33.7µl)
- incubate on ice for 1hr
- spin at 4°C for 10min, remove supernatant with drawn-out Pasteur pipette
- add 200µl of ice cold acetone to TCA pellet
- 15min on ice
- spin at 4°C for 10min
- remove supernatant as before, dry pellet for 1-2min.
- resuspend pellet in 1X PAGE sample buffer → to gel.

If bromophenolblue dye in the 1xPAGE buffer turns yellow after adding to the protein pellet, raise pH by adding few µl of 1M Tris-base.

3.15 Mutagenesis of Myo4-2xGFP
(C. Mund, M. Lopez de Heredia and A. Jaedicke)

- a 2.8 kb SacI / EcoRI fragment including the Myo4p ATPase and actin binding region from pRJ 12 has been subcloned into pRS303
- the resulting plasmid served as template for site directed mutagenesis:
a) for loop mutant: template pRS303-SacI-myo4 head/ATPase-EcoRI construct
primers RJO 1020 / RJO 1021
b) for actin binding mutant: template as above
primers RJO 1022 / RJO 1023
- plasmids have been amplified in *E. coli*, analyzed by restriction digest and sequencing
- positive clones have been linearized with NcoI, followed by integration into RJY 1310 and selection on -his
- transformants have been analyzed by colony PCR using primers RJO 1036 / RJO 1037 for the actin binding mutant, and primers RJO 1037 / RJO 1038 for the P-loop mutant

3.16 Yeast cell breakage with glass-beads
- start with 15-20 OD_{600} units of exponentially growing cells
- spin down cells (2' at 3000rpm) and resuspend cell pellet in 1ml H_20
- transfer to Eppendorf tube, spin down cells 10-15sec at full speed and discard supernatant
→ from now on do everything on ice, using cold solutions

- resuspend the sediment in 2x its volume (i.e. 300-400 µl for 20 OD units) of the breaking buffer: 50 mM Tris pH 7.5, 20 mM DTT, 1x 'Protease Inhibitor Cocktail'
- add a bit less than an equal volume of acid-washed glass beads (there should be some liquid above the beads) and mix on the IKA vibrax at 4 ºC for 2x 3-5 min with a 2 min break on ice
- take 10 µl of the extract for protein determination: spin this aliquot (5 min max. speed), take 0.2 µl (i.e. 1 µl of 1:5 dilution) and 1 µl for the Bio-Rad protein assay
- transfer 120µl of cell extract to new tube and add 40µl of 4xSDS loading buffer, boil for 5 min (or 95 ºC).
- spin down, then take the supernatant. Load 70 µg (minigel) to 150 µg (large pocket gel) per lane, or load 15 or 20µl, respectively, if you have not determined protein concentration

4. Results

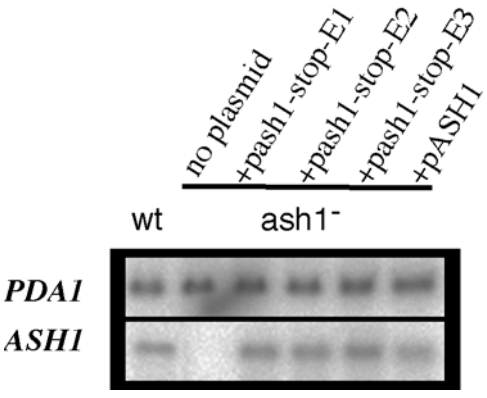
4.1 *ASH1* mRNA localization depends on translation

In earlier site-directed mutagenesis studies it was shown that the position of a premature stop codon directly downstream of the start codon, disruption of the start codon or a premature stop in the middle of the coding sequence severely impairs *ASH1* mRNA anchorage. Interestingly, adding back an exogenous wt *ASH1* copy could not rescue the mislocalization phenotype of the non sense *ASH1* transcript, indicating that *ASH1* mRNA anchorage depends on translation of the Ash1p COOH-terminus (Gonzalez *et al*., 1999). Another hint that tight *ASH1* localization might depend on translation stems from the observation that anchorage is perturbed in cells lacking Khd1p, an RNA-binding protein implicated in translation repression of *ASH1* (Irie *et al*., 2002).

4.1.1 Insertion of premature stop codons into the *ASH1* coding sequence results in aberrant mRNA localization

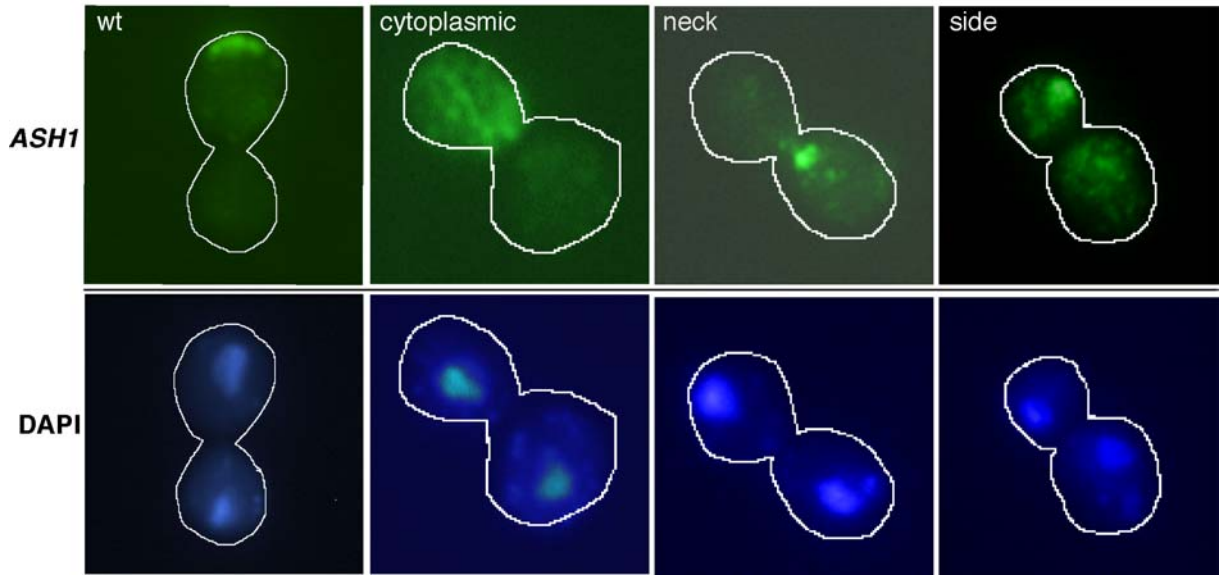
Four localization elements are required for *ASH1* mRNA anchorage (Chartrand *et al*., 1999; Chartrand *et al*., 2002). In order to investigate the role of translation through the localization elements in the anchorage process, premature stop codons were positioned immediately upstream of each localization element by site-directed mutagenesis.

ARS/CEN low copy plasmids harboring the respective *ash1* mutants or wt *ASH1* under transcription control of the *ASH1* promoter were transformed into *Δ ash1* strain RJY 585. Transcription of the respective candidate mutants was verified by Northern blot analysis. Surprisingly, at steady-state non sense *ASH1* mRNA levels were indistinguishable from wt *ASH1*, indicating that the transcripts were stable and not affected by mRNA surveillance mechanisms (reviewed by Vasudevan and Peltz, 2003).



Non sense *ASH1* is transcribed in comparable amounts to wt *ASH1*.
Equal amounts of total RNA derived from wt (RJY 358) and *Δ ash1* (RJY 585), harboring centromeric low copy plasmids with non sense mutant (RJY 621; stop E1, RJY 622; stop E2, RJY 623, stop E3), or wt *ASH1* (RJY 620) were separated by electrophoresis and blotted. The membrane was cut and hybridized against *ASH1*, bottom, and *PDA1*, top, transcripts as loading control.

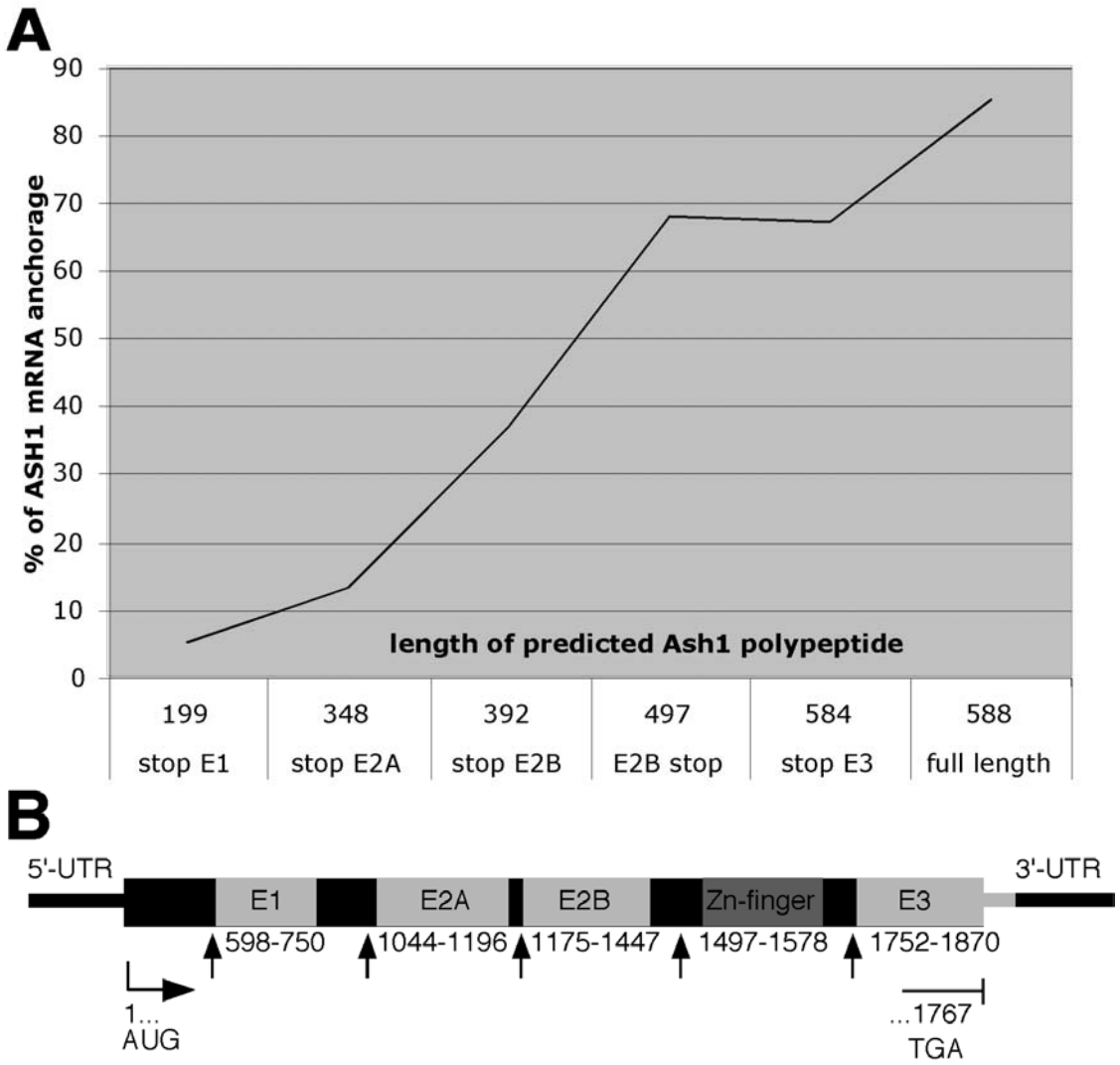
High copy plasmids of the respective non sense *ash1* mutants were transformed into *Δ ash1* strain RJY 585. Compared to wt, non sense *ASH1* mRNA localization was severely impaired, showing a variety of mislocalization phenotypes. Instead of tight anchorage at the bud tip, non sense *ASH1* mRNA was either found as spread over the entire bud- or the mother-bud cytoplasm, concentrated at the mother-bud junction, or mistargeted to the bud side.



Defective localization of non sense *ASH1* mRNA.
High copy plasmids harboring non sense or wt *ASH1* including promoter and terminator regions were transformed into *Δ ash1* strain RJY 585, followed by visualization of the transcripts by FISH in late anaphase cells.
Transcript localization can be described in four mutant phenotypes: localization to the side cortex of the bud, to the neck region between mother and daughter cell, to the bud cytoplasm or to the cytoplasm of both mother and bud (not shown). No signals were detected in *Δ ash1* cells (not shown).

position of stop codon	**stop E1 RJY 602**	**stop E2A RJY 587**	**stop E2B RJY 836**	**E2B stop RJY 666**	**stop E3 RJY 586**	**wt RJY 588**
tight anchorage	**5%**	**13%**	**37%**	**68%**	**67%**	**85%**
mislocalized	**95%**	**87%**	**63%**	**32%**	**33%**	**15%**

Table: Quantitative analysis of nonsense *ASH1* mRNA anchorage.
200 anaphase cells each yeast strain expressing non sense or wt *ASH1* have been analyzed by FISH in double experiments. Anchorage was quantified using the categories 'tight anchorage' and 'mislocalized'.
The localization efficiency of non sense *ASH1* mRNA compared to wt was severely reduced, showing a gradual increase relative to the length of the predicted Ash1 polypeptide. Mutants that harbored a stop codon before the localization elements E1, E2A or E2B failed to anchor the mRNA at the bud tip, whereas the 'E2B stop' and 'stop E3' mutants displayed only modest anchorage defects compared to wt.

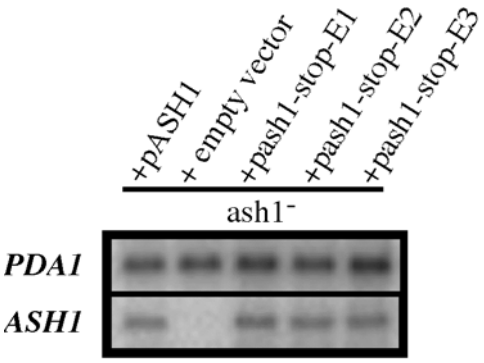


Anchorage efficiency of non sense *ASH1* mRNA as a function of Ash1 polypeptide length.
A) The anchorage efficiency of the generated *ash1* mutants and wt *ASH1* has been plotted against the length of the predicted Ash1 polypeptide. The curve shows a gradual gain until localization element E2B, then drops back to a plateau between E2B and E3, and ends in its maximum for anchorage of full length *ASH1* mRNA.
B) Schematic view of *ASH1* mRNA showing the position of the premature stop codons relative to the localization elements. Note the stretch between E2B and E3 that harbors a region encoding a putative zinc finger motif (see chapter 5.1.1).

In summary, non sense *ASH1* transcripts showed severe mRNA mislocalization defects in proportion to the predicted length of the Ash1 polypeptide: the shorter the truncated protein, the lower the mRNA anchorage efficiency. From this gradual correlation I conclude that *ASH1* mRNA localization depends on translation. Furthermore, and in agreement with Gonzalez *et al*. (1999), the experiment above suggests that translation of the Ash1p COOH-terminus is essential for tight mRNA anchorage.

4.1.2 Mutant *ash1* mRNA harboring a non sense stop codon is stable and remains unaffected by non sense mediated decay

During the course of the experiments the observation that *ASH1* mRNA containing a premature stop codon was as stable as wt *ASH1* transcripts was somehow unexpected.
One possible explanation why mutant *ASH1* mRNA is not a substrate for non sense mediated decay is the binding of the She2 protein to the *ASH1* localization elements (Böhl *et al*., 2000; Long *et al*., 2000). The finding that most mutant *ASH1* mRNA is still transported into the daughter cell but not correctly anchored to the bud cortex led to the assumption that She2p is still able to bind to the mutant *ASH1* mRNA. If so, She2p might in a way compete with other RNA-binding proteins required for non sense mediated decay. She2p binds to all four localization elements, but so far the exact footprint of the protein on the mRNA has not been mapped. Therefore it is possible that this footprint overlaps with the regions in *ASH1* mRNA that contain the inserted premature stop codons. Binding to mutant *ASH1* mRNA might mask the RNA from recognition of factors required for non sense mediated decay.
In order to test for this hypothesis, steady-state mRNA levels were compared to each other in *she2* null mutant background.

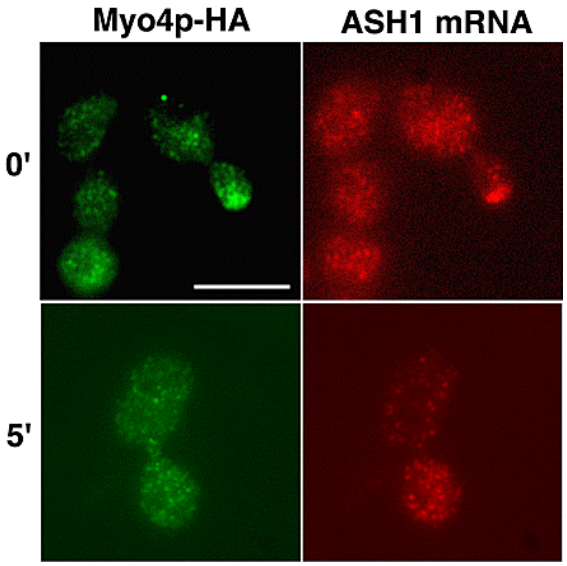


***ASH1* mRNA harboring premature stop codons is as stable as wt *ASH1*, even in *Δ she2* cells.**
Equal amounts of total RNA from a *Δ ash1 Δ she2* strain harboring low copy plasmids with wt *ASH1* (RJY 940), empty vector (RJY 939), *ASH1* stop E1 (RJY 941), *ASH1* stop E2 (RJY 942) and *ASH1* stop E3 (RJY 943) have been separated by gel-electrophoresis, blotted and hybridized with specific probes against *ASH1*, bottom or control *PDA1*, top.

Similar to wt *SHE2* cells in the preceding chapter, non sense *ASH1* mRNA is as stable as wt *ASH1* in *Δ she2*. Though containing premature stop codons, the mRNA is not degraded and remains unaffected by non sense mediate decay. I conclude that She2p is not involved in molecular turnover mechanisms of *ASH1* mRNA.

4.1.3 Brief treatment with the translation inhibitor cycloheximide interferes with tight *ASH1* mRNA- and Myo4p localization to the peripheral bud cortex

If *ASH1* mRNA anchorage is mediated by ongoing, local translation at the cortical ER, an artificial translation block should result in defective *ASH1* mRNA localization. For that purpose cells were treated for 5 minutes with cycloheximide, a drug that arrests nascent polypeptide chains at the ribosome exit channels, thereby freezing the peptide in the state of synthesis (Obrig *et al*., 1971, and references therein).



Cycloheximide addition disrupts *ASH1* mRNA and Myo4p localization.
Cells expressing Myo4-HA_6 and *ASH1* (RJY 1581) were treated with cycloheximide (100µg/ml final concentration). Aliquots were taken before (t = 0') and 5 min (t = 5') after treatment, and processed for imaging of Myo4-HA_6 by indirect immunofluorescence (1^{st} ab 3F10, 1:50, 2^{nd} ab goat anti rat, Alexa 488, 1:100) and *ASH1* mRNA by FISH (right). Bar, 5 µm.

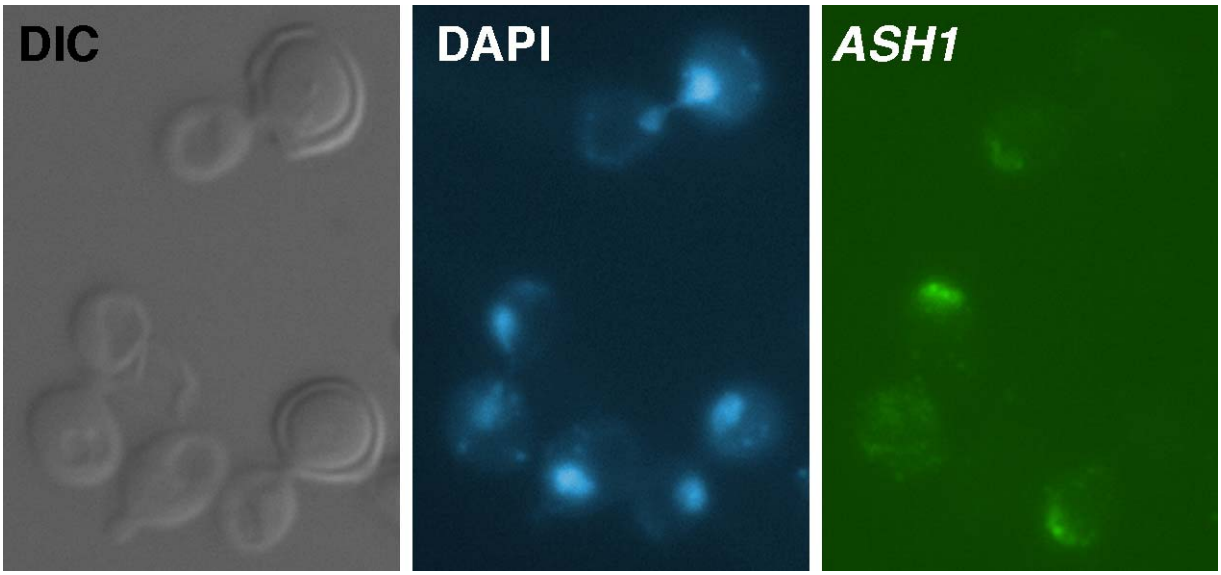
At standard growth conditions *ASH1* mRNA and Myo4-HA_6 partly co-localize at the bud periphery. After 5 minute treatment with cycloheximide neither *ASH1* mRNA nor Myo4-HA_6 remain associated to the bud cortex, but instead distribute as cytoplasmic background staining. This observed mislocalization phenotype is reminiscent of the anchorage defects presented in chapter 4.1.1., again suggesting a requirement for active translation to tightly localize *ASH1* mRNA to the bud cortex.

4.2 RNP-dependent localization of Myo4p

A useful approach to study RNP behavior in living cells is to label one component of the complex with a fluorescent dye, GFP. Although yeast cells are too small to allow micro-injection of *in vitro* labeled RNA directly into the cytoplasm (Cha *et al*., 2001), several approaches have been taken to investigate *ASH1* mRNA *in vivo* dynamics by different means (Bertrand *et al*., 1998; Beach *et al*., 1999; Takizawa *et al*., 2000). The generally used 'green RNA system', initially developed by Bertrand *et al*. (1998) is based on the fusion of GFP to the MS2 RNA-binding protein that becomes tethered to the highly over-expressed *ASH1* 3'-UTR hybrid harboring recognition motifs for MS2. Although it became possible to track GFP signals through the yeast cytoplasm, a major disadvantage is that experimental conditions are very different from the wt situation. For example, strong overexpression of the target mRNA does not reflect cell cycle regulated expression of *ASH1*, (Bobola *et al.,* 1996; Cosma *et al*., 1999). However, since there was reason to speculate on a whole set of mRNAs transported by the She machinery we wanted to investigate Myo4p localization *in vivo*, thereby not focussing on only one cargo but covering the entire She protein-mediated RNP dynamics. GFP-tagging of the motor responsible for the driving force of the RNP and probably the component the furthest away from the target mRNA should minimize sterical competition between factors required to establish a functional RNP.

4.2.1 Myo4-2xGFP localizes to growing bud tips in an RNP-dependent manner

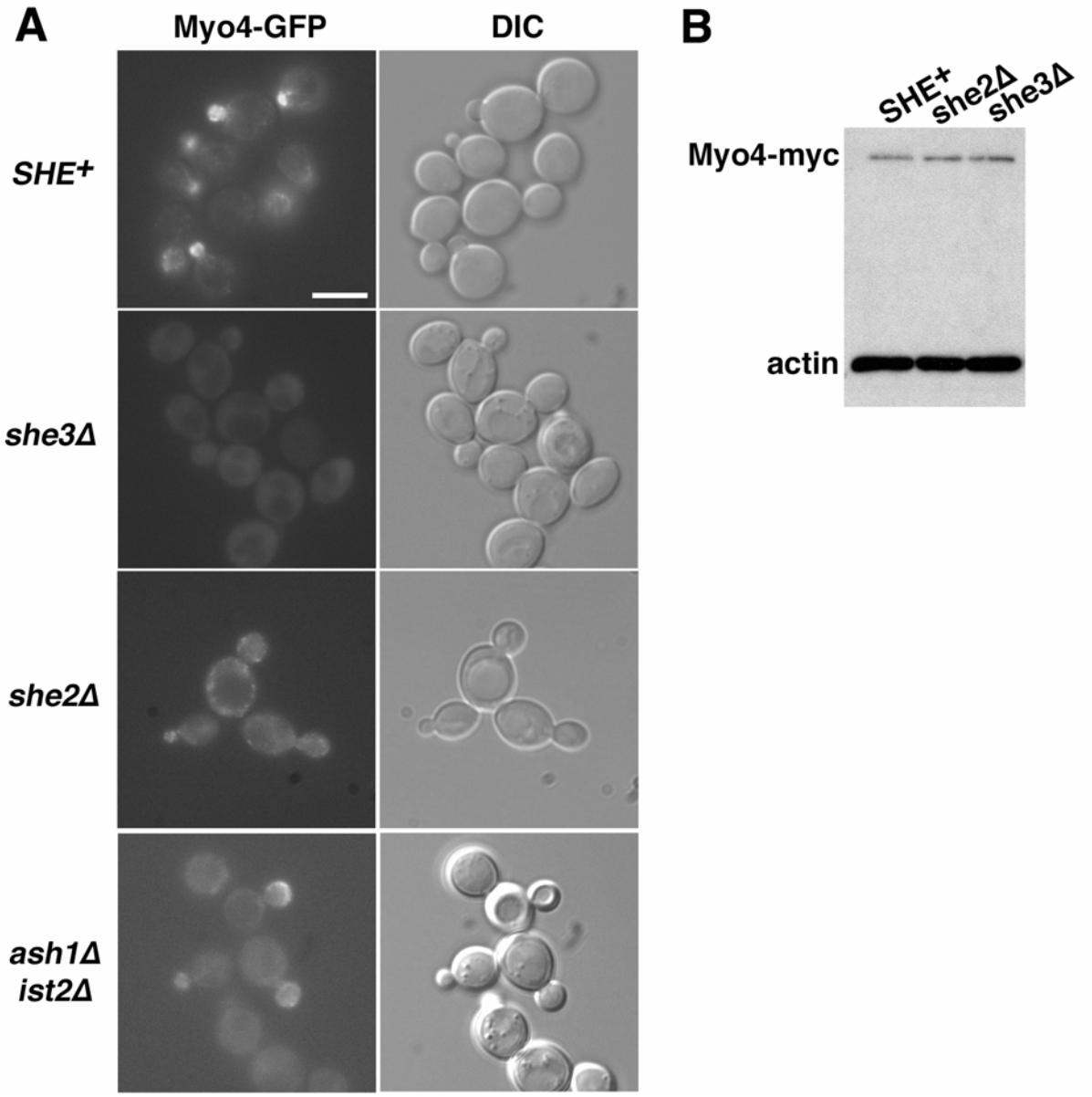
MYO4 was genomically tagged with tandem 2xGFP, resulting in a stably expressed COOH-terminal Myo4-2xGFP fusion protein under transcriptional control of the wt *MYO4* promoter. Prior to Myo4-2xGFP *in vivo* imaging it was tested if the fusion protein is functional in respect to tight *ASH1* mRNA localization.



***ASH1* mRNA localizes as tight crescent at bud tips in a strain stably expressing Myo4-2xGFP.** Cells of strain RJY 1616 have been processed for FISH and hybridized with *ASH1* mRNA-specific DIG-labeled oligos.

Cortical *ASH1* mRNA localization is not impaired by the Myo4-2xGFP fusion, suggesting that the bulky tag does not interfere significantly with interaction partners required for transport or efficient anchorage of *ASH1* mRNA.

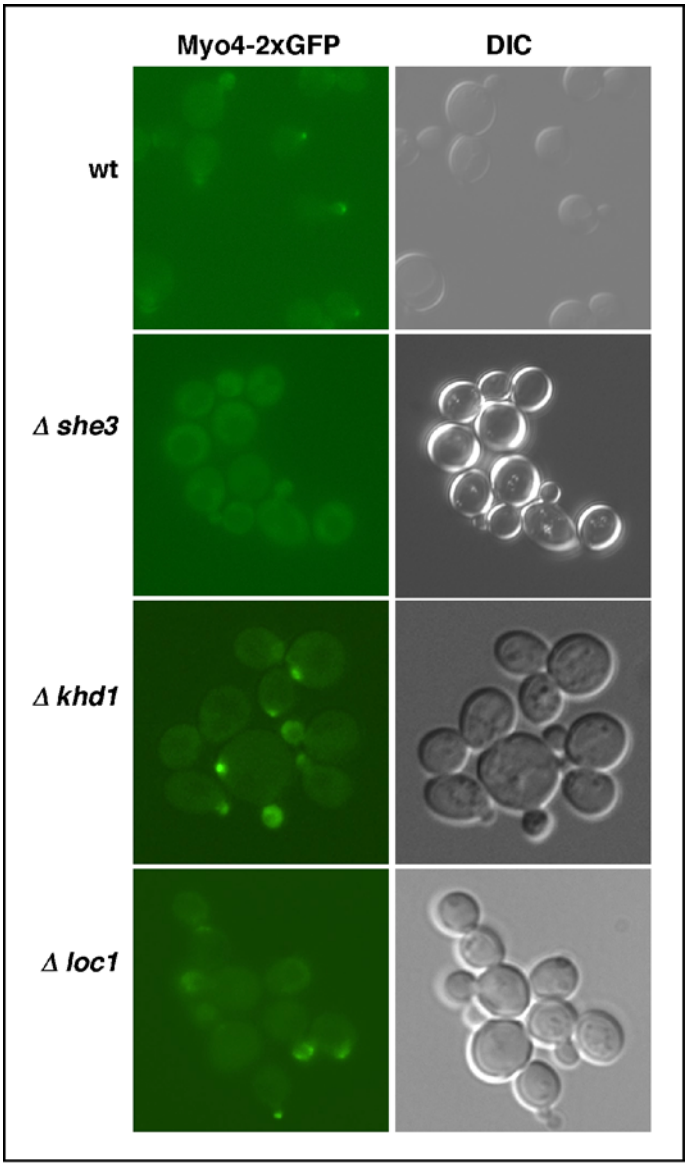
In subsequent experiments, *in vivo* localization of Myo4-2xGFP was imaged in wt cells as well as strains deleted for components of the core She machinery (*SHE2*, *SHE3* or *ASH1 / IST2*), or previously suggested additional She-like factors (*KHD1* and *LOC1*).



She-dependent localization of Myo4-2xGFP (Kruse *et al*., 2002).
A) Live cell imaging of cells expressing Myo4-2xGFP (strains RJY 1310 wt , RJY 1304 *she3 Δ*, RJY 1405 *she2 Δ*, and RJY 1624 *ash1 Δ ist2 Δ*). In wt cells Myo4-2xGFP localizes to the periphery of growing buds. Bud localization is strongly perturbed in *she* mutants, but is largely unaffected by deletions in *ASH1* and *IST2*.
B) Stability of Myo4p is unchanged in *she* mutants. Protein extracts of strains RJY 449 (wt), RJY 968 (*she2 Δ*) and RJY 617 (*she3 Δ*) have been normalized and analyzed by Western blot against Myo4-myc_6 and Act1p, loading control (anti myc: 1st ab 9E10 1:1.000, 2nd ab sheep anti mouse, peroxidase-conjugated 1:5.000; anti Act1p: 1st ab Mab414,1:5.000, 2nd ab sheep anti mouse, peroxidase-conjugated, 1:5.000).

The pattern of the GFP signals strongly resembled the distribution of a COOH-terminal Myo4-myc fusion (Jansen *et al*., 1996; Münchow *et al*., 1999 and unpublished data from C. Kruse), including complete failure to localize to bud tips in *Δ she3* or partially in *Δ she2* mutant cells. However, if deleted for two mRNA cargoes transported by the She machinery,

ASH1 and *IST2* (Takizawa *et al*., 2000), Myo4-2xGFP still localized efficiently to the periphery of growing buds. In addition, Western blot analysis revealed that loss of localized Myo4-2xGFP in *she* mutants was not caused by reduced protein stability compared to wt. It was previously suggested that Khd1p (Irie *et al*., 2002) and Loc1p (Long *et al*., 2001) are required for *ASH1* mRNA localization. Whereas Khd1p can bind to the N element of *ASH1* mRNA, and might function as translation inhibitor, the role of the strictly nuclear factor Loc1p is elusive. Being candidate members of the She machinery, the intracellular distribution of Myo4-2xGFP was followed in cells lacking Khd1p or Loc1p.

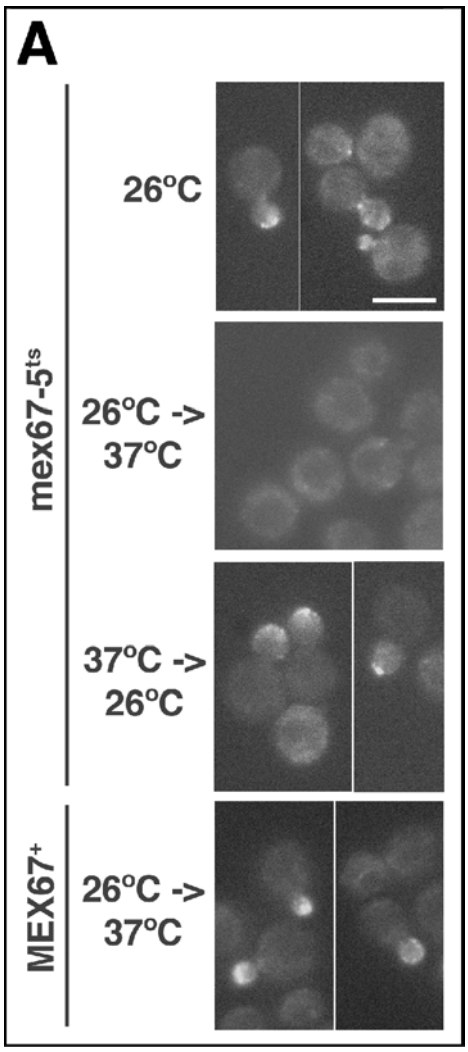


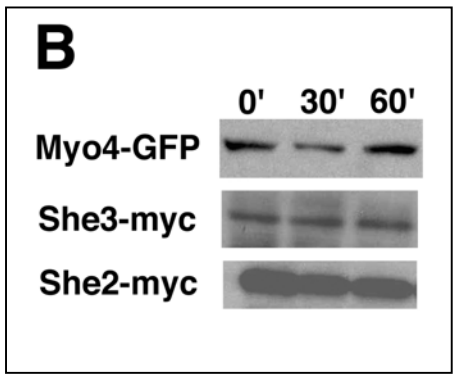
Myo4-2xGFP targeting to sites of polarized growth in cells lacking Khd1p or Loc1p.
Representative snapshots of cells expressing Myo4-2xGFP. Aliquots of liquid cultures from strains RJY 1310 (wt), RJY 1304 (*Δ she 3*), RJY 1933 (*Δ khd1*) and RJY 1579 (*Δ loc1*) were taken and analyzed by *in vivo* fluorescence microscopy. In contrast to *Δ she* mutants, Myo4-2xGFP localization is not affected in cells lacking Khd1p or Loc1p.

Unlike in *she* mutants, Myo4-2xGFP enriches to the periphery of growing buds in *Δ khd1* or *Δ loc1* similar to wt cells. The experiment suggests that the core She machinery is required and sufficient to mediate the cytoplasmic transport to the bud periphery, whereas Khd1p or Loc1p might be considered necessary for anchorage or nuclear packaging, respectively.

4.2.2 Myo4p targeting relies on bulk nuclear mRNA export

Based on the observation that Myo4p / She3p start localizing to growing buds early in G1 phase of the cell-cycle long before *ASH1* transcription is turned on (Jansen *et al*., 1996), and that Myo4-2xGFP localization is not perturbed in cells deleted for the two cargo mRNAs *ASH1* and *IST2*, there was reason to speculate on an additional set of *SHE*-transported mRNAs (Shepard *et al*., 2003; Takizawa *et al*., 2000). In order to investigate the dependence of Myo4p localization on mRNA association, Myo4-2xGFP was placed into a strain mutant for *MEX67* (*mex67-5*) that allows to temporarily block bulk nuclear mRNA export while leaving protein export unaffected (Segref *et al*., 1997; Hurt *et al*., 2000).

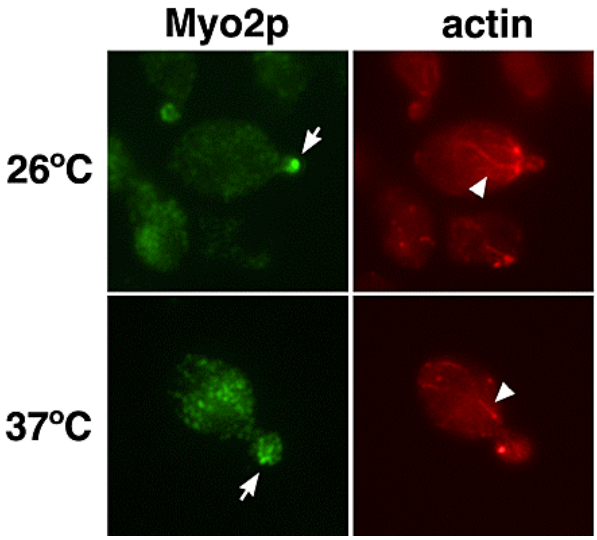




Defective Myo4-2xGFP localization in cells blocked for bulk mRNA export.
A) Shifting *mex67-5* Myo4-2xGFP cells (RJY 1303) from 26°C to the restrictive temperature of 37°C for 30 min completely abolished Myo4-2xGFP localization. When the incubation temperature was shifted back from 37°C to 26°C for 20 min, Myo4-2xGFP again localized to the periphery of growing buds. After temperature shift for 1hr, cortical Myo4-2xGFP localization was unaffected in control cells, *MEX67* (RJY 1343).
B) Temperature shift to 37°C does not affect cellular She protein levels. Western Blot analysis against epitope-tagged She proteins out of crude extracts derived from *mex67-5* cells (RJY 449, RJY 968, RJY 617) shifted to 37°C for 0', 30' and 60' (anti myc: 1st ab 9E10 1:1.000, 2nd ab sheep anti mouse, peroxidase-conjugated 1:5.000, anti GFP: 1st ab B34 1:25.000, 2nd ab sheep anti mouse, peroxidase-conjugated, 1:5.000).

Myo4-2xGFP localization to the bud periphery is lost in cells blocked for bulk mRNA export. The effect is reversible and *mex67-5* dependent since Myo4-2xGFP distribution remains unperturbed in *MEX67* control cells at elevated cultivation temperature. Furthermore it has been shown that steady state She protein levels remained unchanged over the time course of the experiment, ruling out possible localization defects due to limited stability the core She machinery.

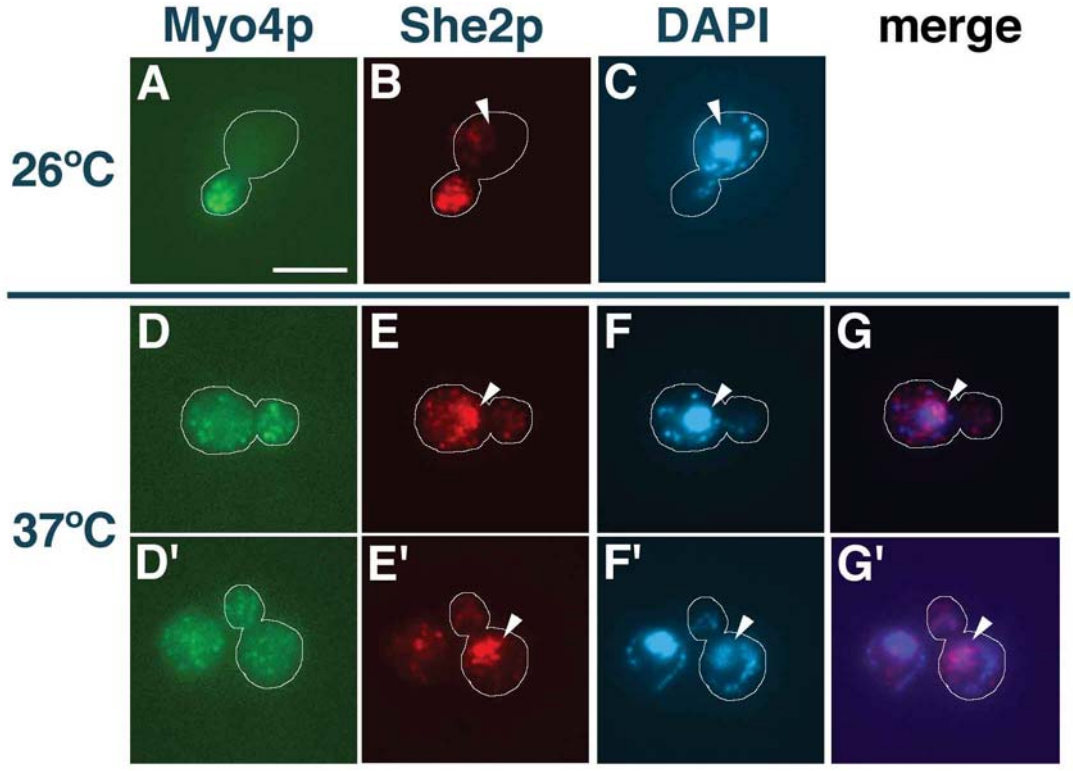
Bud localization of Myo2p and the integrity of actin filaments are not affected by the temperature shift induced mRNA export block.
Conditional *mex67-5* cells of strain RJY 612 have been shifted to 37°C for 30 min and processed for indirect immunofluorescence anti Myo2p and actin (anti Myo2p: 1st ab anti Myo2p tail, 1:1.000, 2nd ab goat anti rabbit, Alexa 488, 1:500; anti actin: phalloidin, rhodamine conjugated, 1:40).



Additional control experiments with *mex67-5* cells demonstrated that - in contrast to Myo4-2xGFP - Myo2p, the second class V myosin in yeast, accumulated in buds under restrictive conditions. Furthermore, polarized actin filaments, supposed to form the tracks for directed myosin-based transport, are still visible at a time when Myo4-2xGFP localization is completely lost. This experiment confirmed that the mislocalisation phenotype in cells blocked for RNA export is specific for Myo4p. Importantly, defective Myo4p localization is caused by a reduced motor activity and not a secondary effect due to cytoskeletal misorganisation.

4.2.3 She2p is a nucleus-cytoplasmic located shuttling mRNA binding protein

Earlier studies have shown that She2p tethers *ASH1* mRNA to the Myo4/She3p complex, and that She2p can be co-detected together with Myo4p / She3p and *ASH1* mRNA at the bud tip only if *ASH1* is highly overexpressed (Böhl *et al*., 2000; Takizawa and Vale, 2000; Long *et al*., 2000). In order to address the question if mRNA export influences on the localization of She2p the cellular distribution of Myo4p, She2p and *ASH1* mRNA was followed in *mex67-5* cells, either at 26°C or at 37°C.

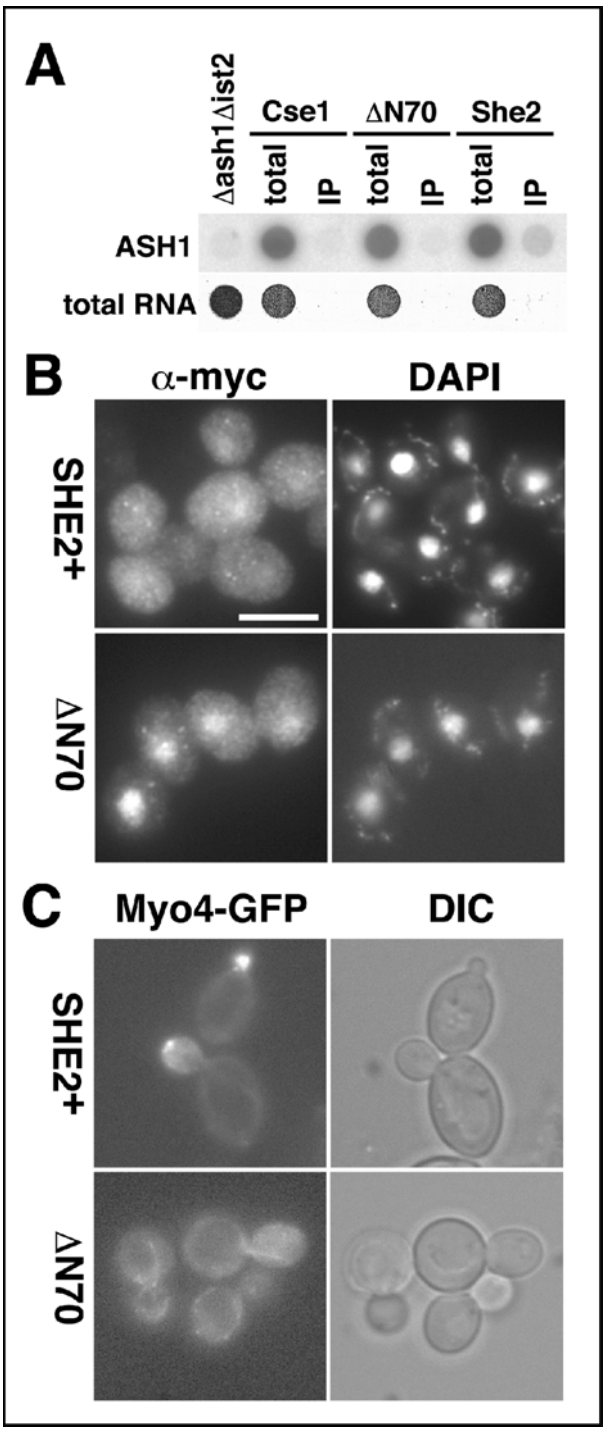


She2p enriches in nuclei upon inhibition of mRNA export.
mex67-5 cells carrying *ASH1* under control of the strong *GAL1* promoter and co-expressing Myo4-HA_6 as well as She2-myc_9 (RJY 1613) were grown at permissive conditions (A-C), then shifted to 37°C for 30 min (D-G) and processed for indirect immunofluorescence (anti HA: 1st ^{ab} 3F10 1:50, 2nd ab goat anti rat, Alexa 488 1:100; anti myc: 1st ab 9E10 1:500, 2nd ab rabbit anti mouse, Alexa 594 1:100). DNA was visualized by DAPI. Arrowheads mark the position of the nucleus.

She2p and Myo4p partly co-localize to the bud at 26°C. This pattern changed dramatically when the cells were shifted to the restrictive condition of 37°C: Both She2p and Myo4p were not found at the cellular periphery any more. Instead Myo4p delocalized to cytoplasmic background staining whereas a fraction of She2p accumulated in nuclei, suggesting that She2p can shuttle between the nucleus and cytoplasm. Cells with a nuclear She2p signal failed to accumulate Myo4p in the bud, indicating a need for She2p being in the cytoplasm in order to function in Myo4p localization to the bud.

4.2.3.1 She2p is exported from the nucleus in an mRNA dependent manner

To further investigate the relationship between proper targeting of Myo4p to the bud and functional RNP formation, I addressed the question if RNA-binding of She2p is essential for nuclear export. A *she2* mutant was generated that had lost the ability to bind *ASH1* mRNA, she2pΔN70 (D.Ferring in Kruse *et al.*, 2002). The idea that She2p's RNA-binding activity might be located within the NH2-terminus based on earlier studies following the localization of GFP-fused NH2-and COOH-terminal truncations of She2p (F. Böhl, unpublished).

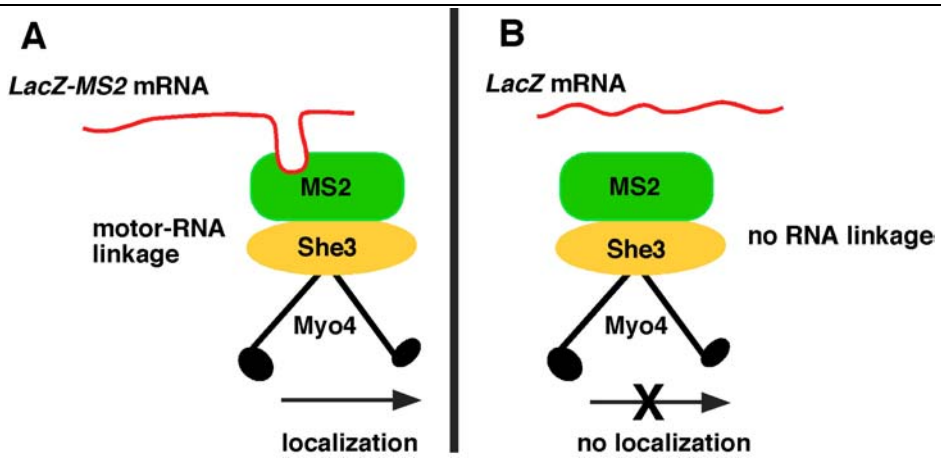


She2p localization relies on its association with mRNA.
A) she2pΔN70 does not bind *ASH1* mRNA.
she2pΔN70, the truncation mutant deleted for the first 70 amino-acids (RJY 1600), and wt She2p (RJY 1599) were expressed as myc-tagged versions in a strain background deleted for *she2*. Immunoprecipitation experiments followed by Northern dot blot analysis against *ASH1* mRNA demonstrated that wt She2p pulled down a substantial amount of *ASH1* mRNA whereas mutant she2pΔN70 as well as the control Cse1 (RJY 375) did not. Binding of the ASH1 probe was specific since no signal appeared for a crude extract derived from a *Δ ash1 Δ ist2* strain (RJY 1004).
B) she2pΔN70 accumulates in nuclei.
Indirect immunofluorescence against myc-tagged versions of wt She2p (RYJ 1599) and she2pΔN70 (RJY 1600) expressed in a *Δ she2* strain, left. A significant amount of she2pΔN70 overlaps with the nuclear DAPI staining whereas wt She2p is predominantly cytoplasmic (1st ab 9E10, 1:1000, 2nd ab goat anti mouse, Alexa 488, 1:500).
C) Myo4-2xGFP is mislocalized in cells expressing she2pΔN70.
Live cell imaging of Myo4-2xGFP in cells expressing she2pΔN70 (RJY 1528) and wt She2p (RYJ 1526). Myo4-2xGFP is targeted to growing buds in *SHE*$^{+}$ cells, whereas *she2ΔN70* cells distribute Myo4-2xGFP uniformly throughout mother- and bud-cytoplasm, left.

Summing up, she2pΔN70, a mutant that does not bind to *ASH1* mRNA anymore, accumulates in nuclei. Furthermore, Myo4-2xGFP fails to localize to growing buds in cells expressing she2pΔN70.

The data suggest that mRNA export is required for efficient cytoplasmic accumulation of She2p, and that RNA-binding of She2p is essential for its function in Myo4p localization.

The fact that localization of Myo4-2xGFP to the bud is perturbed in cells blocked for mRNA export could have been explained by two ways: Either Myo4-2xGFP is mislocalized due to the lack of She2p association with the Myo4p / She3p complex, or due to a lack of She2p-mediated mRNA linkage. Using an experimental system developed by Long *et al*. (2000) it became possible to differentiate between those two possibilities (C. Kruse in Kruse *et al*., 2002):



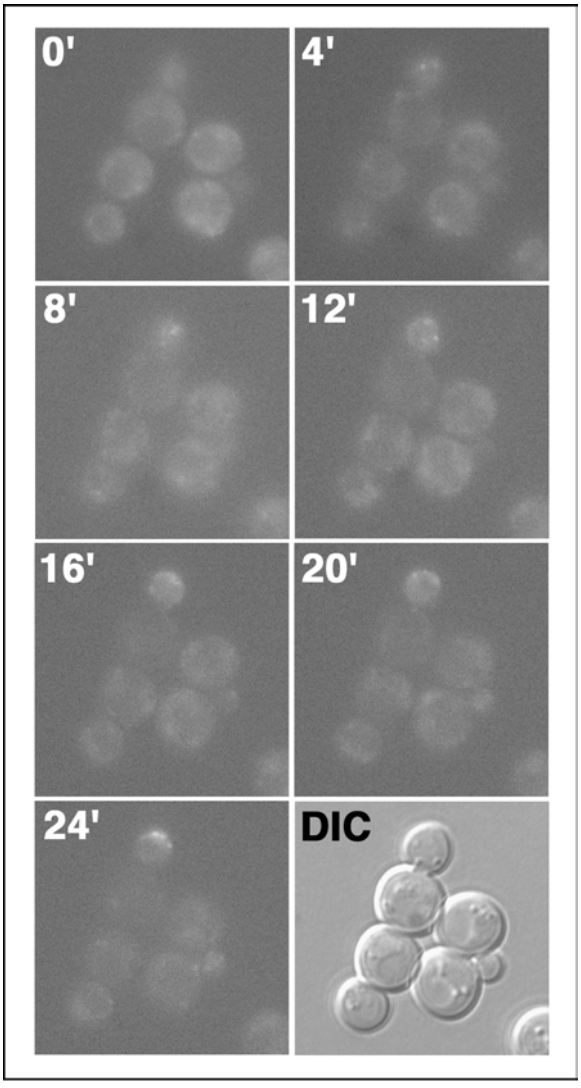
Cartoon: Myo4p targeting to the growing bud periphery depends on RNA association.
A She3-MS2 hybrid protein was expressed together with *LacZ* mRNA (containing a recognition motif for the MS2 RNA-binding protein, A, or blank, B) in a *Δ she2* strain harboring genomic Myo4-2xGFP. Myo4-2xGFP was detected at the tip of growing buds only in situation A, but not in B.

LacZ mRNA harboring an MS2 binding motif is capable to mediate Myo4p enrichment to the bud, in the absence from She2p. The experiment described above suggests that general RNA linkage to the Myo4p / She3p complex is sufficient for Myo4p and hence She RNP localization.

4.2.4 Myo4-2xGFP dynamics in wt *SHE2* versus mutant *she2* cells

4.2.4.1 Reversible and rapid Myo4-2xGFP localization in *mex67-5* cells reconstituted for nuclear mRNA export

It was reported that in *mex67-5* cells block of nuclear mRNA export mediated by a temperature shift was reversible (Segref *et al*., 1996), and thus could be released by shifting the restrictive temperature from 36°C back to permissive 26°C. Now similar conditions were chosen to investigate Myo4-2xGFP localization in dependence on release from mRNA export block. In order to study the dynamics of mRNPs, especially the cortical association in dependence of nuclear mRNA export, the GFP-signal monitoring intracellular distribution of Myo4-2xGFP was tracked by time-lapse microscopy.



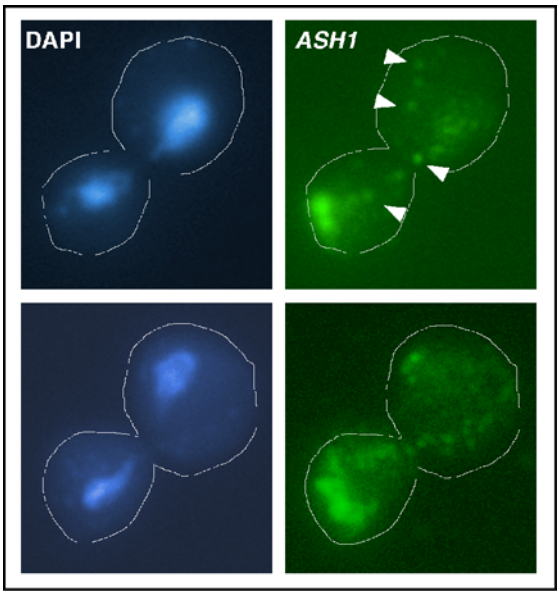
Reversible Myo4-2xGFP localization upon reconstitution of RNA export. *mex67-5* Myo4-2xGFP cells (RJY 1303) were cultivated at 26°C, shifted for 30 minutes to 36°C, then back-shifted to 26°C. Images were taken at indicated timepoints. Myo4-2xGFP fluorescence recovered at the bud tip starting from 8 min after release from RNA export block.

The appearance of Myo4-2xGFP signals at the bud cortex of *mex67-5* cells after backshift to the permissive temperature correlates with the timeframe observed for nuclear mRNA release following export block (Segref *et al*., 1996), presenting further evidence for RNA-dependent Myo4-2xGFP localization. In addition, individual time-lapse images describe cortical Myo4-

2xGFP particles as highly dynamic structures. (see following chapter) Compared to *ASH1* mRNA granules observed by Bertrand *et al*. (1998), Myo4-2xGFP particles could not be tracked in transit all the way from the mother cell cytoplasm through the neck junction, but rather become visible the first time within the vicinity of the cortical periphery of the growing bud.

4.2.4.2 RNA is transported in form of large cytoplasmic granules

The cytoplasmic She RNP has been described as large assembly composed of the minimal transport machinery and the RNA cargo. One tool to visualize the cytoplasmic transport complex in transit has been the 'green RNA' approach (see chapter 4.2). The combination of this method with indirect immunofluorescence against She proteins has allowed a co-staining of protein- as well as RNA-moieties of the She RNP in so-called 'RNA granules'. By video microscopy, Bertrand and co-workers (1998) have tracked a single fluorescent granule on its cytoplasmic transit from the mother into the bud. Furthermore they estimated an average number of 1-4 granules per cells. However, since this technique relies on high overexpression of the target RNA as well as the MS2-GFP detector element, any conclusions about the composition and intracellular dynamics of the wt She RNP must be considered with precaution. In order to investigate cytoplasmic *ASH1* mRNA transport and stay as closely to wt conditions as possible, FISH has been performed in cells overexpressing full length *ASH1* from the endogenous, cell cycle regulated promoter.

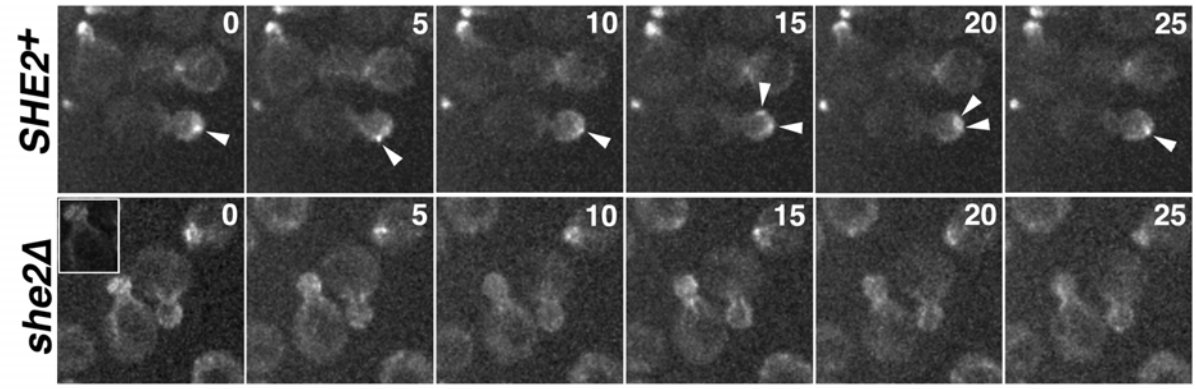


***ASH1* mRNA transport intermediates align on the cytoplasmic route similarly to beads-on-a-string.**
Images of cells from strain RJY 588 that have been processed for FISH against *ASH1* mRNA, right. Arrowheads mark examples of multiple small dots that represent candidates for transport intermediates, aligned in a given direction towards the bud periphery. Left, nuclei stained with DAPI.

Compared to the 'green RNA' approach, FISH has revealed an increased number of potential transport intermediates. Simply speaking, the granules seem to stick to a route that spans the mother cytoplasm and guides the RNP into the bud. As initially suggested by Long *et al*. (1997), this route most likely represents of polarized actin filaments.

4.2.4.3 Cortical Myo4-2xGFP particles are highly dynamic structures

In order to study Myo4-2xGFP dynamics with higher resolution, particles were video-taped by time-lapse confocal microscopy (A. Jaedicke and T. Güttler in Kruse *et al*., 2002). Exploiting the increased accuracy offered by confocal microscopy, it became possible to detect Myo4-2xGFP in both wt and *Δ she2* cells.



Myo4-2xGFP particle dynamics in wt and *Δ she2* cells.
Confocal live imaging of Myo4-2xGFP as described in methods chapter 3.12.
In wt cells (RJY 1310) Myo4-2xGFP particles dynamically roll over the cortical periphery of the growing bud. Large, dot-like assemblies eventually splitted and reformed again into larger dots (arrowheads, see figure 10s-25s interval).
In *Δ she2* cells (RJY 1405) Myo4-2xGFP fails to form discrete transport intermediate granules and can be detected at the bud periphery only if imaged with increased gain settings. Compared to Myo4-2xGFP in wt *SHE2*, particle-like structures in *Δ she2* cells were more in number but therefore smaller. The small dots failed to remain temporarily attached to the cortex, and therefore were predominantly localized to the inner cytoplasm. Eventually assembled into larger aggregates, the highly unstable structures rapidly disintegrated into tiny dots (see figure 5s-10s interval).
Note that the Myo4-2xGFP series in *Δ she2* was performed with increased gain settings over wt *SHE2*. For comparison the insert (in panel *Δ she2*, 0 s) is included with identical settings as those used for wt.

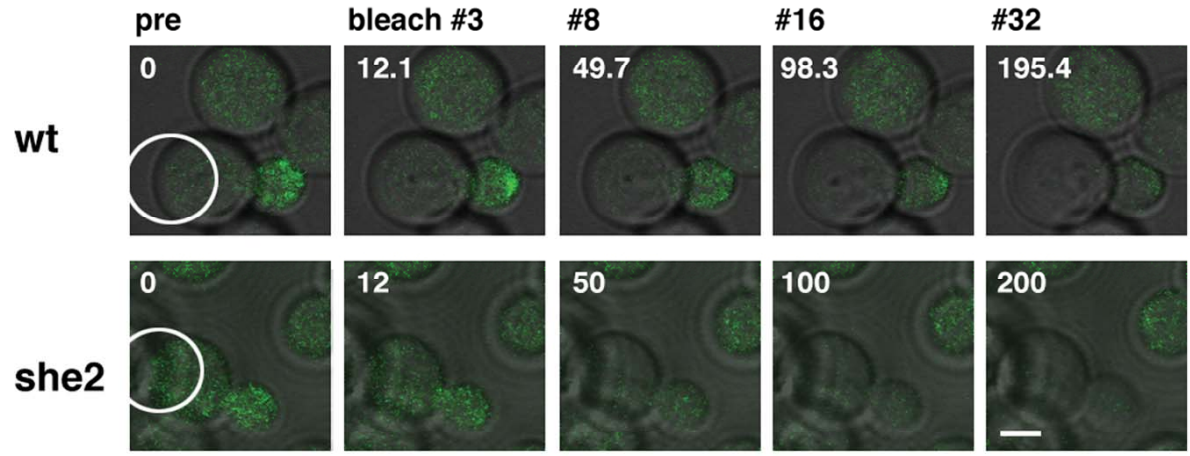
Myo4-2xGFP dynamically localized as cable-like filaments within mother cells. This staining was more intense in *Δ she2* and *she2ΔN70* cells than in wt *SHE2* cells, and was reminiscent to dotty 'beads-on-a-string-staining' for *ASH1* mRNA observed by FISH (see chapter 4.2.4.2 and Münchow *et al*., 1999). However, since it is unlikely that the small dots visualized in *Δ she2* cells contain RNA cargo, they might represent a novel kind of transport intermediates, harboring a cargo different from RNA (see discussion part 5.3.1).

Whereas Myo4-2xGFP in wt *SHE2* cells accumulated in growing buds, dynamically rolling along the cortex, *Δ she2* cells displayed signals of similar intensity throughout the entire mother- and daughter cell cytoplasm. Tracking GFP fluorescence by time-lapse microscopy enabled us to observe a prolonged retention time for enlarged Myo4-2xGFP particles in the bud of wt *SHE2* versus *Δ she2* cells. In addition, particles visualized in wt *SHE2* cells followed a uni-directional movement from the mother cell through the neck into the growing bud, whereas in *she2* mutants we frequently saw a reverse flow-back from buds into the mother.
To summarize those observations, Myo4-2xGFP particles are highly dynamic structures that constantly undergo disassembly into smaller parts, followed by re-formation of larger aggregates. In wt *SHE2* cells the particles remain permanently associated with the bud cortex, whereas in *she2* mutants Myo4-2xGFP stains throughout mother- and daughter cytoplasm.

4.2.4.4 Transient Myo4-2xGFP retention to the bud depends on She2p

Myo4-2xGFP concentration at the peripheral cortex occurred only in wt *SHE2* cells, suggesting that cortical retention most probably prevented the RNP from flow-back into the mother cell. In contrast, *Δ she2* mutants unable to transiently tether the Myo4p particle to the cortex rather displayed a general cytoplasmic fluorescent signal, most likely due to diffusive exchange of smaller particles through the mother-bud junction.
Out of these data it was hypothesized that She2p might be involved in the retention of Myo4-2xGFP to the bud. In order to analyze if She2p carries out a rate-limiting step of exchange of Myo4-2xGFP between the mother and bud compartment, we made a 'fluorescence loss in photo-bleaching', FLIP, assay (Lippincott-Schwartz *et al*., 2001). By comparing the fluorescence residence time in buds of bleached mothers in wt versus *she2* mutant cells, it should be possible to differentiate between a simple diffusion exchange and a She2p dependent, transient retention mechanism.

FLIP was performed on Myo4-2xGFP wt and *Δ she2* cells with middle-sized buds. The mother cell was selectively photo-bleached every 5 s for a total up to 10 min, and the depletion of fluorescence in the bud was followed over time (A. Jaedicke and T. Güttler in Kruse *et al*., 2002).



FLIP of Myo4-2xGFP in wt and *Δ she2* cells.
Frames of confocal images of a representative FLIP experiment using cells expressing Myo4-2xGFP in wt (RJY 1310) and *Δ she2* (RJY 1405). For technical details see methods chapter 3.12.
The bleached region in the mother cell is indicated in the first frame prior to the initial bleach. The number of bleaches and time in seconds are indicated. Note that the contrast for the dimmer *Δ she2* cells was enhanced for visualization. Bar, 2μm.

Compared to wt cells, the bud of *Δ she2* cells lost fluorescence rapidly and completely, with kinetics only slightly slower than the directly bleached mother cell. Wt cells showed a significantly retarded depletion of bud fluorescence under identical conditions, but eventually could also be depleted completely during a 10 min FLIP experiment (data not shown, see Kruse *et al*., 2002). This, and the fact that shorter bleach intervals did not lead to a faster depletion of bud fluorescence (unpublished data), strongly argued that the depletion of Myo4-2xGFP was only limited by the exchange between bud and mother.

In order to describe a possible retention mechanism that could lead to a better understanding how the concentration of Myo4-2xGFP in buds of wt cells is achieved, the depletion kinetics were analyzed with the help of a computer simulation (J. Beaudouin in Kruse *et al*., 2002), from which two main conclusions were drawn: First, the exchange of *free* Myo4-2xGFP between bud and mother is limited by diffusion. Second, the retaining interaction of Myo4-2xGFP in the bud depends on She2p, is short lived (< 100 s), and in constant exchange with the soluble pool in both bud and mother cell.

4.3 Biochemical purification of the She RNP complex using TAP

The She proteins, factors required for *ASH1* mRNA localization but not for cell viability, have been identified using genetic screens. At least one additional protein required to establish Ash1p asymmetry, Khd1p, has been found by other means (Irie *et al*., 2002). In order to identify possible other physically attached factors that might have been missed during the genetic screen - such as factors required for cell viability - and to allow a detailed biochemical as well as functional characterization of the RNP complex, I decided to purify the 'She RNP complex'.

Since the success of a biochemical purification procedure is based on the choice for the purification strategy and the optimal starting material, various aspects had to be considered in advance. Based on the experience of successful efforts in genome-wide high throughput screenings mapping protein-protein interactions of the yeast proteome (Gavin *et al*., 2002), the method of choice for purification of native protein complexes has been 'TAP, tandem affinity purification' (reviewed by Puig *et al*., 2001).

Since the aim was the purification of the complete and intact She RNP particle, protein and RNA degradation events were reduced to a minimum. For that purpose the cell-breakage conditions as well as buffer compositions were optimized. For example, to keep RNA intact cells were shock-frozen and mechanically lysed in liquid nitrogen (Schultz, 1999).

Transcription from the *ASH1* locus is cell cycle regulated (Bobola *et al*., 1996), the transcript short-lived and the She RNP-based transport of *ASH1* mRNA into the bud is accomplished within a very narrow time window (Bertrand *et al*., 1998). To circumvent those obstacles, the initial starting material for the purification was particularly enriched for She RNP particles in the state of transportation using point mutants of the motor protein Myo4p. By that means the She RNP was artificially frozen within a transit state, unable to reach its final destination, namely the bud tip, but free and sterically accessible as bait (see below).

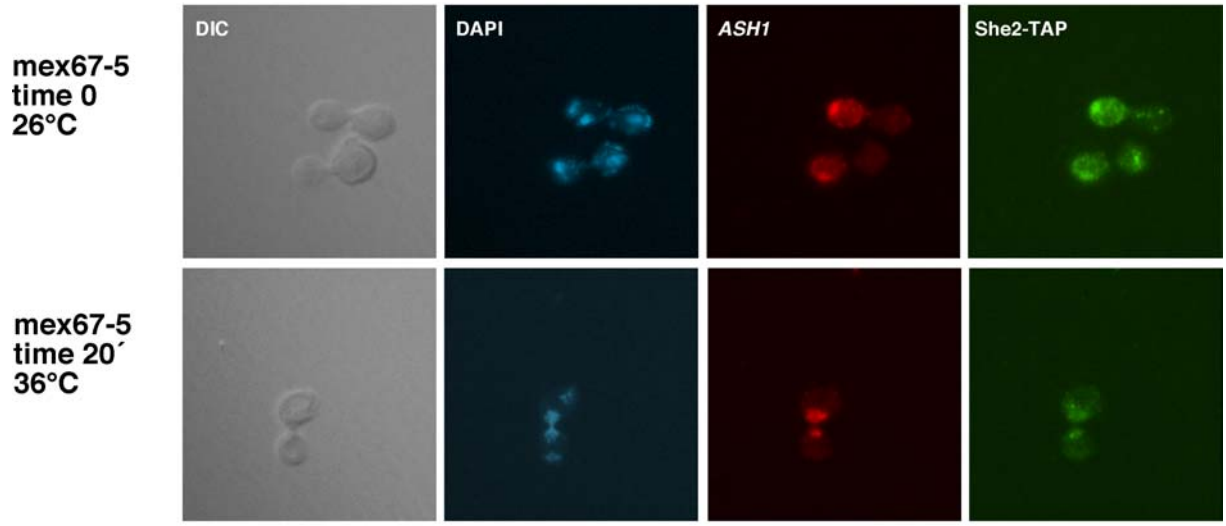
The components of the She machinery are expressed in low copy number (Huh *et al*., 2002). In order to allow the purification of sufficient material for the following identification of protein factors by mass-spectrometry, the culture volume had to be increased to about 40 liters, roughly 20fold compared to the initial introduction of the TAPtag by Rigaut and colleagues (1999).

Increasing the starting material on the one hand allows not only a quantitatively more efficient and specific affinity-based purification of the target protein complex. On the other hand it also results in higher background-binding of disturbing, unspecific contaminants to the chosen affinity matrix. Those have to be clearly distinguished from the actual components of the

purified assembly. For that purpose the result of each purification was confirmed by at least one control experiment under identical experimental conditions. To be able to sort out contaminants, unspecific binding was monitored by parallel purification from a TAPtag-less yeast strain, starting with 1.5fold increased material over the actual She-TAP experiment. In addition, using two already characterized components of the same complex as bait in separate purifications should principally yield a similar, if not identical, protein pattern. Thus, as internal control, Myo4p and She2p both were used as bait proteins for TAP.

4.3.1 She2-TAP is fully functional in respect to *ASH1* mRNA localization and nuclear accumulation upon mRNA export block

Prior to the preparative purification of the She RNP complex it had to be assured that the fusion of the TAPtag to the bait protein - She2p or Myo4p - did not interfere with the protein's cellular function. In co-localization studies the cellular distribution of She2-TAP and *ASH1* mRNA was monitored, both in wt and *mex67-5* cells blocked for nuclear mRNA export.



RNA export block results in nuclear accumulation of *ASH1* mRNA and She2-TAP.
Cells of strain RJY 983 have been shifted to the indicated temperatures and processed for combined FISH against *ASH1* and indirect immunofluorescence against She2-TAP (1st ab anti protein A, 1:100.000; 2nd ab goat anti rabbit, Alexa 594 coupled, 1:500).
In order to check for localization and dynamics of She2-TAP a temperature sensitive allele of the essential mRNA export factor *MEX67* has been used, *mex67-5*. Upon *ASH1* overexpression induced from the strong *GAL1* promoter, She2-TAP and *ASH1* mRNA co-localized to the peripheral cortex of large budded cells at permissive temperature. After 20-min shift to the restrictive temperature of 36°C, both She2-TAP and *ASH1* mRNA enrich in the nucleus.

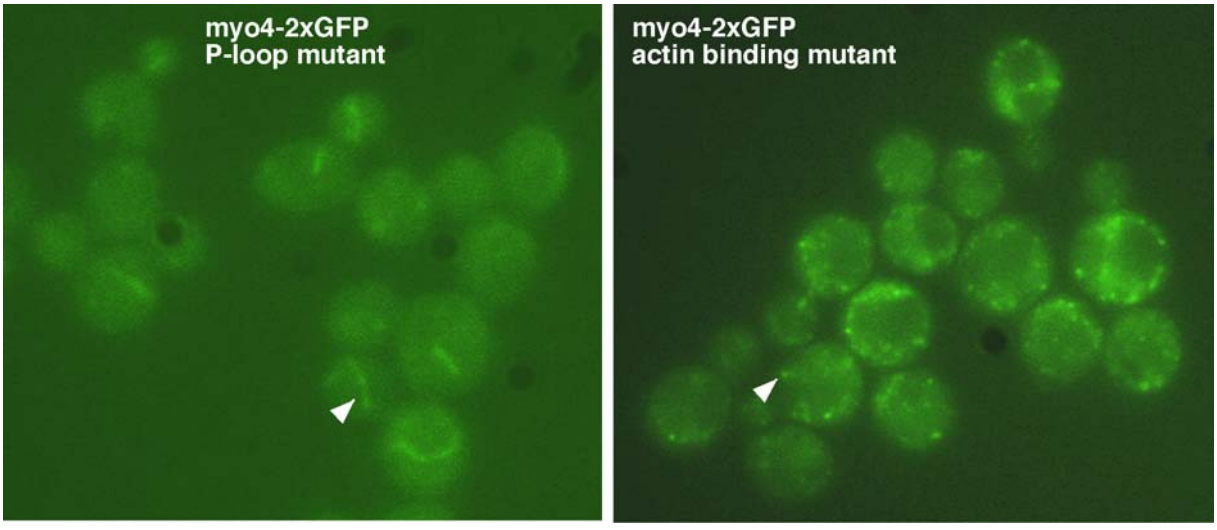
She2-TAP behaved indistinguishable from She2-myc (see chapter 4.2.3), suggesting that the fusion to the TAPtag did not block any known cellular function of She2p. Thus, She2-TAP can serve as bait to purify the She RNP complex.

In similar experiments cells harboring Myo4-TAP have been analyzed in double-experiments together with wt cells (data not shown).

4.3.2 myo4p mutants allow an enrichment for the She RNP complex in transit

As mentioned earlier, transport of the She RNP complex is accomplished within only a brief period of time before anaphase of the cell cycle. The Myo4p motor protein is regarded as the complex' molecular locomotive responsible to deliver the driving force for uni-directional movement along polarized actin-filaments from the mother into the bud. Generally speaking, myosin force generation is carried out by a number of ordered conformational changes within the actin-binding ATPase, induced by rounds of hydrolysis of ATP (see chapter 1.4.3.2). Thus, essentially, one can state that two features are crucial: binding to actin and ATP hydrolysis.

To block myosin function without changing the binding parameters to potential cellular interaction partners, two sets of *myo4* mutants were generated by site-directed mutagenesis (see methods part 3.15). Target for the genetic manipulation of *MYO4* were regions within the ATPase domain: the actin-binding site and the P-loop, a region required for ATP hydrolysis. In order to be able to directly track the cellular distribution of the generated myosin mutants, mutagenesis was performed on strains harboring Myo4-2xGFP.

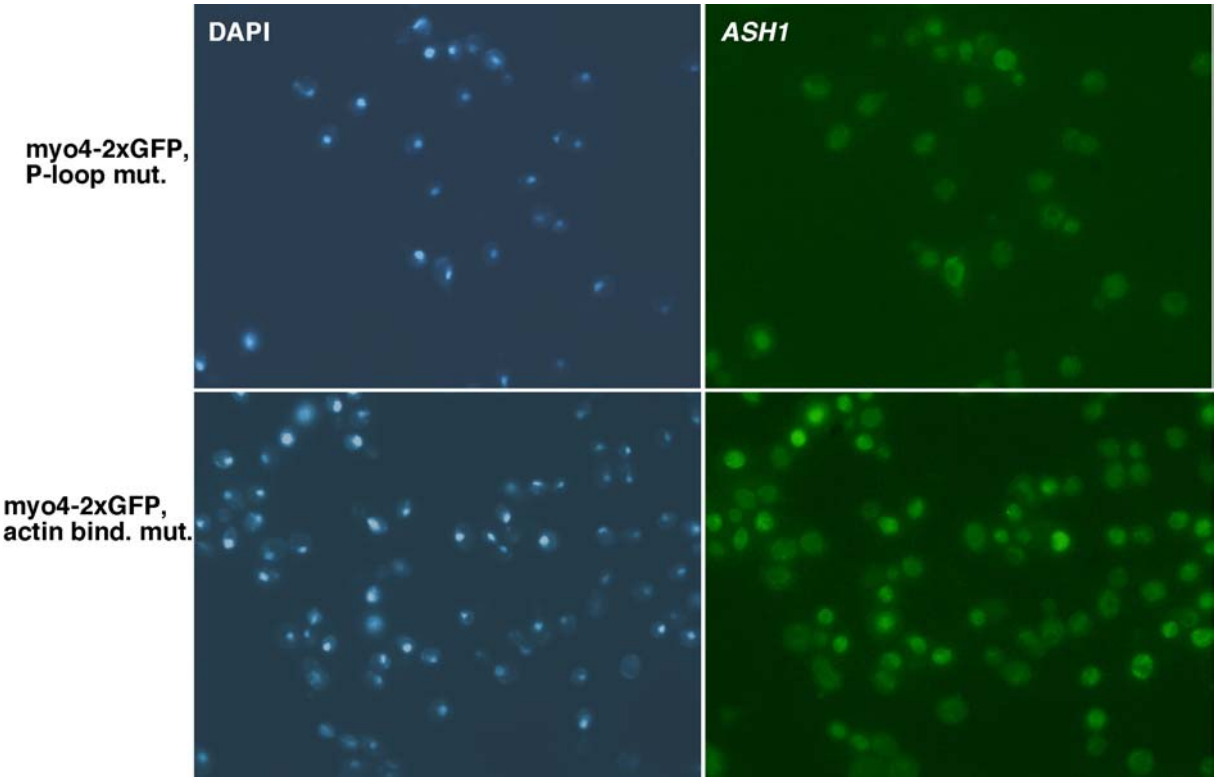


myo4-2xGFP ATPase mutants fail to accumulate at the bud tip.
The myo4p P-loop mutant (RJY 1529) localizes to structures reminiscent of actin filaments, whereas the myo4p actin-binding mutant (RJY 1531) accumulates in a granular pattern at the cell periphery (depicted by white arrowheads).

Both myo4p mutants generated by site-directed mutagenesis fail to localize to the bud tip. The fluorescent signal from the P-loop mutant (also called 'rigor mutant') stains rod-like structures spanning the cytoplasm, an observation that fits well with the idea that this mutant - having lost its capability to release and exchange ADP against ATP - might stick in an immobile state to actin filaments. The actin-binding mutant instead is seen uniformly distributed as cytoplasmic dots at the cell periphery.

Since the mutagenized region within Myo4p is most likely not involved in cargo-binding but only in force generation (Reck-Peterson *et al*., 2000), both localization patterns seem to reflect the She RNP complex in a 'frozen' but completely assembled intermediate state, not capable to deliver its mRNA cargo to the bud tip.

In order to test the generated *myo4* ATPase mutants for mRNA localization, cells expressing myo4-2xGFP and *ASH1* were processed for FISH.



Defective *ASH1* mRNA localization in *myo4* mutants.
Cells of strains RJY 1616 (Myo4-2xGFP, wt), RJY 1940 (myo4-2xGFP, P-loop mutant) and RJY 1941 (myo4-2xGFP, actin-binding mutant) have been processed for FISH against *ASH1*. Strain RJY 1616 localized *ASH1* mRNA as tight crescent to the periphery of large buds (not shown, see chapter 4.2.1). In contrast, cells harboring the myo4p actin-binding- or P-loop-mutation fail to localize *ASH1* mRNA, but instead display cytoplasmic background staining.

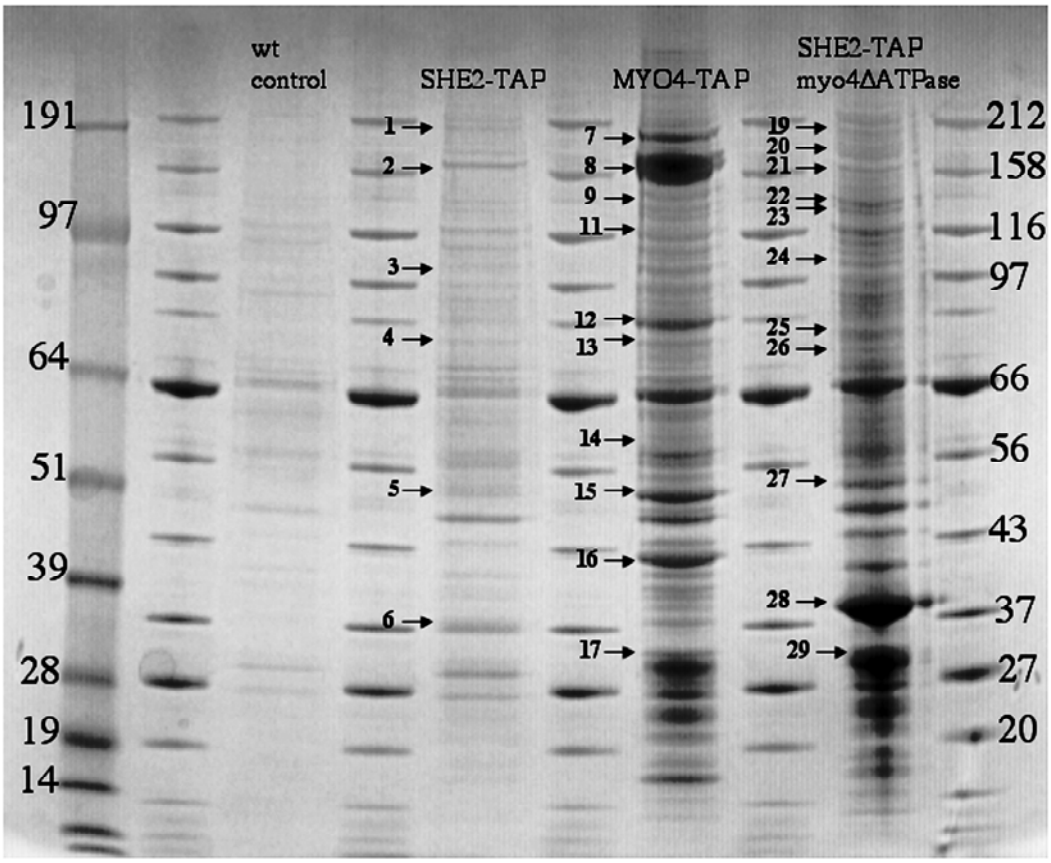
Both *myo4* ATPase mutants fail to localize ASH1 mRNA to growing buds, indicating that cytoplasmic She RNP trafficking has been successfully blocked in an intermediate state.

4.3.3 Biochemical purification of the She RNP complex

Having created a possible tool to enrich for She RNPs in transit, the genetic background of a myosin motor mutant combined with She2-TAP seemed to reflect a suitable source for biochemical purification.

For comparison of protein pattern distribution, large scale extracts of four different yeast strains were generated and served as starting material for affinity purifications: She2-TAP, Myo4-TAP, She2-TAP in *myo4* P-loop mutant background, and wt control. The final eluates were analyzed for protein composition as well as mRNA association.

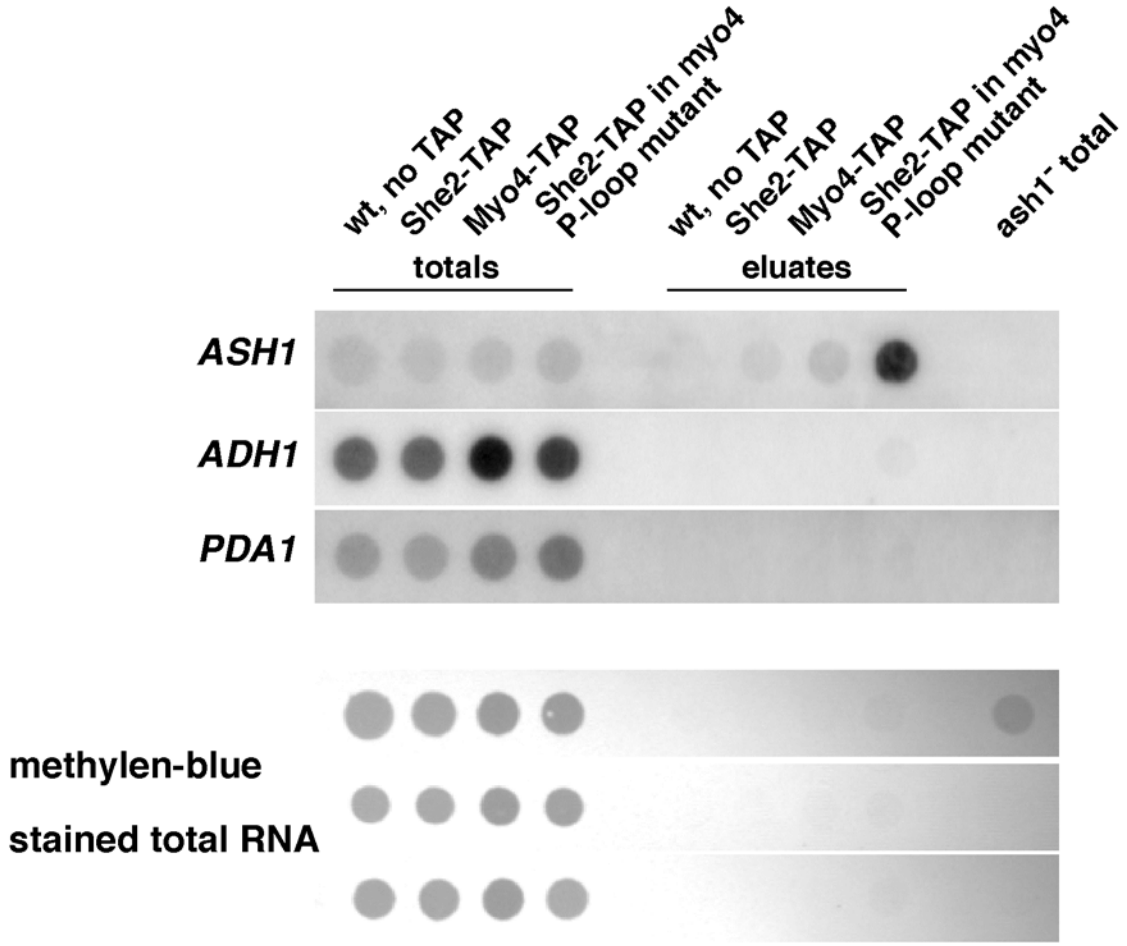
For a detailed protocol see chapter 3.10.



Large scale TAP eluates.
TAP eluates from strains RJY 358 (wt control), RJY 953 (She2-TAP), RJY 1420 (Myo4-TAP) and RJY 1939 (She2-TAP, *myo4* P-loop mutant) have been loaded onto a 4-12% Bis-Tris gradient gel and stained by colloidal Coomassie blue. The control eluate derived from a purification using a 60-liter culture (1.5 fold) of a wt-strain lacking a TAPtag served to specifically sort out protein bands resulting from unspecific binding to the affinity matrix. Bands that were judged as lacking from the control lane were cut out and identified by mass-spectrometry, as indicated by numbered arrows. Results are listed in the following table.

Band 1	FAS1	fatty acid synthase	
Band 2	**MYO4**		
Band 3	RNR1	ribonucleotide reductase	
Band 4	YHR020w		
Band 5	**SHE3**		SHE2-TAP
Band 6	**SHE2**		
	L10E	rib. protein	
	ASC1	WD repeat protein, suggested function in translational initiation, interacts with Scp160p	
Band 7	MYO1		
	YFR016c		
Band 8	MYO2		
	MYO4		
Band 9	*GIN4*	protein kinase, septin ring, interacts with Ptc4p	MYO4-TAP
Band 11	*SYP1*	hypothetical ORF, localizes to bud tip	
	SVL3	endocytosis, interacts with Bnr1p	
	RFC1	processivity factor for DNA polymerase delta and epsilon	
Band 12	CRN1	crosslinks actin and MTs, inhibits actin nucleation	
Band 13	YHR020w		
	EAP1	eIF-4E associated protein, transl. Initiation	
Band 14	PDC1	pyruvate decarboxlase	
	DBP2	RNA helicase, localizes to bud-neck, suggested function in NMD pathway	
	TCP1	member of TRiC complex, chaperone	
Band 15	**SHE3**		
Band 16	ACT1		
Band 17	TPM1	tropomyosin	
	RPS4b	rib. protein	
	RPS3	rib. Protein	
Band 19	URA2	pyrimidin biosynthesis, contaminant?	
Band 20	KEM1	cytopl. mRNA dedradation, contaminant?	
	RPA190	subunit of RNA pol. I	
Band 22	TIF4631	eIF-4gamma, transl. initiation	
	FUN12	eIF-5B, transl. initiation	
	RPA135	subunit of RNA pol.I	
	RRP12	ribosome biogenesis	
Band 24	RFC1	processivity factor for DNA polymerase delta and epsilon	
	SVL3	endocytosis, interacts with Bnr1p	
Band 25	PWP1	periodic tryptophan protein, WD40 repeats	
	EAP1	eIF-4E associated protein, transl. Initiation	
	PUF6	Pumilio-homology domain family, unknown function, localised to nucleolus?	
	YHR020w		
	MSN4	transcription factor, stress response	
	NRP1	putative RNA binding protein	
Band 26	**SHE2**		
	GCD2	eIF-2B, transl. Initiation	
	MSN4	transcription factor, stress response	SHE2-TAP
	NOG1	nucleolar G-protein	in myo4 ATPase mutant
Band 27	**SHE3**		
	TY1-15	transposon	
	NOP13	nucleolar protein	
Band 28	**SHE2**		
Band 29	RPL4a	rib. protein	
	RPS3	rib. protein	
	RPS4b	rib. protein	
	L5	rib. protein	
	RPS1a	rib. protein	
	L4	rib. protein	
	S4	rib. protein	

Protein analysis of She TAP eluates.
List of the protein factors found in She TAP eluates as analyzed by mass-spectrometry (C. Thurk, MPI for psychiatry, Munich).



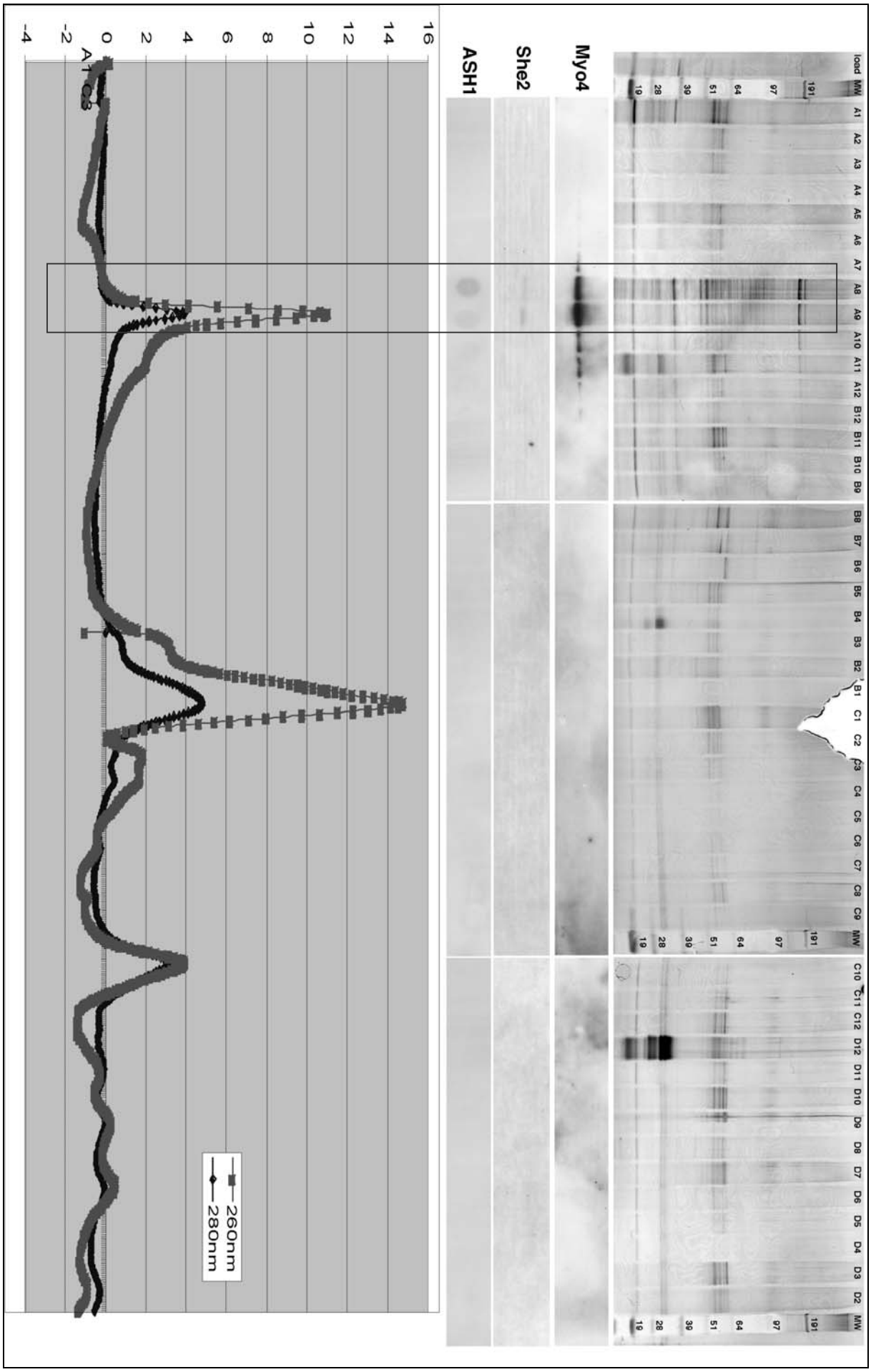
***ASH1* mRNA is a specific component of the purified She RNPs.**
RNA aliquots of the TAP eluates from strains RJY 358 (wt control), RJY 953 (She2-TAP), RJY 1420 (Myo4-TAP) and RJY 1939 (She2-TAP, *myo4* P-loop mutant) and totals have been subjected to phenol/chloroform extraction followed by DNaseI digest, a second round of phenol/chloroform extraction and ethanol precipitation. Equal amounts have been spotted onto a positively charged nylon membrane using a dot-blotter and hybridized with probes specific for *ASH1*, *PDA1* and *ADH1*, top. Total RNAs have been stained with methylenblue dye, bottom. A total extract of an ash1⁻ strain, RJY 1629, served as control to rule out unspecific binding of the ASH1 probe.

ASH1 mRNA can be detected in all extracts that have been used for TAP. *ASH1* mRNA is also present in all eluates from She RNP purifications but is absent from the wt control eluate. Furthermore no signal could be detected in the ash1⁻ total. The by far most intense spot corresponds to the eluate from the She2-TAP in the *myo4* 'rigor mutant' background. Two highly abundant control mRNAs, *PDA1* and *ADH1*, cannot be found in any eluate.
In summary, the She RNP complex could be purified from yeast extracts using a TAP-based strategy. All protein components of the core She machinery as well as *ASH1* mRNA could be identified.

4.3.4 In gel-filtration experiments the She RNP complex elutes as a single assembly at a size of approximately 4.5 MDa

Though with Myo4p, She3p and She2p all components of the core She machinery could be identified within the purified material, it was still unclear if the proteins were indeed members of a single complex, or alternatively established multiple sub-complexes. In order to be able to differentiate between these possibilities, the eluate derived from a large-scale She2-TAP purification in a *myo4* actin-binding mutant background was further analyzed by gel-filtration chromatography.

See next page: Proteins and nucleic acids contained within the eluate of a large scale She2-TAP from a *myo4* actin-binding mutant fractionate in two defined peaks.
The eluate of a She2-TAP, *myo4* actin-binding mutant (RJY 1938) purification from a 60-liter culture has been loaded onto a 'superose 6' gel-filtration column, and 0.75ml fractions have been collected. Fractions have been analyzed as follows:
$1/10^{th}$ of each fraction has been separated by SDS-PAGE on 4-12% Bis-Tris gradient gels and analyzed by Western blot against Myo4-2xGFP and She2p (anti GFP: 1^{st} ab B34 1:25.000, 2^{nd} ab sheep anti mouse, peroxidase-conjugated, 1:5.000; anti She2p: 1^{st} ab anti She2p 1:1.000, 2^{nd} ab donkey anti rabbit, peroxidase-conjugated, 1:5.000).
RNA of $1/20^{th}$ of each fraction has been extracted with phenol / chloroform, ethanol precipitated and spotted onto a nylon-membrane using a dot-blotter. The membrane has been hybridized with probes specific for *ASH1* and *ADH1*, control (not shown).
The remaining fraction was TCA-precipitated, separated by SDS-PAGE on 4-12% Bis-Tris gradient gels and subjected to silver staining.
Note that the void volume of the column was approximately 8ml, and that the exclusion limit of the chosen matrix is < 5 MDa. Spectra for 260nm and 280nm were taken and plotted against the corresponding elution volume. Two peaks were detected within the profile, and both absorption spectra peak in the same fractions. The rectangle highlights fractions A8 and A9 in which the core She machinery has been identified. Corresponding fraction numbers are given at the top of the silver-stained gel.


load MW A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12 B12 B11 B10 B9
B8 B7 B6 B5 B4 B3 B2 B1 C1 C2 C3 C4 C5 C6 C7 C8 C9 MW
C10 C11 C12 D12 D11 D10 D9 D8 D7 D6 D5 D4 D3 D2 MW
191 97 64 51 39 28 19
Myo4
She2
ASH1
A1-C3
260nm
280nm

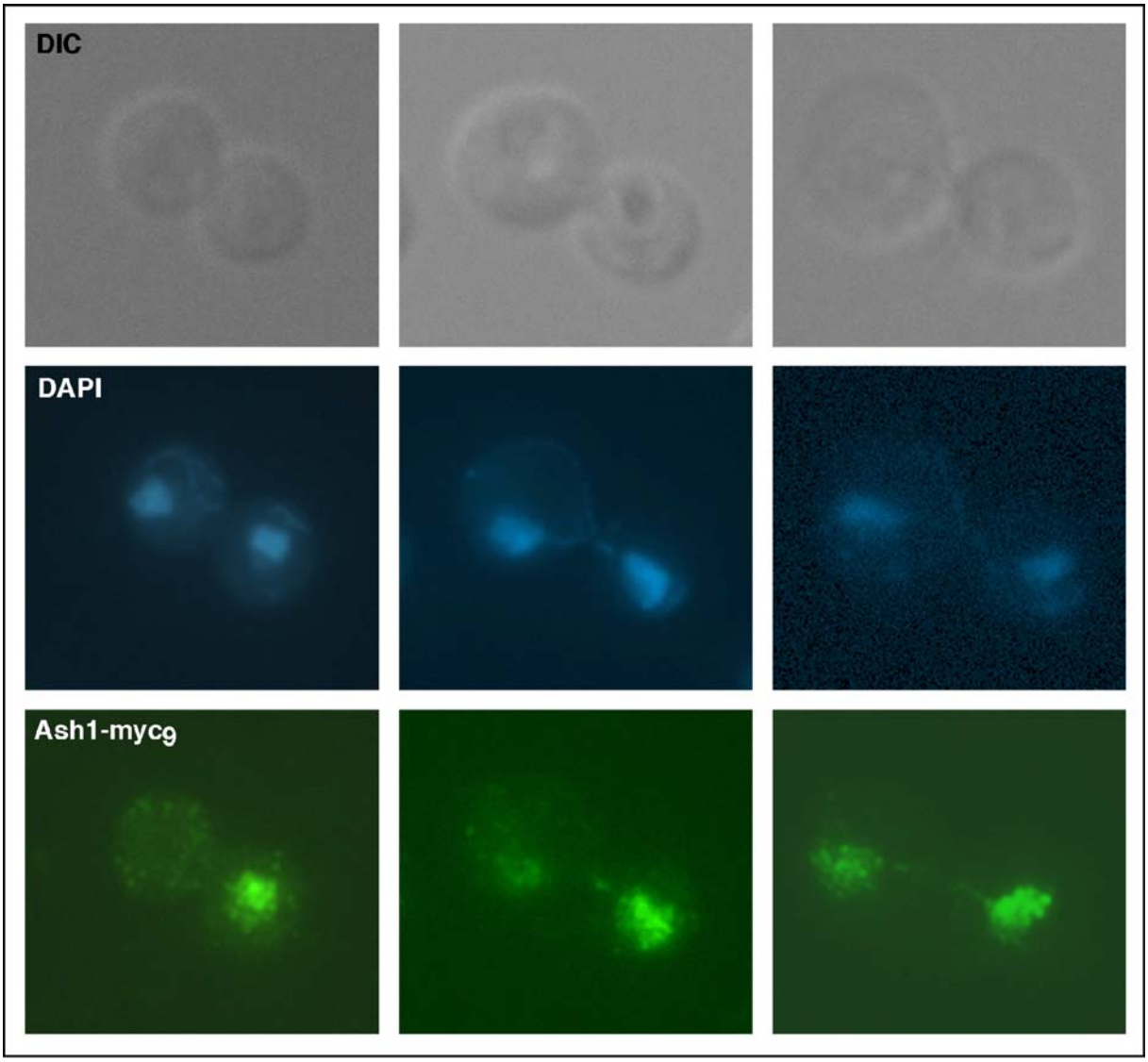
The elution profile from the 'superose 6' size exclusion chromatography column shows two peak fractions for both 260nm and 280nm. She2p, Myo4-2xGFP and *ASH1* mRNA have been detected only in fractions corresponding to the first peak, indicating the purified She RNP complex. The size of the She RNP of approximately 4.5 MDa has been estimated from the elution pattern of molecular size marker proteins.
The co-detection of the She core machinery and *ASH1* mRNA in the same elution fraction from the gel-filtration experiment strongly suggests the successful biochemical purification of the native She RNP transport complex.

4.4 Initial characterization of Eap1p and Gin4p, two new candidate factors potentially involved in cytoplasmic She RNP transport

4.4.1 The eIF 4E-binding protein Eap1p affects Ash1p localization

4.4.1.1 Cells lacking Eap1p fail to restrict Ash1p to daughter cell nuclei

One of the identified factors within the purified material from both Myo4-TAP and She2-TAP experiments has been Eap1p (see chapter 4.3.3). Eap1p has been characterized as inhibitor of translation initiation due to its ability to bind and sequester eIF4E from eIF4G (Cosentino *et al.*, 2000). Thus an attractive possible implication for Eap1p in cytoplasmic She RNP transport would be a function as translation initiation inhibitor for Ash1p.
In order to investigate if cells lacking Eap1p display a She-like phenotype on *ASH1* mRNA or Ash1p distribution, *Δ eap1* cells have been analyzed by FISH and indirect immuno-fluorescence, respectively. In initial double experiments against wt cells, *Δ eap1* cells displayed no detectable *ASH1* mRNA localization defects (data not shown).



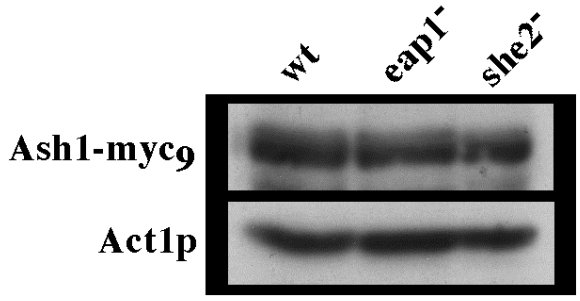
	asymmetric Ash1p	partly symmetric Ash1p	symmetric Ash1p
wt	(36/50) 72%	(7/50) 14%	(7/50) 14%
Δ she2	(12/45) 27%	(2/45) 4%	(31/45) 69%
Δ eap1	(19/65) 29%	(26/65) 40%	(20/65) 31%

Defective localization of Ash1-myc9 in cells lacking Eap1p.
Cells of strains RJY 2068 (*Δ eap1*, Ash1-myc$_9$), RJY 2069 (wt, Ash1-myc$_9$) and RJY 2070 (*Δ she2*, Ash1-myc$_9$) have been processed for indirect immunofluorescence (1st ab 3F10, 1:50; 2nd ab goat anti rat, Alexa 488, 1:100). Nuclear Ash1-myc$_9$ localization was classified into the categories 'asymmetric' (left), 'partly symmetric' (middle), and 'symmetric' (right), as shown by sample images for DIC, DAPI and Ash1-myc9, respectively.
For quantification in triple experiments, late anaphase cells still connected but with separated nuclei have been selected in the DAPI channel before switching to the FITC channel, showing Ash1-myc$_9$ distribution.

Compared to wt, *Δ eap1* or *Δ she2* cells fail to restrict Ash1-myc$_9$ to daughter cell nuclei. Whereas the majority of *Δ she2* cells accumulate Ash1-myc$_9$ in equal mounts symmetrically to both nuclei, *Δ eap1* cells display a somewhat milder phenotype: The predominant amount of *Δ eap1* cells showed only a partial staining of mother cell nuclei with still a major amount of Ash1-myc$_9$ localized to the daughter cell nucleus.

4.4.1.2 Ash1p steady-state levels are unchanged in *Δ eap1* cells

As Eap1p has been shown to function as negative regulator of translation initiation, the partial mislocalization phenotype of Ash1-myc$_9$ in *Δ eap1* cells might be due to an increased level of Ash1p compared to wt.



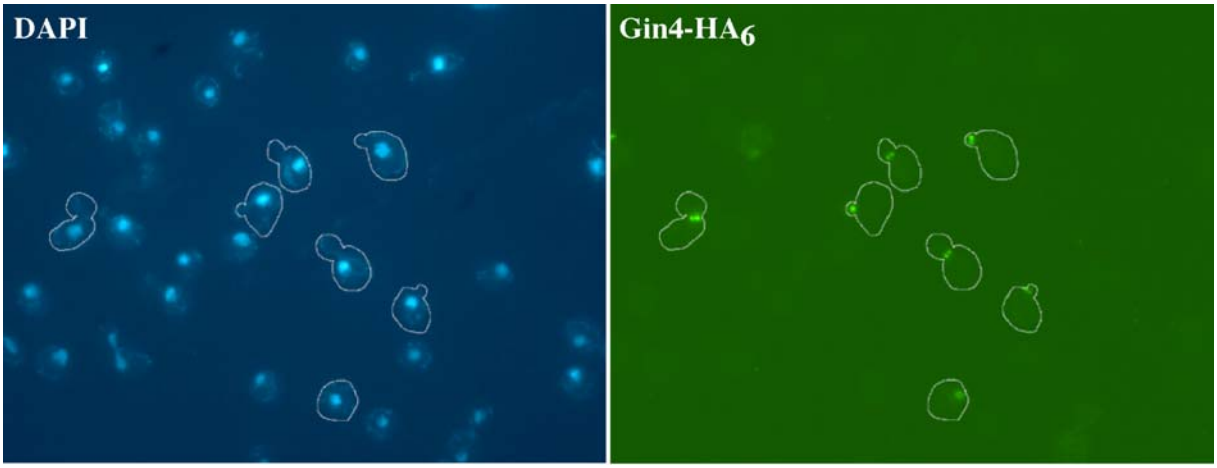
Eap1p does not influence on total Ash1-myc$_9$ levels.
Protein extracts of cells from strains RJY 2068 (*Δ eap1*, Ash1-myc$_9$), RJY 2069 (wt, Ash1-myc$_9$) and RJY 2070 (*Δ she2*, Ash1-myc$_9$) have been prepared by the glass-bead method. Extracts have been normalized with the 'Bio-Rad protein assay' and equal amounts have been analyzed by Western blotting against Ash1-myc$_9$ and Act1p, control (anti Ash1-myc$_9$:1st ab 9E10, 1:1.000, 2nd ab sheep anti mouse, peroxidase-conjugated, 1:5.000; anti Act1p:1st ab Mab414, 1:5.000, 2nd ab sheep anti mouse, peroxidase-conjugated, 1:5.000).

Western blot analysis has revealed comparable amounts of Ash1-myc$_9$ in all three extracts, indicating that the *Δ eap1* mutation does not effect on intracellular Ash1p steady-state levels. Taken together, the eIF4E binding protein Eap1p has been identified in an affinity purification-based biochemical approach together with the core She machinery and *ASH1* mRNA. Mutants that lack Eap1p fail to efficiently restrict Ash1p to daughter cell nuclei, albeit Ash1p levels are unchanged.

4.4.2 The Ser-/Thr-specific protein kinase Gin4p localizes to the mother-bud junction throughout the cell cycle

Another factor that has been identified in the Myo4-TAP experiment is Gin4p. Gin4p is a 130 kDa protein with Ser-/Thr-specific kinase activity (Barral *et al*., 1999; Dobbelaere *et al*., 2003), and localizes to the septin ring at the mother-bud junction (reviewed by Faty *et al*., 2002). The septin ring has been suggested to function as diffusion barrier for integral plasmamembrane proteins, thereby separating the mother from the daughter cell compartment (Takizawa *et al*., 2000). Gin4p has been picked as candidate for initial characterization due to its given potential for post-translational modifications of members of the cytoplasmic She RNP. However, initial FISH experiments have revealed that *ASH1* mRNA localization was unperturbed in cells lacking Gin4p (data not shown).

In order to confirm the suggested intracellular distribution by indirect immunofluorescence, an HA_6 epitope has been fused to the Gin4p COOH-terminus and integrated by homologous recombination into the yeast genome.



Gin4-HA_6 localizes to the mother-bud junction throughout all cell cycle stages.
Representative snapshots of cells expressing Gin4-HA_6 from strain RJY 2010 that have been processed for indirect immunofluorescence (1st ab 3F10 anti HA, 1:50; 2nd ab goat anti rat Alexa 488, 1:100). Gin4-HA_6 is not detectable in unbudded cells, but enriches at the presumptive budding site as soon as the bud emerges in G1 phase. In cells with small buds Gin4-HA_6 forms a ring-like structure separating the mother from the growing bud. This structure is duplicated to a double-ring in large budded cells. It has been suggested that cytokinesis in turn allows the inheritance of one ring to the daughter cell whereas the other remains associated with the mother (Faty *et al*., 2002).
The Gin4-HA_6 fusion has been regarded as functional since *Δ gin4* cells are elongated and display cytokinesis defects (Barral *et al*., 1999).

The intracellular distribution of Gin4-HA_6 correlates with *in vivo* data, as Gin4-GFP has been reported to localize to the bud neck (Huh *et al*., 2003). A possible functional implication of this localization pattern for cytoplasmic She RNP transport is discussed in chapter 5.5.4.

5. Discussion

The generation of diversity from a pool of genetically identical cells is a key feature during development of multi-cellular organisms. The localization of a specific mRNA in a cell cytoplasm and subsequent asymmetric cell division is one mechanism by which cell fate determinants can be sorted between sister cells. In order to avoid mistargeting the exact positioning of such mRNAs is often coupled to their translation regulation, so that unlocalized transcripts either become degraded or remain translationally silent. Thus post-transcriptional regulation of gene expression during development can occur by a functional linkage between mRNA localization and translation control. Importantly, this molecular connection is not only restricted to early development but is also used to establish synaptic plasticity. Synaptic activation can induce the translation initiation of locally stored neuronal RNA granules in dendrites (Krichevsky and Kosik, 2001).

5.1 Tight *ASH1* mRNA localization to the peripheral cortex of the daughter cell depends on translation

5.1.1 Defective anchorage of *ASH1* mRNA containing premature stop codons

In *Saccharomyces cerevisiae, ASH1* mRNA is localized to the peripheral bud cortex employing an active transport process along polarized actin filaments. This work has presented several pieces of evidence that suggest a direct role for the translation apparatus in the tight attachment of *ASH1* mRNA to the bud cortex, named 'anchorage'.
Insertion of a premature stop codon upstream of the *ASH1* ZIPcodes E1, E2A or E2B leads to a gradual loss of localization efficiency, whereas 'E2B stop' and 'stop E3' localizes nearly indistinguishably from wt. The detected abnormalities seem to be due to insufficient anchorage rather than to faulty cytoplasmic transport, since the localization elements remained unaffected by the chosen mutagenesis protocol. Furthermore, FISH against non sense *ASH1* transcripts revealed the predominant amount of mRNA in the bud cytoplasm.

Below I want to discuss two possibilities that might explain this phenomenon. The first postulates a reduced anchorage deficiency due to a reduced ribosome association, the second involves the loss of a potential protein-RNA interaction.
After correct delivery the mRNA might fail to be anchored at the cortex due to insufficient ribosome association. Shorter open reading frames predict a reduced number of active ribosomes per mRNA, maybe too few. If the anchorage was mediated predominantly by

ongoing translation, it might be that an mRNA encoding a short polypeptide could not be effectively retained simply because a threshold number of active ribosomes might be needed to prevent the message from detachment and diffusion into the cytoplasm.
Inspired from studies in *Drosophila*, where *oskar* mRNA localization requires Oskar protein (Rongo *et al*., 1995), one can imagine an alternative explanation for the reduced anchorage efficiency of non sense *ASH1* mutants. Since the steady-state rates of non sense *ASH1* mRNA are comparable to wt, there is no reason why the mRNA should not be translated into COOH-terminally truncated Ash1p. The observed localization defects of non sense *ASH1* mRNA mutants are in agreement with earlier findings proposing that translation of the Ash1p COOH-terminus is required for cortical retention of *ASH1* mRNA (Gonzalez *et al*., 1999).
Interestingly, the anchorage of an *ASH1* non sense mutant transcript could not be rescued by co-expression of wt *ASH1*, indicating a direct involvement of the translation apparatus.
Importantly, the GATA-1-like zinc finger motif that mediates the interaction with the *HO*-promoter is contained within the Ash1p COOH-terminus (Maxon and Herskowitz, 2000), in between elements E2B and E3 at position 499-526, corresponding to nucleotides1497-1578 of the mRNA (see chapter 4.1.1). Structurally related motifs have been reported to efficiently bind to RNA. CPEB binding to the CPE within the target mRNA involves two canonical 'RNA recognition motifs', RRMs, as well as a C2C2H2-type zinc-finger, and the integrity of all three motifs is required for efficient recruitment (Hake *et al*., 1998). Furthermore a C2C2-type zinc-finger motif has been found to mediate RNA association of eIF2 (Laurino *et al*., 1999). Insertion of a premature stop codon before E1, E2A, E2B or after E2B predicts a polypeptide that lacks the zinc-finger motif, whereas insertion before E3 should result in a truncation mutant that still contains the putative RNA-binding region. Thus a model that implies Ash1p in *ASH1* mRNA anchorage *in cis* would be that the zinc-finger motif confers some RNA-binding activity to the Ash1p COOH-terminus. An easy way to test this hypothesis would be to use the loss-of-function ash1p-C502R mutant (Maxon and Herskowitz, 2000) and check for *ASH1* mRNA anchorage.
However, the model cannot explain the finding that already the E2B stop *ASH1* mutant, which is positioned upstream of the predicted zinc-finger motif, localizes as efficiently as the stop E3 mutant. One way to explain this would be the existence of a so far unknown motif encoded within E2B, which - similarly to the zinc finger motif - might confer RNA-binding activity to the nascent Ash1 polypeptide.

Another indication for a direct role of the translation apparatus in *ASH1* mRNA anchorage comes from the observation that brief treatment with the translation inhibitor cycloheximide disrupts *ASH1* mRNA and Myo4p localization. Cycloheximide causes active ribosomes to stall during elongation and thereby freeze the transcript together with the unfinished polypeptide chain in a ternary complex. Since it is known that the Ash1p COOH-terminus is needed for RNA retention *in cis*, a likely explanation for how the drug might affect mRNA localization could be that the majority of polypeptide intermediates lack the Ash1p COOH-terminus and therefore fail to restrict the mRNA to the cortex. In support of this idea over-expressed Ash1p can be detected at the bud tip shortly before diffusion into the daughter cell nucleus. In addition, the observation that cortical Myo4p localization is affected as dramatically as *ASH1* mRNA makes it feasible to speculate that the retention of the motor depends solely on the association with the mRNA. Once the mRNA becomes disconnected from the cortical polyribosomes, both *ASH1* mRNA and Myo4p diffuse into the cytoplasm. However, until the complete functional She RNP has not been characterized biochemically it is not possible to rule out the existence of a so far unknown accessory factor that is needed specifically for cortical transcript retention.

5.1.2 Non sense *ASH1* mRNA does not trigger non sense mediated decay

Consistent with the idea that mutant *ASH1* mRNA might not represent a target for 'non sense mediated decay', NMD, is the finding in mammalian cells that NMD activation depends on translation. Intron-less mRNAs containing a premature translation stop codon are often unaffected by NMD (reviewed by Maquat, 2004). Since in yeast only a very limited set of transcripts bear introns, NMD must be regulated differently (reviewed by Mitchell and Tollervey, 2001; Vasudevan and Peltz, 2003). Instead of splice-sites, yeast mRNAs contain poorly conserved sequence elements recognized by nuclear hnRNP-like proteins. This mark in turn can serve as binding site for the yeast surveillance complex, or alternatively concerts a stabilizing function onto the RNA. The finding that non sense *ASH1* mRNA does not trigger NMD even in *Δ she2* cells rules out such a stabilizing function of She2p towards *ASH1* mRNA (see part 4.1.2). Thus, the simplest explanation would be that *ASH1* mRNA is not susceptible to NMD, as such exceptions to the rule have been reported to exist (Maquat and Li, 2001; Brocke *et al*., 2002; Chester *et al*., 2003).

Another possible explanation why non sense *ASH1* mRNA is stable comes from the idea that NMD generally requires translation. Especially the first round of translation (the so-called 'pioneer round') seems to be critical, and occurs in the vicinity of the nuclear pores. Assuming

that Ash1 polypeptide synthesis involves control mechanisms that delay translation initiation and protein release to the bud tip, *ASH1* mRNA could escape from such a surveillance mechanism and remain stable. The issue of *ASH1* translation control will be discussed in more detail below.

5.2 She2p is a shuttling protein that most likely associates with *ASH1* mRNA already in the nucleus

Earlier studies in our lab and others have shown that She2p directly and specifically binds to *ASH1* mRNA, thereby linking the transcript to the Myo4p / She3p transport machinery. Under wt conditions She2p distributes uniformly throughout the cytoplasm but under harsh ASH1 overexpression conditions She2p can be detected together with *ASH1* mRNA at the bud tip (Böhl *et al*., 2000).

Immuno-cytological co-localization as well biochemical pulldown experiments have indicated a direct and specific association of She2p with *ASH1* mRNA. Several experiments included in this work reveal that the intracellular She2p distribution seems to be governed by its RNA association.

In temperature sensitive *mex67-5* cells temporarily blocked for bulk mRNA export, She2p can be observed to accumulate in the nucleus. Immunofluorescence imaging under unperturbed RNA export conditions has revealed a localization pattern for She2p slightly overlapping with DAPI staining, indicating that even under wt conditions She2p is not strictly excluded from the nuclear compartment. This and the fact that Mex67p has been exclusively implicated in mRNA export make it unlikely that Mex67p might be involved directly in She2p export. The connection between nuclear She2p localization and RNA export block rather indicates the possibility that She2p might be maintained in the nucleus only if RNA export is blocked. In other words, She2p can only be exported together with mRNA. In order to test for this idea, an NH2-terminal She2p truncation mutant has been designed that does not bind detectable amounts of *ASH1* mRNA *in vivo* anymore, she2pΔN70. As predicted from the model, indirect immunofluorescence imaging of she2pΔN70 under unperturbed RNA transcription and -export conditions revealed a predominantly nuclear localization. This change in intracellular steady-state distribution of She2p on behalf of its RNA-binding capacity leads to the hypothesis that She2p is a shuttling protein that is exported only if bound to RNA. Assuming that She2p binds to *ASH1* mRNA already in the nucleus, it might therefore constitute an early cue that pre-determines an RNP to be subsequently localized in the cytoplasm. In simple terms, this early nuclear separation of the wheat from the chaff would

allow later on for a clear distinction between mRNAs to be sorted to the bud periphery and bulk cytoplasmic transcripts. Nuclear shuttling of factors involved in cytoplasmic mRNA localization is not an exception but rather seems to be commonly used (Farina and Singer, 2002). For instance, in embryonic chicken fibroblasts the localization of *β-actin* mRNA to the leading edge is mediated by ZBP1 which specifically binds to a ZIPcode element within the 3'-UTR (Ross *et al*., 1997). Recent unpublished data not only revealed a temporal nuclear localization of ZBP1 but also have shown that phosphorylation of ZBP1 blocks premature translation of *β-actin* mRNA (S. Hüttelmaier, personal communication).

In a publication from the year 2003 touching similar aspects of She RNP dynamics, Gonsalvez and colleges could elegantly confirm that the association of She2p with *ASH1* mRNA depends on a functional NH2-terminus. Nevertheless, the authors did not detect She2p within the nucleus, and this disagreement was explained by subtle differences in the experimental setup. Endorsing evidence for She2p being a nuclear protein, however, stems from a proteome-wide localization approach, in which She2p was found to localize to the nucleus (Huh *et al*., 2002).

5.2.1 She2p is actively imported into the nucleus

Further support for She2p being a nuclear protein comes from the finding that She2p has been detected in complex with Srp1p, the yeast importin α (Ito *et al*., 2001). This finding together with a second observation made in the present study argues for an active nuclear import mechanism for She2p: Whereas the RNA-binding activity of She2p has been roughly mapped to the NH2-terminus, a putative nuclear localization signal remained unaffected by the truncation procedure. Alternatively the truncation procedure might have cut down the size of the protein in such an extent that the shortened mutant allele she2pΔN70p gains the ability to enter the nucleus by free diffusion. However, a nuclear accumulation of she2pΔN70 clearly beyond the diffusion equilibrium was demonstrated, speaking against a passive import by diffusion of the larger full-length She2p. Thus it is likely that She2p is imported into the nucleus using an active transport mechanism.

The chosen immuno-cytological protocol used in this study was based on fixed cells, and thus has allowed to gain insight only on intracellular steady state distributions of She2p. Much progress has been made during the last decade in the establishment of microscopic live-cell imaging techniques using GFP-tagged versions of the target protein (Lippincott-Schwartz *et*

al., 2001). In order to be able to monitor She2p *in vivo* dynamics in more detail, GFP-tagged versions of She2p had been constructed. Unfortunately so far none of the She2p-GFP chimeras was able to complement a *Δ she2* mutant when tested for *ASH1* mRNA localization.

5.3 RNP-dependent localization of Myo4p

The observation that She2p is a shuttling protein was made in conjunction with studies on the intracellular localization of Myo4p. As already mentioned earlier, Myo4p constantly enriches at sites of cell growth, that is predominantly at the peripheral cortex of the growing bud. At the time course of the experiments presented herein the sole known cargo for Myo4p were mRNAs that become asymmetrically distributed to the bud. Recently it has been suggested that cortical ER inheritance relies on functional Myo4p (Estrada *et al*., 2003), an issue that will be discussed in more detail in part 5.3.1. Using a Myo4-2xGFP chimeric protein it became possible to investigate *in vivo* dynamics of Myo4 dependent transport processes. Comparing the *in vivo* localization pattern of Myo4-2xGFP to that obtained by indirect immunofluorescence using fixed cells made clear that the bulk fluorescent tag at the Myo4 COOH-terminus did not interfere with the cellular protein function. As a second control Myo4-2xGFP was able to complement *Δ myo4* for *ASH1* mRNA localization. Thus, in contrast to She2-GFP, the Myo4-2xGFP chimera was regarded as fully functional.
Earlier experiments have revealed that the association of Myo4p with mRNA depends on She3p and She2p, and that this association is required for the targeting of the motor protein to the bud (Jansen *et al*., 1996; Münchow *et al*., 1999). Concomitantly, the intracellular distribution of Myo4-2xGFP to the growing bud tip is blocked in *Δ she2* or *Δ she3* mutants. In a working model based on reports from several labs She2p is thought to bridge the mRNA cargo to the She3p / Myo4p complex (Kwon and Schnapp, 2001). Myo4p and She3p have been shown to constantly co-localize throughout the cell cycle (Jansen *et al*., 1996), whereas the association with She2p seems to be dependent on mRNA association and more transient (Böhl *et al*., 2000). Unlike the core She machinery, neither Khd1p nor Loc1p are required for Myo4-2xGFP localization, suggesting that in *Δ loc1* or *Δ khd1* mutants Myo4p remains efficiently associated with its mRNA cargo. Khd1p has been shown to bind *ASH1* and co-localize with RNA granules in the 'green RNA' approach (Irie *et al*., 2002). However, efficient targeting of Myo4p to the bud in *Δ khd1* mutants suggests that Khd1p is not needed for linking *ASH1* mRNA to Myo4p. Still, under wt conditions, Khd1p might be transported together with *ASH1* mRNA but is not required for cytoplasmic transit. Alternatively, as

postulated by Irie and collegues (2002), Khd1p might affect *ASH1* mRNA localization not at the transport but at the anchorage step.

Yet, the question how Myo4p targeting is regulated on the molecular level remained unanswered. Myo4-2xGFP localization in a strain deleted for the two by that time established mRNA cargoes *ASH1* and *IST2* is rather unaffected, a finding that allowed an argumentation for additional cargo mRNAs, as potentially introduced by Takizawa *et al*. (2000). Though *mex67-5* cells blocked for bulk mRNA export fail to localize Myo4-2xGFP to growing buds, the unexpected finding that She2p co-enriches in nuclei under the same conditions has left open two possibilities: Either Myo4p activity is regulated by association with the linker protein She2p or alternatively by the cargo mRNA. Considering that the *mex67-5* mediated mRNA export block has been reported as reasonably tight (Segref *et al*., 1997), whereas substantial amounts of She2p can be detected in the cytoplasm even under restrictive conditions, a more likely candidate for Myo4p steering was the mRNA. On the other hand all cells with a nuclear She2p accumulation have failed to localize Myo4p to the growing bud. A key to that question could be found in using an MS2-based reporter system in which Myo4-2xGFP was efficiently targeted to sites of polarized growth in the absence of She2p (Kruse *et al*., 2002). Hence, functional She RNP formation is regulated by She2p-mediated mRNA cargo-loading, a feedback mechanism ensuring that on behalf of cellular energy only a functional RNP that is fully assembled will be moved to the bud. Since She2p can be artificially replaced it is likely that the protein functions solely as handle which accomplishes the physical association with the RNA cargo.

How the RNA cargo mediates particle formation remains to be determined. There are arguments that speak against a cross-linking function mediated by the RNA or the RNA-binding protein, since a single RNA-protein interaction motif within *LacZ* turns out to be sufficient for particle formation, and the MS2 mutant protein that has been used was designed to minimize homo-dimerization (Peabody and Lim, 1996).

Motor protein targeting by cargo-association is a widespread phenomenon (reviewed by Karcher *et al*., 2002). For example in *Xenopus laevis* melanophores, CaM-kinase II-mediated phosphorylation of myosin V during mitosis abrogates the association to its cargo, the melanosomes, thereby breaking the transport complex and down-regulating motor activity (Rogers *et al*., 1999). Another example for mRNA cargo based motor targeting has been found in *Drosophila*. Cytoplasmic dynein mediated *hairy* mRNA localization includes the regulation of motor activity by the association with *hairy* mRNA (Bullock *et al*., 2003).

5.3.1 Retention of Myo4p in the bud depends on association with a She2p RNP

Confocal imaging with increased sensitivity over widefield optics has revealed that considerable amounts of Myo4-2xGFP fluorescence are contained within the bud cytoplasm. Compared to wt, the Myo4-2xGFP signals detected in *Δ she2* cells are much smaller in size and highly unstable, as monitored by the transient formation of larger assemblies that readily disintegrate into smaller sub-populations. Moreover the tiny dots have the potential to pass the neck region and thereby change the cytoplasmic compartments in a diffusive way, leading to a more or less even fluorescence distribution throughout both mother and bud. Wt particles containing She2p, however, are found concentrated at the peripheral bud cortex. A constant dynamic association-dissociation/attachment-detachment performance to and from the cortex has been detected, as if the dots were rolling over a limited peripheral region within the bud cortex. FLIP, a method used to detect compartment retention time differences of fluorescently labeled proteins, could demonstrate a significantly prolonged bud retention time of wt particles over those lacking She2p. Thus She2p not only is required as handle to tether the RNA cargo to the Myo4p/She3p complex, but also seems to mediate a transient retention of the RNP with the bud cortex.

As Myo4-2xGFP localization to the growing bud cortex in *Δ she2* cells strictly depends on the expression of the hybrid *LacZ-MS2* reporter RNA (Kruse *et al*., 2002, see cartoon in chapter 4.2.3.1), the transient retention at the bud tip is ultimately mediated by the RNA cargo. It is conceivable that this temporal retention to the cortex, a cellular region containing rough ER structures, is at least partly decisive for the cargo mRNA to be accurately handed over from the transport complex to the local protein synthesis apparatus.

However, the ability to differentiate between a simple diffusion exchange and a She2p-dependent transient retention mechanism did not answer the question how Myo4p is targeted to the bud in the absence of She2p. As mentioned earlier She2p is required for linking the RNA to the Myo4p / She3p complex, thereby regulating the activity of the motor protein. Myo4p / She3p has been consolidated as a general transport machinery for RNAs that all become tethered by a single RNA binding protein, She2p (Takizawa *et al*., 2000; Shepard *et al*., 2003). Taking this into account, the tiny dots representing Myo4-2xGFP in cells lacking She2p cannot contain RNA cargo, but still they keep the ability to move into the bud. Essentially this suggests the existence of an additional, RNA-independent type of cargo for Myo4p that is bound to the motor complex independently from She2p. Furthermore, it is evident that this type of cargo is transported independently from RNA, and in form of smaller dots rather than large granules as observed for cytoplasmic RNA transport (chapter 4.2.4.3).

Endorsing evidence for an additional type of cargo for Myo4p stems from the observation that cortical ER inheritance is mediated by the Myo4p / She3p complex (Estrada *et al*., 2003). In more detail the authors report that cortical ER transport into the bud is dependent on Myo4p / She3p but occurs separately from She2p and transcript localization. It is thus conceivable that, apart from mRNAs, Myo4p / She3p might constitute a more widely used transport machinery that carries various cargoes to the growing bud, analogous to Myo2p, the other family member of class V myosins in *S. cerevisiae*. Myo2p is known to transport vacuoles and secretory vesicles, and cargo differentiation occurs by the employment of specific adapter proteins. However, a postulated ER adapter protein functionally resembling She2p remains to be identified. Using a highly sensitive microscopic imaging setup only small amounts of Myo4-2xGFP can be detected in the absence from She2p at the bud tip, in total sufficient to mediate cortical ER inheritance.

5.4 Biochemical purification of the She RNP

The molecular identification of protein components within the She RNP complex has finally involved the mass-spectrometric analysis of protein samples, performed in collaboration with Dr. C. Thurk, MPI for Psychiatry, Munich, and with Dr. T. Ruppert, ZMBH, Heidelberg.

5.4.1 Principle considerations before starting the biochemical purification

The She proteins, factors required for *ASH1* mRNA localization have been identified using genetic screens, in which only non-essential candidates could be identified. A detailed biochemical characterization including potentially essential components of the mRNP complex harboring the *ASH1* mRNA cargo, the 'She RNP', is lacking. A widely used method to purify native protein assemblies has been TAP (Rigaut *et al*., 1999).

5.4.1.1 The mRNA as structural component of the She RNP

As discussed in detail above, the retention of the She RNP at the bud cortex seems to be mediated by the RNA moiety of the complex. Furthermore, experiments with a She3p-MS2 hybrid substituting for She2p have revealed that the RNA has a scaffold function within the She RNP: Myo4-2xGFP bud localization in bright particles was rescued only by co-expression of the chimeric *LacZ-MS2* cargo RNA (Kruse *et al*., 2002). Cells in which *LacZ-MS2* transcription was repressed did not only fail to localize Myo4-2xGFP, but also lacked granular Myo4 staining, suggesting that the RNA cargo has a structural role within the RNP.

She2p has been shown to bind to all four localization elements within *ASH1* mRNA in vitro (Böhl *et al*., 2000), and all four elements are required to efficiently anchor *ASH1* mRNA at the bud tip (Chartrand *et al*., 2002). Since the ZIPcodes within the *ASH1* transcript comprise large secondary structural elements, it is feasible that binding of She2p induces a conformational change within the mRNA, and that this molecular switch now enables additional trans-acting factors - such as Khd1p - to assemble. In that way She2p would act as 'RNA chaperone'.
Hence, in order to be able to purify the entire functional She RNP complex, the TAP-protocol had to be adapted in such a way that RNA degradation was minimized.

5.4.1.2 The choice of the ideal starting material for the She RNP purification, and the exclusion of contaminants from the true She RNP components

The successful purification of the She RNP predicts the identification of the minimal transport machinery within the purified material as an essential prerequisite for the credibility of this work. This goal has been accomplished in repeated experiments, indicating that the TAP strategy is a feasible method not only for protein complex but also for RNP purifications.
As an internal control for the integrity of the complex, the choice of the bait protein has varied in either She2-TAP or Myo4-TAP. In principle, since the She proteins are constituents of a single RNP both purification approaches should introduce the same pattern in protein composition. Nevertheless, Myo4-TAP yielded a much higher amount of factors associated with the motor protein than She2-TAP. The simplest explanation for this observation would be the direct cytoskeletal concatenation of Myo4p, resulting in the pulldown of a number of contaminants cross-linked via the actin cytoskeleton (*ACT1*, *CRN1*, *TPM1*). Another additional explanation could be that the motor protein is much better exposed, allowing for a more effective bait pulldown, whereas She2p is thought to be packed tightly within the core of the RNP. As the mock purification yield has been relatively poor, all extra protein bands in She-TAP experiments must be specific interaction partners. In order to be able to distinguish between cytoskeletal contaminants (or others) and true She RNP components, She2-TAP experiments have been performed in a *myo4* mutant background. Two *myo4* mutants have been designed in a way that both block the cytoplasmic transport of the She RNP (chapter 4.3.2) and thereby enrich the purification starting material for She RNPs in transit to the bud: Whereas the 'rigor mutant' phenotypically sticks to the actin filaments, the 'actin-binding mutant' cannot bind cables anymore. She2-TAP in a *myo4* rigor mutant background thus not only yielded a more profound band pattern but also a more complex protein composition than

in wt strains. Mass-spectrometric analysis has revealed a number of additional proteins that have not been detected in the purification from wt material, mirroring the She RNP enrichment of the *myo4* mutant over the wt strain. The fact that mutant myo4p has not been identified within the purified material is most likely due to the COOH-terminal fusion with two GFPs, rendering the hybrid protein to a molecular size of more than 200 kDa, a range that might be too bulk to efficiently migrate into the chosen gel matrix. The observation that the protein composition again varied between She2-TAP from wt and myo4p rigor mutant strains prompted to the speculation that a She2-TAP from a myo4 actin-binding mutant might be the ideal choice for future experiments. This source should be free from cytoskeletal contaminants, but still enriched for the target She RNP. Indeed, a final large scale She2-TAP from the myo4 actin-binding mutant background yielded an intense band pattern, both in quality and quantity. Unfortunately these protein samples could not be analyzed due to a systematic error during the mass-spectrometric analysis. Before that accident all visible protein bands had yielded solid results, indicating an extremely sensitive detection procedure.

5.4.2 Qualitative analysis of the purified material

5.4.2.1 The She RNP transport complex has been purified as single protein/RNA assembly with an approximate molecular mass of 4.5 MDa

Although the She core-machinery could be identified within the purified material, it was still not clear whether the proteins comprise a single assembly or several smaller sub-complexes. In order to determine the approximate molecular size of the She RNP complex the purified material from a large scale She2-TAP from a *myo4* P-loop mutant background has been analyzed by size exclusion chromatography.

Western- and Northern blot analysis have revealed Myo4p, She2p and *ASH1* mRNA in the same fraction, whereas a highly expressed control RNA could not be detected. She3p has not been analyzed here, but since Myo4p has been shown to constantly co-migrate with She3p by glycerol gradient fractionation (Böhl *et al*., 2000), She3p can be regarded as part of the complex.

5.4.2.2 Identification of the She RNP transport complex in the purified material

In a model mRNA localization involves three separate steps, namely nuclear packaging, cytoplasmic transport and cortical anchorage. As discussed above, She2p is likely to be associated with *ASH1* mRNA in the nucleus, in the cytoplasm and during anchorage. The

material that has been subjected to size exclusion chromatography stemmed from a She2-TAP in a *myo4* actin-binding mutant background, a mutation that interferes with actin binding. Loosing actin-binding capacity this myo4p mutant is unable to drive She RNP into the bud. As a consequence the purified material should be free from a postulated anchorage complex. In order to separate the transport- form a postulated nuclear complex involving She2p, the preparation of the starting material included a 100.000g spin from which only the supernatant has been taken for TAP. Finally the identification of Myo4p together with She2p in the same fraction argues for a cytoplasmic rather than a nuclear complex.

She2p has been detected in two elution fractions from the gel-filtration column, and the extremely sensitive Northern dot blot has revealed *ASH1* mRNA exclusively in the same fractions. She2p bridges *ASH1* mRNA to She3p, and RNA association triggers She2p binding to She3p (Böhl *et al*., 2000). Thus it is likely that the two identified fractions containing both She2p and *ASH1* mRNA represent the She RNP transport complex. However, the finding that Myo4p elutes in a broader range over several fractions whereas She2p and *ASH1* mRNA can be detected only in two fractions argues for a limited transient She2p association with the She3p / Myo4p transport machinery. This result might attest Myo4p/She3p as being involved in additional cytoplasmic transport processes apart from mRNA localization, as suggested by Estrada *et al*. (2003).

The finding that the She core machinery together with *ASH1* mRNA elutes from the gel-filtration column as a single RNP of approximately 4.5 MDa finally demonstrated the integrity of the purified material. This result has not only ruled out substantial possible degradation but also confirmed earlier genetic, immuno-histological and biochemical data implying the She proteins in the cytoplasmic localization process of *ASH1* mRNA.

In addition to the She proteins several proteins have been identified which have not been implicated to function in cytoplasmic mRNA localization earlier. Among the pool of additional proteins mass-spectrometric analysis identified factors implicated in translation regulation and mRNA biogenesis as well as ribosomal proteins.
Despite the identification of the core She machinery, *ASH1* mRNA and a number of additional factors, other proteins like Loc1p, Khd1p or Puf5p that previously have been reported to be involved in *ASH1* mRNA localization have not been found in any purified material. This might be due to their rather transient association with the cytoplasmic She

RNP, or to a function earlier during nuclear RNP packaging. Also, no ER-resident proteins could be identified, though Myo4p has been implicated in cortical ER inheritance (Estrada *et al*., 2003).

5.4.3 The translational inhibitor Eap1p, and its possible implication in cytoplasmic *ASH1* mRNA transport

5.4.3.1 The translational inhibitor Eap1p co-purifies with the She RNP

As an example for a new factor, Eap1p was found to be associated with both She2- and Myo4-TAP. Eap1p belongs to the family of eIF4E-binding proteins, 4EBPs, that act as translational repressors during protein synthesis initiation (reviewed by Gingras *et al*., 1999). Whereas CPE-mediated translation control seems to be restricted to higher metazoans (Mendez and Richter, 2002), the 4EBP family has orthologs in numerous lower eukaryotic organisms including *Dictyostelium* and yeast. Eap1p has been shown to associate with the cap-binding protein eIF4E, thereby blocking the eIF4E-eIF4G interaction that is required for the recruitment of the small ribosomal subunit to the mRNA (Cosentino *et al*., 2000).
As a second characteristic Eap1p has been implicated in spindle pole body function, since cells lacking Eap1p display chromosomal instability defects and are synthetically lethal with mutations in *ndc1*, a gene required for spindle pole body duplication (Chial *et al*., 2000). Interestingly the synthetic lethality can be rescued by eap1-Y109A, an allele that harbors a loss-of-function mutation within the conserved eIF4E-binding domain (also present in eIF4G). Thus, the eap1-Y109A allele allows to uncouple translation repression from spindle pole body duplication. This dual function might explain why Eap1p, 70 kDa, is much larger in size than other eIF4E-binding proteins, 10 kDa.
Analysis of the Eap1p primary sequence has revealed several characteristics apart from the 4E-binding motif (Cosentino *et al*., 2000). Similar to its human ortholog 4E-T, which has been shown to shuttle 4E into the nucleus (Dostie *et al*., 2000), the Eap1p coding sequence harbors a putative bipartite nuclear localization signal. Furthermore Eap1p contains a Walker A consensus motif, indicating the potential for purine nucleotide binding, and a proline-rich domain in the COOH-terminus. Proline-rich stretches have been suggested to represent docking sites for SH3-domains, a type of domain that is often found in proteins involved in signal transduction pathways. The finding that Eap1p is highly phosphorylated *in vivo* suggests that kinases are upstream effectors of Eap1p (Cosentino *et al*., 1999, and references therein).

An extremely interesting feature is that Eap1p has been shown to interact with Scp160p (Mendelsohn *et al*., 2003). A deletion in *scp160* has been shown to be synthetically lethal with *eap1* mutants, and this lethality can be partly rescued by the eap1-Y109 allele. Even more, both proteins interact physically in an RNA-dependent manner.

5.4.3.2 Cells lacking Eap1p correctly localize *ASH1* mRNA, but accumulate Ash1p to some extent in both mother and daughter cell nuclei

As Eap1p has been implicated in translation regulation and interacts with Scp160p, the protein has been regarded as an outstanding attractive candidate for a subsequent characterization concerning a function in *ASH1* mRNA localization.
In preliminary results that have to be confirmed cells lacking Eap1p display a mild *she*$^-$ phenotype on Ash1p-distribution, whereas *ASH1* mRNA localization remains unaffected. This partial mislocalization phenotype seems to be independent from total Ash1p expression, since initial quantitation experiments have revealed no significant differences of Ash1p levels in *Δ eap1* versus wt cells. The rather gentle effect is most probably the reason for why *EAP1* was missed in the original *SHE* screen. Nonetheless, the *Δ eap1* phenotype allows a comparison with other secondary factors like Khd1p or Scp160p that have been proposed to function in Ash1p translation regulation.

5.4.3.3 *Drosophila* Cup is a functional Eap1p homologue, that couples *oskar* mRNA localization to local translation

During the course of this work, Cup has been identified as 147 kDa component of an 8 protein RNP including *oskar* mRNA in *Drosophila* oocytes (Wilhelm *et al*., 2000). Like Eap1p, Cup belongs to the 4EBP family and functions as translation repressor for *oskar* during cytoplasmic transport to the oocyte posterior pole. It has been reported that Cup recruits Barentsz and is therefore required to assemble the *oskar* mRNA localization complex. Being apparently involved in both processes it has been suggested that Cup might be a regulatory target to couple mRNA localization and translation (Wilhelm *et al*., 2003).
Strikingly similar to our preliminary results, cup mutants from a different fly line lacking the 4E binding-site display pre-mature *oskar* translation, though *oskar* mRNA localization to the posterior remains unperturbed. In addition it has been shown that Cup directly interacts with Bruno (Nakamura *et al*., 2004). Hence, *Drosophila* Cup can be regarded as functional homologue of Eap1p, linking mRNA localization to translation control.

Summing up, Eap1p is a member of the 4EBP family, which is characterized by the ability to repress cap-dependent translation initiation due to the sequestration of eIF 4E. Eap1p affinity is likely to be regulated by post-translational modifications, so that hypo-phosphorylation induces 4E binding whereas hyper-phosphorylation releases Eap1p and allows translation initiation.

5.5 Building a new model for *ASH1* mRNA localization

5.5.1 Postulated mechanism for Eap1p function during cytoplasmic *ASH1* mRNA transport

The idea that *ASH1* mRNA is translated during the cytoplasmic transport stems from a careful systematic site-directed mutagenesis study affecting the four localization elements (Chartrand *et al*., 2002). The ZIPcodes have been replaced by elements that disrupted the secondary structure of the RNA but left the Ash1p primary sequence unchanged, resulting in a construct named 'ASH1-mut'. Asymmetric mRNA localization as well as Ash1p distribution was completely abolished. Adding back the wt ZIPcodes into the 3'-UTR of *ASH1*-mut could rescue tight asymmetric mRNA localization to the bud tip, but not Ash1p distribution to both nuclei, indicating a positional effect of the localization elements on Ash1p distribution. From these and other observations the authors suggested that the localization elements are required within the coding sequence in order to impede the elongating ribosome as molecular obstacles. Video microscopy of fluorescently labeled particles containing the *ASH1*-E3 element has revealed a cytoplasmic transport time of roughly 240 seconds (Bertrand *et al*., 1998). At a given general protein synthesis rate of 10 amino-acids per second the 588 amino-acids of Ash1p would be terminated before the She RNP has reached the bud and diffuse into the mother cell nucleus. Thus the secondary structures of the localization elements within the Ash1 coding sequence have been proposed to represent essential features for translation inhibition that are required to slow down the synthesis rate and thereby enable the She RNP to localize to the bud before translation termination (Chartrand *et al*., 2002).

A supplementary level of Ash1p translational control is added in this study, precisely the control of translation initiation by the 4EBP-family member Eap1p. Eap1p has been found to be associated with She2-TAP in a *myo4* ATPase mutant background, indicating that Eap1p is part of the She RNP transport complex. The translation initiation factors 4E, 4G and 4A form the multifunctional eIF-4F subunit, required for recruitment of the 43S complex including the small ribosomal subunit. Eap1p is known to sequester 4E from binding to 4G, and 4A,

thereby repressing translation initiation. Since Eap1p is thought to compete with 4G for binding to 4E, this finding indicates that the purified material contains a mix of both Eap1p-4E and 4G-4E complexes. This mixture reflects the translation status of *ASH1* mRNA during cytoplasmic transport: some transcripts are blocked, while others seem to be already initiated for translation to occur. This interpretation is further strengthened by the specific co-purification of ribosomal proteins, whereas the TAP control experiment using 1.5 fold the amount of starting material of a TAP-less wt strain did not yield substantial band intensities in the molecular range of 10-40 kDa. However, the major indication for Eap1p being involved in Ash1p translation initiation comes from the initial observation that in cells lacking Eap1p *ASH1* mRNA localizes indistinguishably from wt to the bud tip, whereas Ash1p to some extent distributes symmetrically to both mother and daughter nuclei. This phenomenon mirrors the localization pattern of *ASH1*-mut observed by Chartrand *et al*., 2002 and suggests an 'early translation defect'. In translation assays based on an inducible promoter the observed protein mis-localization defect of Ash1-mut has been related to a three-fold premature protein accumulation compared to wt Ash1 (Chartrand *et al*., 2002). Thus premature synthesis has enabled Ash1p diffusion in substantial amounts into the mother cell nucleus. Taking over this argumentation line in respect to the observed defects in *Δ eap1* cells, *ASH1* mRNA is most likely translated 'earlier' during transport than in wt cells. Finally, an 'earlier' translation than in wt cells predicts a mechanism controlling *ASH1* translation initiation.
Summing up this part, Eap1p is likely to interact indirectly or directly with a number of factors implicated in translation control. Similarly to *Drosophila* Cup, Eap1p might function as adapter between 4E at the mRNA cap and additional trans-acting factors, thereby transducing upstream signals from Ser- / Thr-kinases.

5.5.2 Functional implication of additional She RNP associated factors

As part of the She RNP complex Eap1p seems to be involved in the control of *ASH1* translation initiation. It has been shown that Eap1p is highly phosphorylated *in vivo*, suggesting a post-translational regulation mechanism. The primary sequence reveals a number of potential phosphorylation target sites in the vicinity of the 4E-binding region. Within the purified material from a Myo4-TAP the Ser-/Thr-specific septin kinase Gin4p has been identified as potential upstream regulator of Eap1p. Gin4p has been shown to co-localize together with the septin ring, a ring-like structure that forms at the mother-bud junction during bud growth (chapter 4.4.2; Barral *et al*., 1999). This intracellular distribution renders Gin4p as an ideal upstream candidate kinase for Eap1p.

Asc1p, a ribosome-associated WD40 repeat protein, has been identified as an additional She2-TAP associated factor. Since Asc1p has not been detected in the purified material from the She2-TAP in a *myo4* ATPase mutant, it is possible that Asc1p is solely involved in the anchorage process. Interestingly, during an initial She2-TAP we identified the Eap1p-interacting protein Scp160p as component of the purified material (data not shown). As mentioned earlier cells lacking Scp160p display mild *ASH1* mRNA localization defects. Surprisingly, mass-spectrometric analysis identified peptides exclusively contained within the Scp160p COOH-terminus, a region that has recently been reported to be required for interaction with Asc1p (Baum *et al*., 2004). Together the two proteins have been suggested to function as signaling platform regulating ribosome activity, and therefore might be implicated in Ash1p translation. However, like *Δeap1* cells, initial experiments with strains deleted in asc1 have not revealed a detectable defect in *ASH1* mRNA localization (data not shown).

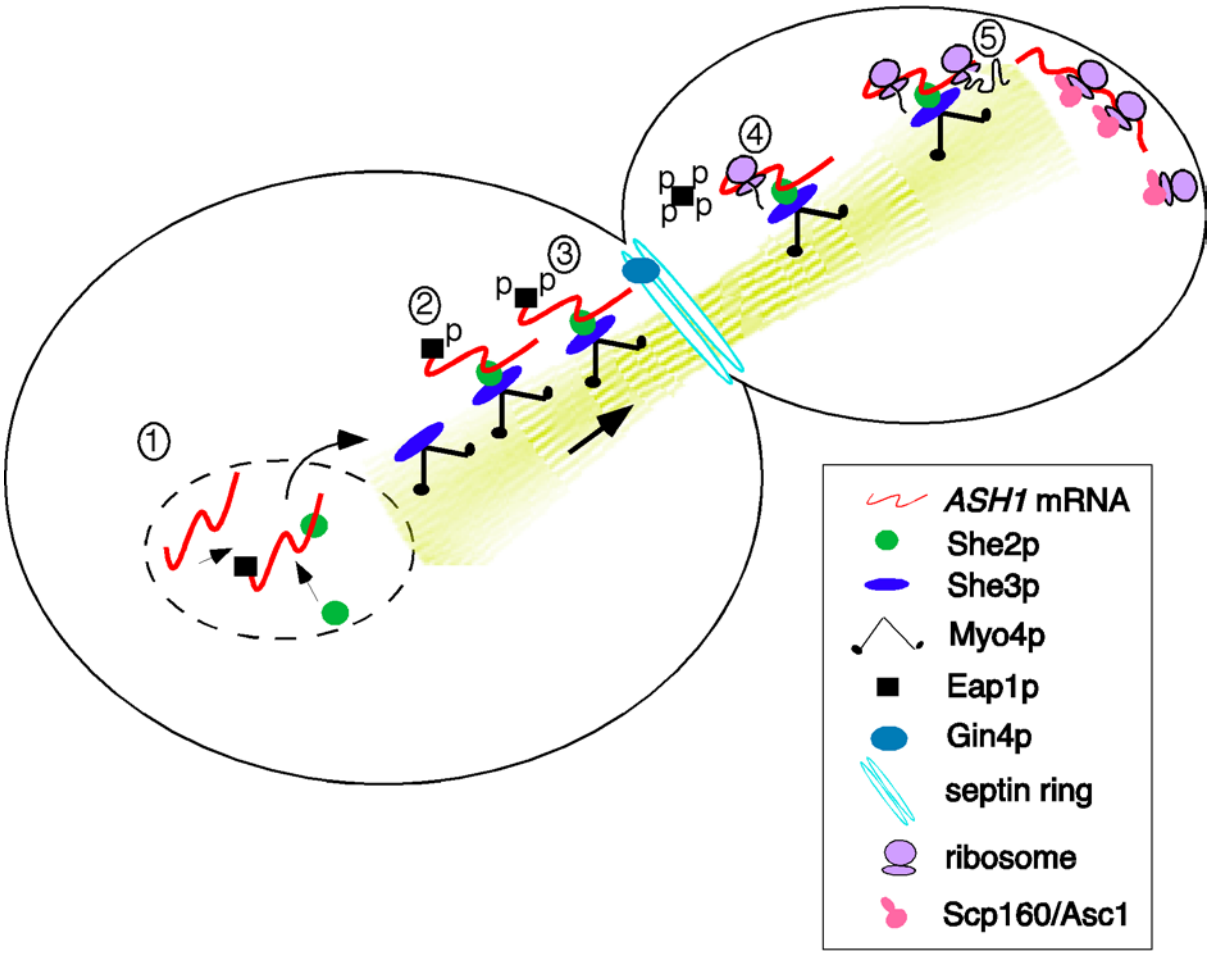
5.5.3 The idea of local termination versus local synthesis

Integration of various results proposes that the bud tip is the ideal location to terminate *ASH1* translation, since only released from that cellular location Ash1p has the biggest chance to diffuse into the daughter cell nucleus instead into the mother. It has been proposed that the translation elongation rate is retarded due to the presence of molecular obstacles, secondary structures within the coding sequence of the RNA. Eap1p phosphorylation during transport might be an additional regulatory mechanism that delays the onset of translation and thereby contributes to local Ash1p translation termination at the bud tip.

The idea is based on the results from a set of purifications yielding the She RNP transport complex. A separate purification for a postulated distinct anchorage complex has not yet been performed. Thus the model can only describe processes that might occur during transport, but leaves open the question if there is an additional local translation re-initiation mechanism at the bud tip.

The relatively small size of the yeast organism against the *Drosophila* ovary implies a shorter travelling distance for the She RNP compared to the *oskar* RNP. A shorter travelling distance implies a faster completion of the journey, so that Ash1p translation elongation can be initiated before ending up at the bud tip. In order to assure the correct positioning of Oskar it is likely that more sophisticated control mechanisms have emerged in metazoan organisms, including CPE-like translation repression and local release.

5.5.4 Gedankenexperiment: A functional implication of new She protein-like factors into the 3-step model for *ASH1* mRNA localization



The following model combines the results from this study and others. Factors that have been biochemically co-purified together with the She RNP complex are integrated, as well as a speculation on their functional contribution to *ASH1* mRNA targeting.

The model illustrates the journey of the She RNP from the mother into the bud cytoplasm, where it eventually becomes anchored to the peripheral cortex.

Upon nuclear packaging (step 1), She2p and *ASH1* mRNA - possibly in conjunction with Eap1p – are co-exported into the cytoplasm, where the nuclear *ASH1* RNP meets the cytoplasmic Myo4p / She3p transport machinery. Together both sub-complexes form a solid cytoplasmic She RNP that can be readily detected by immuno-histological imaging techniques ('beads-on-the-string', step 2). *ASH1* mRNA translation is repressed due to Eap1p bound to 4E at the *ASH1* mRNA cap. During the passage through the bud neck Gin4p mediated Eap1p phosphorylation induces release of the repressor (step 3), and allows formation of the 4E-4G interaction. Ribosome assembly onto the travelling mRNA marks the onset of Ash1p synthesis (step 4). Elongation is remarkably retarded due to the bulk secondary structures formed by the localization elements. The RNP reaches the bud cortex

before translation termination occurs. Before completion of the first translation round the zinc-finger motif within the Ash1p-COOH terminus mediates the hand over of *ASH1* mRNA cargo from the transport complex onto membrane bound ribosomes at the cortical ER (step 5). The latter step might be assisted by the Scp160p / Asc1p complex that is associated with the 40S ribosomal subunit. Whereas Ash1p is released and diffuses into the daughter cell nucleus, the transport complex disassembles and floats back into the mother cell.

5.6 Future experiments

The model for *ASH1* mRNA localization presented here assumes a number of direct and indirect protein-protein as well as protein-RNA interactions, which have to be confirmed by biochemical and co-localization studies. It is essentially important to confirm the integrity of the She RNP, repeat the large scale She2-TAP in a *myo*4 loop mutant and have enough purified material for a gel-filtration experiment. Not before this is accomplished a TAP-based protocol can be worked out in order to purify the nuclear *ASH1* RNP or the anchorage complex.

Trans-acting factors like She2p, Eap1p or Khd1p have to be characterized in more detail, which hopefully will finally allow the determination of the precise order of recruitment onto the mRNA. Again a division of the localization process into three parts can be of great use. Here the *mex67-5* mutant will be helpful for further studies on nuclear packaging and shuttling. The role of the Ash1p COOH-terminus in *ASH1* mRNA retention has to be characterized in more detail. In order to do so point mutations that disrupt the Ash1p DNA-binding capacity will be helpful.

Generally it can be worked out if the localization of other mRNAs in yeast follows the same principles as *ASH1*. Hereby of special interest is the issue of the linkage between mRNA localization and translation.

A central issue of this work has been the biochemical purification of the She RNP complex, and the identification of so far unknown She protein-like factors that take part in the cytoplasmic mRNA transport process. As a first candidate for further characterization Eap1p was picked by its virtue to be involved in the translation initiation process. Though the Eap1 protein seems to function as repressor of Ash1p synthesis, the conclusions from the initial experiments presented here have to be confirmed by other experiments. On top of the list is a validation of the direct interaction with the She RNP, and its functional implication on *ASH1* mRNA as well as Ash1p distribution. In this context, Eap1p overexpression might result in reduced Ash1p levels. A postulated shuttling behavior can be visualized by

immunohistological means using the *mex67-5* mutant. Assuming that this is the case, one can ask the question if the shuttling depended on an RNA interaction, and perform the same experiment with the *eap1-Y109A* mutant allele. In support of the model, the Eap1p hyper-phosphorylation must be analyzed, and tested if the septin kinase Gin4p can indeed act as potential upstream regulator. Similarly to *Drosophila* Cup and Bruno, Eap1p might also directly interact with Khd1p in immunoprecipitation experiments. Furthermore the relatively mild *SHE* phenotypes of mutations in eap1, scp160, asc1, khd1 might be augmented in combination with each other. In this respect it will be interesting to analyze Ash1 protein levels in *in vivo* and *in vitro* assays, and compare *ASH1* against *ASH1-mut* intensities.

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7. Abbreviations

ab	antibody
ADP	adenosine diphosphate
ARS	autonomous replicating sequence
ASH	asymmetric synthesis of *HO*
ATP	adenosine triphosphate
ATPase	adenosine triphosphate hydrolase
CEN	centromer
CPE	cytoplasmic polyadenylation element
CPEB	cytoplasmic polyadenylation element binding protein
CPSF	cleavage and polyadenylation specificity factor
Da	Dalton
DAPI	diamidino-2-phenylindol dihydochloride
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DIC	differential interference contrast
E1, E2…	mRNA localization element
E. coli	*Escherichia coli*
eIF	eukaryotic (translation-) initiation factor
eIF4E-BP	eukaryotic initiation factor 4E binding protein
ER	endoplasmatic reticulum
et al.	*et alii*, latin, meaning 'and others'
FLIP	fluorescence loss in photo-bleaching
FISH	fluorescence *in situ* hybridization
GDP	guanosine diphosphate
GFP	green fluorescent protein
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HA	hemagglutinin
hnRNP	heterogeneous nuclear ribo-nucleoprotein
HO endonuclease	homothallic switching endonuclease
hr	hour
kb	kilo bases
min	minute
mRNA	messenger ribonucleic acid
NMD	nonsense mediated decay
OD	optical density
oligo	oligonucleotide
ORF	open reading frame
Pi	inorganic phosphate
poly-(A)	polyadenylic acid
PAP	poly-(A) polymerase
pre-mRNA	precursor messenger ribonucleic acid

RNA	ribonucleic acid
RNP	ribo-nucleoprotein
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
s	second
S	Svedberg unit
S. cerevisiae	*Saccharomyces cerevisiae*
SHE	Swi5p-dependent *HO* expression
TAP	tandem affinity purification
TCA	tri-chlor acetic acid
TOR	target of rapamycin
UTP	untranslated region
wt	wildtype

8. Publications

Kruse C, Jaedicke A, Beaudouin J, Böhl F, Ferring D, Güttler T, Ellenberg J, Jansen RP. Ribonucleoprotein-dependent localization of the yeast class V myosin Myo4p.
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Presentations

Jaedicke A and Jansen RP. Translation dependent anchorage of *ASH1* mRNA in *S. cerevisiae*. 40th Annual Meeting of the American Society for Cell Biology, December 2000, San Francisco

Kruse C, Jaedicke A and Jansen RP. RNA-controlled Myo4p localization in *S. cerevisiae*. Annual ELSO Meeting, June 2002, Nice

Jaedicke A, Kruse C and Jansen RP. RNA-controlled Myo4p localization in *S. cerevisiae*. 42nd Annual Meeting of the American Society for Cell Biology, December 2002, San Francisco

Jaedicke A and Jansen RP. 'Purification and analysis of the She-RNP complex from *S. cerevisiae*. Annual Meeting of the SFB 352, October 2003, Heidelberg

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