ASH1 mRNA Anchoring Requires Reorganization of the Myo4p/She3p/She2p Transport Complex*

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1The abbreviations used are: 3'-UTR, 3'-untranslated region; DAPI, 4',6'-diamidino-2-phenylindole; ER, endoplasmic reticulum; IP, immunoprecipitation; mRNA, messenger RNA; OAc, acetate; PCR, polymerase chain reaction; RNP, ribonucleoprotein; RT-PCR, reverse transcriptase-PCR; SDS-PAGE, sodium dodecyl sulfate
polyacrylamide gel electrophoresis; TAP, tandem affinity purification

Running title: Characterization of the ASH1 mRNA transport RNP.
One mechanism by which cells post-transcriptionally regulate gene expression is via intercellular and intracellular sorting of mRNA. In *S. cerevisiae*, the localization of *ASH1* mRNA to the distal tip of budding cells results in the asymmetric sorting of Ash1p to daughter cell nuclei. Efficient localization of *ASH1* mRNA depends upon the activity of four *cis*-acting localization elements and also upon the activity of *trans*-factors She2p, She3p and Myo4p. She2p, She3p and Myo4p have been proposed to form an *ASH1* mRNA localization particle. She2p directly and specifically binds each of the four *ASH1* *cis*-acting localization elements, while She3p has been hypothesized to function as an adaptor by recruiting the She2p/mRNA complex to Myo4p, a type V myosin. The Myo4p/She3p/She2p heterotrimeric protein complex has been proposed to localize mRNA to daughter cells using polarized actin cables. Here we demonstrate that while the predicted Myo4p/She3p/She2p heterotrimeric complex forms *in vivo*, it represents a relatively minor species compared to the Myo4p/She3p complex. Furthermore, contrary to a prediction of the heterotrimeric complex model for *ASH1* mRNA localization, *ASH1* mRNA artificially tethered to She2p is not localized. Upon closer examination we found that mRNA tightly associated with She2p is transported to daughter cells but is not properly anchored at the bud tip. These results are consistent with a model whereby anchoring of *ASH1* mRNA requires molecular remodeling of the Myo4p/She3p/She2p heterotrimeric complex, a process that is apparently altered when mRNA is artificially tethered to She2p.
The establishment and maintenance of cellular polarity is a salient feature of eukaryotic cells and involves the asymmetric sorting of proteins to distinct compartments or regions of the cell. One mechanism by which proteins can be sorted in a variety of eukaryotic cell types is via mRNA localization, a process by which mRNA is specifically localized to a particular region of a cell (1-3). A primary step in the process of mRNA localization is the identification of the localization substrate by trans-acting factors, resulting in the formation of a localization mRNP. The mRNP localization complexes are dynamic structures undergoing molecular reorganization at various stages of the localization pathway (4-6). RNA localization can result from a number of distinct mechanisms involving direct transport of the mRNA to the site of localization, generalized degradation of the mRNA with localized protection or random diffusion followed by entrapment of the mRNA at the site of localization (1,2). Ultimately, translation of the mRNA at the site of localization leads to the asymmetric distribution of the protein in the cell.

The asymmetric sorting of Ash1p in *Saccharomyces cerevisiae* is a paradigm for investigating the asymmetric segregation of proteins via mRNA localization. Ash1p is a transcriptional repressor that is asymmetrically sorted to daughter cells resulting in differential gene expression between mother and daughter cells (7-9). The unequal segregation of Ash1p results from the localization of *ASH1* mRNA at the distal tip of budding yeast cells during anaphase of the cell cycle (10-12). Localization of *ASH1* mRNA depends upon four cis-acting localization elements, named E1, E2A, E2B and E3, and each element is sufficient for localizing a heterologous reporter RNA to daughter cells (10,13,14).
In addition to the *cis*-acting localization elements, efficient *ASH1* mRNA localization also requires several *trans*-acting factors: She1-5p, Loc1p, Khd1p, Bud6p, Puf5p, Puf6p, Scp160p and a polarized actin cytoskeleton (11,12,15-20). The proteins apparently most directly involved with the localization of *ASH1* mRNA are Myo4p/She1p, She2p and She3p. Myo4p is a type V non-processive myosin that directly transports the *ASH1*-RNP particle to daughter cells (16,21-23). She2p interacts directly and specifically with each of the four *cis*-acting elements in *ASH1* mRNA and also associates with She3p (24,25). She3p interacts with Myo4p as well as She2p; consequently, She3p has been proposed to function as an adaptor by linking the She2p/*ASH1* mRNA complex onto the motor protein, Myo4p (24-26). The current model for *ASH1* mRNA localization proposes the formation of a Myo4p/She3p/She2p/*ASH1* mRNA complex *in vivo* (24-26). Once formed, the *ASH1*-RNP is transported to daughter cells on polarized actin cables. Interestingly, the function of Myo4p and She3p is not restricted to mRNA localization. Myo4p and She3p are also required for inheritance of cortical ER, but in contrast to the *ASH1* mRNA localization pathway, cortical ER sorting is independent of She2p (27).

In this report we present evidence that She3p can simultaneously associate with Myo4p and She2p resulting in the Myo4p/She3p/She2p heterotrimeric complex and analyze the role of this heterotrimeric complex in the *ASH1* mRNA localization pathway. In comparison to the Myo4p/She3p complex, we found that the heterotrimeric complex was present at a relatively lower abundance. Closer inspection revealed that the heterotrimeric complex which is sufficient for transport of mRNA to daughter cells is insufficient for anchoring the mRNA at the bud tip.
These results therefore favor a model whereby anchoring of *ASH1* mRNA requires reorganization of the Myo4p/She3p/She2p/ASH1 transport mRNP.
EXPERIMENTAL PROCEDURES

Yeast Strains, Media and Plasmids

The yeast strains used in this study are listed in Table 1. Yeast cells were either grown in rich media or in defined synthetic media lacking the indicated nutrients (28). Yeast cells were transformed using lithium acetate, and gene deletions were created as described previously, using PCR products generated from plasmid pUG6 (29,30). Silent deletions were subsequently generated using plasmid pSH47 (30). Endogenous yeast genes were tagged with myc\textsubscript{13} or 3HA epitopes using PCR products as described elsewhere (31).

Plasmids used in this study are listed in Table 2 and were constructed using standard molecular techniques (32). Details regarding plasmid construction are available upon request.

Soluble Lysate Preparation and Immunoprecipitation

Exponentially growing yeast cultures corresponding to \(3 \times 10^8\) cells were harvested for each immunoprecipitation, and the cell pellets were resuspended in lysis buffer containing 50 mM HEPES-KOH, pH 7.3, 20 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, 5% glycerol, 0.1 mg/ml chymostatin, 2 mg/ml aprotinin, 1 mg/ml pepstatin, 0.5 mg/ml leupeptin and 0.01 mg/ml benzamidine. Glass beads were added to the suspension, and cells were broken by vortexing. The extracts were cleared by centrifugation at 5000 x g for 2 min at 4\( ^\circ \)C. The soluble fraction was recovered and added to antibody bound Protein A beads (Pierce). Anti-myc
immunoprecipitations were performed using 7.5 µg of monoclonal 9E10 antibody (Roche). Anti-HA immunoprecipitations were performed using 10 µg of monoclonal HA.11 antibody (Convance). The soluble lysate was incubated with the Protein A beads for 2 hr at 4°C with gentle agitation. The beads were subsequently washed four times with 500 µl of wash buffer (50 mM HEPES-KOH, pH 7.3, 100 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100 and 5% glycerol). Bound proteins were eluted by boiling the Protein A-antibody complexes in Laemmli buffer. Equivalent amounts of cell extract (Total) and bound proteins (IP) were separated by SDS-PAGE and analyzed by Western-blot. For the salt titration experiment, the above wash buffer containing potassium acetate at concentrations ranging from 100 mM to 800 mM was used. Each experiment was performed at least twice and quantitative values were determined with either AlphaImager 2200 (AlphaInnotech Corp.) or FluroChem 8900 (AlphaInnotech Corp.) software.

**Immunoprecipitation/RT-PCR**

With few modifications, immunoprecipitation/RT-PCR reactions were performed as previously described, using antibody bound Protein A beads (33). For immunoprecipitation experiments using the myc epitope 7.5 µg of anti-myc 9E10 was used. For immunoprecipitation of endogenous She2p, 20 µl of anti-She2p rabbit antiserum was used. The RT-PCR reactions were performed using the Access/RT-PCR kit (Promega) according to the directions provided.
by the manufacturer and were performed under conditions to ensure that amplifications were in
the linear range of the assay.

**RNP Purification**

Yeast cells expressing the desired constructs were harvested by centrifugation, and a
soluble yeast lysate was prepared as described in the above immunoprecipitation section. The
soluble lysate was added to 20 µl of FLAG-Agarose beads (ANTI-FLAG® MS2 Affinity Gel,
Sigma) and incubated for 2 hr at 4°C with gentle agitation. The beads were recovered by
centrifugation at 500 x g for 2 min at 4°C, and the beads were washed four times with 600 µl of
wash buffer (50 mM HEPES-KOH, pH 7.3, 100 mM potassium acetate, 2 mM EDTA, 0.1%
Triton X-100 and 5% glycerol). The bound proteins were eluted by heating the beads at 65°C
for 10 min in 100 µl of elution buffer (50mM Tris-HCl, pH 8.0, 100mM NaCl, 10mM EDTA
and 1% SDS). The soluble lysate (Total) and eluate (IP) fractions were subsequently separated
by SDS-PAGE and analyzed by Western-blot. Myc tagged proteins were detected using the
anti-myc 9E10 monoclonal antibody, while the 4XFLAG-MS2 fusion protein was detected
using ANTI-FLAG M2® Monoclonal Antibody – Peroxidase Conjugate (Sigma).

**In situ Hybridization and Fluorescent Microscopy**

The *in situ* hybridizations were performed as described previously using *ASH1*-Cy3 or *lacZ-*
Cy3 DNA oligonucleotide probes (11,34). Cells were subsequently stained with DAPI and mounted on slides with mounting media containing phenylenediamine. Images were captured using a Nikon Eclipse 600 epifluorescence microscope equipped with a 60X N.A. 1.4 objective, interfaced to a Micromax-Interline Transfer CCD Camera (Princeton Instruments, Inc.) and MetaMorph Imaging Software (Universal Imaging Corp.).
RESULTS

She2p associates with Myo4p dependent on She3p-Previous work demonstrated that She3p is capable of associating in vivo with both Myo4p and She2p (24,25), potentially resulting in two complexes: Myo4p/She3p (complex II) and She3p/She2p (complex III) (Fig. 1A). Based on the presumed ability of She3p to simultaneously interact with both Myo4p and She2p, it was hypothesized that a Myo4p/She3p/She2p heterotrimeric complex (complex IV) could also form in vivo (Fig. 1A) (24-26). Therefore, we investigated the presence of the heterotrimeric complex by immunoprecipitating Myo4p-myc13 from a yeast lysate and analyzing the ability of She2p to co-immunoprecipitate with Myo4p-myc13 (Fig. 1B). She2p was found to specifically co-precipitate with Myo4p dependent on She3p (Fig. 1B compare lanes 2 and 3). These results support the conclusion that a fraction of She2p is a component of a Myo4p/She3p/She2p heterotrimeric complex (complex IV) and are in agreement with results obtained from a genome-wide TAP analysis (35).

In vivo analysis of She3p-containing protein complexes-Given that we could detect an in vivo association between Myo4p and She2p, we sought further insight into the steady state abundance of She3p-containing complexes II, III and IV (Fig. 1A). Following immunoprecipitation of Myo4p-3HA and Western-blotting, we observed at least a 20-fold lower signal for She2p-myc6 compared to the signal corresponding to She3p-myc6 (Fig. 1C, lane 4). In this experiment, the
signal for She2p-myc\textsubscript{6} following immunoprecipitation was more easily detected upon over-exposure of the blot (data not shown). Assuming each Myo4p-3HA containing complex has an equivalent ability of being immunoprecipitated, this result suggests that the Myo4p/She3p complex (complex II) predominates over the heterotrimeric complex (complex IV).

We next determined the relative abundance of the She3p/She2p complex (complex III) in comparison to the heterotrimeric complex (complex IV). Two isogenic yeast strains were used for this analysis, one strain expressing endogenous levels of Myo4p-myc\textsubscript{13} and the other strain expressing endogenous levels of She3p-myc\textsubscript{13}. Immunoprecipitations were performed, and the abundance of co-precipitating She2p was determined by Western-blot (Fig. 1D). We observed 2-fold more She2p co-precipitating with She3p-myc\textsubscript{13} compared to Myo4p-myc\textsubscript{13}. Since She2p can associate with She3p \textit{in vivo} in two potential complexes: the She2p/She3p complex (complex III) and the heterotrimeric complex (complex IV), the level of She2p that co-precipitates with She3p under these conditions represents the sum of complexes III and IV. Consequently, our results suggest that complex III and complex IV are present at nearly equivalent levels.

It is also possible that the various complexes have different stabilities under the experimental conditions. The salt-stability of the various complexes was therefore examined. All complexes were found to be equally stable at 100 mM KOAc (Fig. 2). However, the She3p/She2p complex and the heterotrimeric complex were not stable at salt concentrations
higher than 200 mM KOAc (Fig. 2B and C). In contrast the Myo4p/She3p complex was stable up to 800 mM KOAc (Fig. 2A). Consequently, we conclude that the apparent lower abundance of She2p-containing complexes is not due to differences in the stabilities of the complexes under the ionic conditions used to prepare the cell lysates.

*ASH1* mRNA is associated with similar amounts of Myo4p, She3p and She2p. She3p and Myo4p have been shown to associate with *ASH1* mRNA in vivo, and the association of *ASH1* mRNA with She3p and Myo4p is dependent on She2p, suggesting that She3p and Myo4p associate with *ASH1* mRNA via heterotrimeric complex formation (22,26). A prediction made by the current model is that She2p associates with *ASH1* mRNA prior to associating with She3p and Myo4p. Thereby more *ASH1* mRNA could be associated with She2p than with She3p or Myo4p. Examining the level of *ASH1* mRNA associated with She2p, She3p and Myo4p tested this prediction. She2p-myc13, She3p-myc13 and Myo4p-myc13 were immunoprecipitated from yeast lysates, and co-precipitating *ASH1* RNA was analyzed by RT-PCR (Fig. 3A). A similar amount of *ASH1* mRNA was found to co-precipitate with She2p-myc13, She3p-myc13 and Myo4p-myc13 under these conditions, consistent with the hypothesis that very little, if any, *ASH1* mRNA associated with any of these three proteins exists free of the heterotrimeric complex.

To more quantitatively assess the association of Myo4p, She3p and She2p with *ASH1* cis-acting localization elements, mRNPs containing *ASH1* cis-acting elements were purified, and
the levels of She2p-myc\textsubscript{13}, She3p-myc\textsubscript{13} and Myo4p-myc\textsubscript{13} that co-purified with the mRNP were determined by Western-blot. To purify mRNPs, a \textit{lacZ} mRNA construct containing MS2 stem loops as well as the \textit{ASH1} E3 \textit{cis}-acting localization element was constructed (Fig. 3B). For a negative control, \textit{lacZ-MS2} mRNA lacking the E3 \textit{cis}-acting localization element was used. Expression of the fusion mRNA constructs in a strain that also expresses a 4x FLAG-MS2 fusion protein enables the purification of the MS2-containing mRNPs via the MS2 protein/MS2 RNA interaction (36). Following enrichment of the MS2-RNPs, the presence of Myo4p-myc\textsubscript{13}, She3p-myc\textsubscript{13} and She2p-myc\textsubscript{13} was determined by Western-blot analysis (Fig. 3C). When normalized to the amount of immunoprecipitating 4x FLAG-MS2 we observed equivalent levels of She2p and She3p co-purifying with the mRNP. In contrast, we observed a slight, 1.8-fold, enrichment for co-purifying Myo4p when compared to She2p (Fig. 3C). Using this purification scheme Myo4p, She3p and She2p are only co-precipitated from strains expressing the \textit{lacZ-MS2-E3} fusion mRNA construct (Fig. 3C). These observations indicate that the \textit{in vivo} association and purification of these proteins are specific for \textit{ASH1} \textit{cis}-acting localization elements. The results from the mRNP purification experiment are consistent with the IP/RT-PCR analysis, suggesting that the majority of \textit{ASH1} mRNA associated with Myo4p, She3p or She2p is present in the heterotrimeric complex. Furthermore, from our observation that approximately two fold more Myo4p is associated with \textit{ASH1} mRNA compared to She3p and She2p leads us to hypothesize that the stoichiometry of the Myo4p/She3p/She2p complex may
be 2:1:1, assuming that all She2p that is associated with ASH1 mRNA is also present in the heterotrimeric complex.

**Association of mRNA with the heterotrimeric complex is not sufficient for mRNA localization**

The observations that the heterotrimeric complex is present in yeast cells and that Myo4p, She3p and She2p associate with ASH1 mRNA at similar levels suggest that association of mRNA with the Myo4p/She3p/She2p heterotrimeric complex is necessary and sufficient for mRNA localization to daughter cells. In the heterotrimeric complex model, She2p functions as a molecular scaffold to interface mRNA localization substrates with the Myo4p/She3p transport complex. If the role of She2p in mRNA localization is limited to its scaffold function, then any mRNA tethered to She2p should localize to daughter cells.

To examine this detail of the model, a version of ASH1 mRNA containing six MS2 stem loop structures was constructed. This ASH1-MS2 mRNA is not compromised for mRNA localization since in wild-type yeast cells it is sorted to the distal tip of the bud (Fig. 4A). When the ASH1-MS2 mRNA is expressed in cells expressing She2p-MS2, the ASH1-MS2 mRNA has the potential to associate with either the She2p or MS2 component of the fusion protein. Compared to cells expressing She2p, we observed that the percentage of cells with localized ASH1-MS2 mRNA is decreased 2-fold in cells expressing She2p-MS2 (Fig. 4A). The reduction in ASH1-MS2 mRNA localization is not a consequence of the MS2 domain interfering with She2p mRNA localization activity since wild-type ASH1 mRNA lacking MS2 sequences is
localized at equivalent levels in cells expressing either She2p or She2p-MS2 (data not shown). Consequently, these results imply that when \textit{ASH1-MS2} mRNA is associated with the She2p portion of the She2p-MS2 fusion protein the mRNA is capable of being correctly localized, but when this mRNA is associated with the MS2 portion of the fusion protein, the localization substrate is not able to be localized.

This hypothesis was tested by investigating the ability of \textit{ASH1-MS2} mRNA to localize to daughter cells when associated exclusively with the MS2 portion of the She2p-MS2 fusion protein. To achieve exclusive binding of \textit{ASH1-MS2} mRNA through the MS2 RNA-binding domain, She2p mutant R63K, which specifically lacks \textit{ASH1} mRNA-binding activity, was fused to MS2 and tested for the ability to localize \textit{ASH1-MS2} mRNA (33). When compared to cells expressing She2p, we observed that localization of \textit{ASH1-MS2} mRNA is reduced 20-fold in cells expressing She2p-R63K-MS2 despite the ability of She2p-R63K-MS2 to associate with Myo4p and \textit{ASH1-MS2} mRNA (Fig. 4B and data not shown). This finding suggests that the mechanism for \textit{ASH1} mRNA localization is not as simple as She2p functioning as a molecular scaffold to interface mRNA localization substrates to the Myo4p/She3p complex.

\textit{mRNA artificially tethered to She2p is transported to daughter cells}-It is likely that \textit{ASH1} mRNA localization involves a transport component and an anchoring component. The inability of \textit{ASH1-MS2} mRNA bound exclusively to the MS2 portion of the She2p-MS2 fusion protein to localize to daughter cells could be due to the inability of the She2p-MS2 fusion protein to
transport mRNA to daughter cells, and/or the inability of the fusion protein to anchor mRNA at the bud tip. We reasoned that if we could pinpoint where in the localization pathway that the MS2 mRNA-binding activity could not substitute for She2p mRNA-binding activity, we might gain further insight into the mechanism of mRNA localization to daughter cells.

In an effort to distinguish between an mRNA localization transport defect or an anchoring defect related to MS2 mRNA-binding activity, we sought to separate transport from anchoring. By investigating the distribution of mRNA localization substrates throughout the entire cell cycle, the transport and anchoring components could be individually investigated. Transport and anchoring of mRNA dependent on MS2 mRNA binding activity throughout the cell cycle was studied by fusing wild-type She2p to MS2 and individually monitoring the intracellular distribution of galactose-inducible lacZ mRNA, lacZ-ASH1 mRNA or lacZ-MS2 mRNA (Fig. 5). Since She2p-MS2 is unable to associate with lacZ mRNA, as expected this mRNA is uniformly distributed between mother and daughter cells at all stages of the cell cycle (Fig. 5/top panel-small budded cells and bottom panel-cells at anaphase). Furthermore, as expected lacZ-ASH1 mRNA was observed to localize to daughter cells during all stages of cell cycle since this mRNA exclusively associates with the She2p portion of the She2p-MS2 fusion protein. Upon examining the distribution of lacZ-MS2 mRNA, we observed that this mRNA was correctly sorted to daughter cells prior to anaphase (Fig. 5/top panel). However, at anaphase when ASH1 mRNA is normally localized, lacZ-MS2 mRNA is uniformly distributed between mother and daughter cells (Fig. 5/bottom panel). Consequently, these results suggest that prior to anaphase
She2p-MS2 is able to participate in the transport of *lacZ-MS2* mRNA to daughter cells, but is not able to properly anchor *lacZ-MS2* mRNA in anaphase cells.
DISCUSSION

Previously, a model for ASH1 mRNA localization was proposed that featured a Myo4p/She3p/She2p heterotrimeric complex and ASH1 mRNA was hypothesized to associate with the heterotrimeric complex via She2p (24-26). The basis for this model came largely from two observations: the ability of She3p to interact with Myo4p and She2p as well as the ability of Myo4p and She3p to associate with ASH1 mRNA dependent on She2p (22,24-26). While these observations provided the foundation for this model, it remained to be determined if She3p simultaneously interacts with Myo4p and She2p resulting in the formation of the heterotrimeric complex. Furthermore, the heterotrimeric complex model does not satisfactorily explain the observation that unlike endogenous She2p, which is uniformly distributed between mother and daughter cells, Myo4p and She3p are asymmetrically sorted to daughter cells dependent on associated mRNA (8,22,33,37). Consequently, it was conceivable that She2p might have a role in ASH1 mRNA localization that does not involve the formation of the Myo4p/She3p/She2p complex.

In support of the heterotrimeric complex model, we observed that Myo4p and She2p associate dependent on She3p (Fig. 1). Further examination of She3p-containing complexes revealed that the Myo4p/She3p complex is more abundant than either the She3p/She2p complex or the heterotrimeric complex (Fig. 1). One possible explanation for the greater abundance of the Myo4p/She3p complex in comparison to the She2p-containing complexes is that She2p may be expressed at a much lower level than either She3p or Myo4p. However, She2p is approximately
2-fold more abundant than Myo4p and 4-fold more abundant than She3p (38). Therefore, the predominance of the Myo4p/She3p complex over the other two complexes is apparently independent of concentration differences of these three proteins and is apparently related to intrinsic differences in the ability of She3p to associate with Myo4p versus She2p. Consistent with this assertion is our finding that the She3p/She2p association is more sensitive to ionic strength compared to the Myo4p/She3p interaction (Fig. 2). Based on these results we hypothesize that She3p may have differential affinities for Myo4p and She2p, thus resulting in the preferential formation of the Myo4p/She3p complex. Alternatively, formation of the three protein complexes could be subject to post-translational regulation. If formation of the heterotrimeric complex or the She3p/She2p complex is subject to regulation, it is apparently unrelated to the ability of mRNA to associate with She2p. We found no difference in the amount of She2p associated with Myo4p or She3p when She2p is unable to associate with mRNA localization substrates or when mRNA localization substrates are over-expressed (data not shown).

An implicit prediction of the heterotrimeric model is that RNA artificially tethered to She2p should localize to daughter cells in an analogous fashion to ASH1 mRNA. Even though She2p-R63K-MS2 is capable of associating with Myo4p and ASH1-MS2 mRNA, ASH1-MS2 mRNA is delocalized in anaphase cells (Fig. 4). A trivial explanation for the inability of ASH1-MS2 mRNA to localize to daughter cells dependent on She2p-R63K-MS2 is that fusing MS2 to She2p could interfere with a She2p function in ASH1 mRNA localization. However, wild-type
She2p-MS2 is fully competent for localizing ASH1 mRNA (data not shown), indicating that the MS2 RNA-binding domain does not impede the ability of wild-type She2p to localize ASH1 mRNA.

The precise defect in ASH1 mRNA localization associated with tethering mRNA to She2p through MS2 appears to be related to anchoring. A completely artificial mRNA localization substrate, lacZ-MS2 mRNA, localizes to small and medium sized buds dependent on She2p-MS2 (Fig. 5). Consequently, She2p-MS2 is able to participate in the delivery of RNA localization substrates during this period of the cell cycle when the actin cytoskeleton is highly polarized for efficient delivery of various cargos to the bud. In contrast, She2p-MS2 is unable to localize lacZ-MS2 mRNA during anaphase when ASH1 mRNA is normally localized. We hypothesize that upon reaching the bud tip She2p-MS2/lacZ-MS2 mRNA is unable to mimic a She2p/ASH1 mRNA molecular remodeling event that results in the anchoring of the mRNA localization substrate. Since in contrast to She2p, mRNA tethered to She3p is localized in anaphase cells, anchoring may require transfer of mRNA from She2p onto an anchoring factor that is part of the Myo4p/She3p complex (Fig. 6) (24). By artificially tethering mRNA to She2p through high affinity binding, the transfer of mRNA required for anchoring may be abrogated. Once the mRNA has been transferred from She2p onto the anchoring factor, She2p may dissociate from the anchoring complex, thus resulting in the observed uniform cellular distribution of She2p.

If She2p disassociates from the anchoring complex, it is possible that in addition to the
heterotrimeric complex, Myo4p and She3p also associates with \textit{ASH1} mRNA in complexes that are devoid of She2p. Thus in the IP/RT-PCR experiments there is the potential for purifying transport complexes containing She2p as well as anchoring complexes that are devoid of She2p (Fig. 3). Similarly, in the mRNP purification assay all \textit{ASH1} containing mRNPs should be purified. Thus if Myo4p and She3p also associate with \textit{ASH1} mRNA in complexes devoid of She2p, it is possible for similar amounts of \textit{ASH1} mRNA to be associated with Myo4p, She3p and She2p, despite the observation that the heterotrimeric complex represents a relatively minor species. Moreover if the sole purpose of the Myo4p/She3p/She2p heterotrimeric complex is to transport \textit{ASH1} mRNA to daughter cells, the observed lower abundance of this complex in comparison to the Myo4p/She3p complex may in fact be indicative of the transitory nature of the heterotrimeric complex.

Previous evidence for molecular remodeling of mRNA localization complexes has been observed in oocytes. In \textit{Drosophila} oocytes a protein complex containing Swa and PABP have been shown to associate with \textit{bicoid} mRNA (5). Like \textit{bicoid} mRNA, both proteins accumulate in the anterior region of the oocyte, suggesting a potential role for these proteins in anchoring \textit{bicoid} mRNA at the anterior cortex of the oocytes (5,39,40). In addition, the Mod and Nod proteins have also been shown to associate with \textit{bicoid} mRNA and to form a complex with Swa and PABP \textit{in vivo} (5). However unlike Swa and PABP, Mod and Nod do not accumulate at the anterior cortex of the oocyte, thus implicating these proteins in the transport phase of \textit{bicoid} mRNA localization (5,41,42). Therefore the proper localization of an mRNA might be mediated
by distinct transport and anchoring steps involving the remodeling of the RNP at various stages during the course of localization. In yeast, certain proteins like She2p may be a unique component of the transport complex while other proteins like She3p and Myo4p may be present in both transport and anchoring complexes.

In conclusion, this work provides new evidence that the Myo4p/She3p/She2p heterotrimeic complex participates in the transport of \textit{ASH1} mRNA, but is not actively involved in anchoring \textit{ASH1} mRNA at the bud tip. Furthermore since She3p and Myo4p appear to have roles in both transport and anchoring, this data suggests that \textit{ASH1} mRNA may be transferred from She2p onto a factor that is part of the Myo4p/She3p complex. The identity of the anchoring factor and the precise mechanism by which \textit{ASH1} mRNA is anchored at the bud tip awaits further characterization.
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FIGURE LEGENDS

FIG 1. The Myo4p/She3p/She2p heterotrimeric complex forms in vivo. A, Schematic representation of potential She3p-containing complexes. B, She2p associates with Myo4p in a She3p dependent manner. Yeast strains YLM1309 (lanes 1 and 2) and YLM1406 (lane 3) were transformed with plasmid pRL453 (YCplac111-She2p). Transformants were grown in synthetic media devoid of leucine, and lysates were prepared for immunoprecipitation of Myo4p-myc\textsubscript{13}. Immunoprecipitations were performed in the absence (lane 1) and presence (lanes 2 and 3) of anti-myc antibody. An aliquot of the lysate (Total) and the pellet (IP) fractions was analyzed by Western-blots for the presence of Myo4p-myc\textsubscript{13} using the anti-myc antibody and for the presence of She2p using a rabbit polyclonal She2p antiserum. In lanes 1 and 2, Myo4p-myc migrates with a greater mobility than the Myo4p-myc present in lane 3. This difference is due to a slight difference in the strains used. While both strains were initially transformed with the myc\textsubscript{13}:kanMX6 cassette, strain YLM1309, as confirmed by PCR, has undergone an intragenic recombination event reducing the number chromosomally integrated myc tags and thus reducing the molecular weight of Myo4p-myc. C, Myo4p associates with a greater amount of She3p in vivo in comparison to She2p. Yeast strain YLM1320 was transformed with the following combination of plasmids: pRL460 (YCplac22/She3p-myc\textsubscript{6})/pRL199 (YCplac111/She2p-myc\textsubscript{6}) (lanes 1 and 4), YCplac22 (vector)/pRL199 (lane 2), and pRL460/YCplac111 (vector) (lane 3). Transformants were grown in synthetic media lacking leucine and tryptophan. Lysates were
prepared for immunoprecipitation of Myo4p-3HA. Immunoprecipitations were performed in the absence (lane 1) and presence (lanes 2-4) of anti-HA antibody. An aliquot of the lysate (total) and the pellet fractions (IP) was analyzed by Western-blot for the presence of Myo4p-3HA using the anti-HA antibody and for the presence of She3p-myc6 and She2p-myc6 using the anti-myc antibody. D, The abundance of the She3p/She2p complex is equivalent to the heterotrimeric complex. Yeast strains W303-She3p-myc (lanes 1 and 2) and W303-Myo4p-myc (lane 3) were grown in YEPD media, and lysates were prepared for immunoprecipitation of She3p-myc13 and Myo4p-myc13. Immunoprecipitations were performed in the absence (lane 1) and presence (lanes 2 and 3) of anti-myc antibody. An aliquot of the lysate (total) and pellet fractions (IP) was analyzed by Western-blot for the presence of She3p-myc13 and Myo4p-myc13 using the anti-myc antibody and for the presence of She2p using a rabbit polyclonal She2p antiserum.

FIG. 2. The Myo4p/She3p complex is more resistant to ionic strength than the She3p/She2p complex and the heterotrimeric complex. A, Yeast strain YLM1320 was transformed with plasmids pRL460 (YCplac22/She3p-myc6) and pRL453 (YCplac111/She2p). The transformant was grown in synthetic media lacking leucine and tryptophan, and a soluble lysate was prepared for immunoprecipitation of She3p-myc6. Immunoprecipitations were performed in the absence
(lane 1) and presence (lanes 2-6) of anti-myc antibody. Following immunoprecipitation, the beads were washed with buffer containing 100 mM KOAc (lanes 1 and 2), 200 mM KOAc (lane 3), 400 mM KOAc (lane 4), 600 mM KOAc (lane 5) and 800 mM KOAc (lane 6). An aliquot of the lysate (total) and pellet fractions (IP) was analyzed by Western-blot for the presence of Myo4p-3HA using the anti-HA antibody and for the presence of She3p-myc\textsubscript{6} using the anti-myc antibody. B, The experiment was repeated as in Fig.2A, but the total (total) and pellet fractions (IP) were analyzed by Western-blot for the presence of She3p-myc\textsubscript{6} using the anti-myc antibody and She2p using a polyclonal She2p antiserum. C, Yeast strain W303-Myo4p-myc was grown in YEPD, and a soluble lysate was prepared for immunoprecipitation of Myo4p-myc\textsubscript{13}. Immunoprecipitations were performed in the absence (lane 1) and presence (lanes 2-6) of anti-myc antibody. Following immunoprecipitation, the beads were washed with buffer containing 100mM KOAc (lanes 1 and 2), 200mM KOAc (lane 3), 400mM KOAc (lane 4), 600mM KOAc (lane 5) and 800mM KOAc (lane 6). An aliquot of the lysate (total) and pellet fractions (IP) was analyzed by Western-blot for the presence of Myo4p-myc\textsubscript{13} using the anti-myc antibody and for the presence of She2p using a rabbit polyclonal She2p antiserum.

FIG. 3. **She2p, She3p and Myo4p are associated with similar amounts of ASH1 mRNA.** A, IP-RT/PCR analysis of ASH1 mRNA associated with She2p, She3p and Myo4p. Yeast strains W303-She2p-myc (lanes 1 and 2), W303-She3p-myc (lane 3) and W303-Myo4p-myc (lane 4) were grown in YEPD, and a soluble lysate was prepared for immunoprecipitation of She2p, She3p and Myo4p, respectively. Immunoprecipitations were performed in the absence (lane 1) and presence (lanes 2-6) of anti-myc antibody. Following immunoprecipitation, the beads were washed with buffer containing 100mM KOAc (lanes 1 and 2), 200mM KOAc (lane 3), 400mM KOAc (lane 4), 600mM KOAc (lane 5) and 800mM KOAc (lane 6). An aliquot of the lysate (total) and pellet fractions (IP) was analyzed by Western-blot for the presence of She2p, She3p and Myo4p using the anti-myc antibody and for the presence of Myo4p-myc\textsubscript{13} using the anti-myc antibody.
4) were grown to mid-log phase, and lysates were prepared for IP/RT-PCR. Immunoprecipitations were performed in the absence (lane 1) and presence (lanes 2-4) of anti-myc antibody, and the resulting immunoprecipitates were subsequently used for RT-PCR analysis using \textit{ASH1} specific primers. RT-PCR reactions corresponding to an aliquot of the lysate prior to immunoprecipitation (Total) as well as reactions corresponding to the immunoprecipitate in the presence (+RT) or absence (-RT) of reverse transcriptase are shown. Additionally, an aliquot of the immunoprecipitate was analyzed by Western-blot using the anti-myc antibody.

B, Schematic representation of the RNA and fusion protein constructs used for mRNP purification. The mRNA constructs were engineered to contain six MS2 stem loop structures. In addition, the experimental mRNA construct also contained the E3 \textit{ASH1} \textit{cis}-acting localization element. The fusion protein construct was designed to express a 4xFLAG-MS2 fusion protein. All constructs were galactose inducible.

C, \textit{ASH1} RNP purification. Yeast strain W303-She2p-myc was transformed with the following combination of plasmids: pRL804 (pESC-LEU/4xFLAG-MS2)/pRL856 (YEplac195/\textit{lacZ-MS2-ADHII}) (lane 1), and pRL804/pRL857 (YEplac195/\textit{lacZ-MS2-E3-ADHII}) (lane 2). Yeast strain W303-She3p-myc was transformed with the following combination of plasmids: pRL804/pRL856 (lane 3), and pRL804/pRL 857 (lane 4). Yeast strain W303-Myo4p-myc was transformed with the following combination of plasmids: pRL804/pRL856 (lane 5), and pRL804/pRL857 (lane 6). Transformants were grown to mid-log phase in synthetic media containing 2% raffinse but lacking leucine and uracil. Expression of the reporter mRNA and fusion protein constructs was
subsequently induced by the addition of galactose to a final concentration of 2% and incubation for an additional three hours. Subsequently, the cells were harvested and lysates were prepared for immunoprecipitation. The RNP complexes were purified using anti-FLAG antibody beads, and the co-precipitating proteins were separated by SDS-PAGE and analyzed by Western-blots using anti-myc and anti-FLAG antibodies.

FIG. 4. **ASH1 mRNA tethered to She2p-R63K-MS2 is not localized.** A, Yeast strain YLM1309 was transformed with the following combination of plasmids: pRL171 (YEplac181/She2p)/pRL859 (YEplac112-ASH1-MS2), pRL666 (YEplac181/She2p-MS2)/pRL859, and pRL768 (YEplac181/She2p-R63K-MS2)/pRL859. Transformants were grown in liquid synthetic media devoid of leucine and tryptophan to mid-log phase. Subsequently, the cells were harvested and processed for *in situ* hybridization using Cy3 conjugated complementary DNA probes for *ASH1* mRNA. Representative images for *in situ* hybridization, DAPI straining and Nomarski optics are shown. The Cy3 images corresponding to She2p/ASH1-MS2 mRNA and She2p-MS2/ASH1-MS2 mRNA are representative of mRNA localization, and the Cy3 image corresponding She2p-R63K-MS2/ASH1-MS2 mRNA is representative of unlocalized mRNA. Also indicated is the percentage of cells with asymmetric signal for *ASH1* mRNA (n=100 cells). B, Yeast strain YLM1309 was transformed with the following combination of plasmids: pRL171/pRL859 (lanes 1 and 2), pRL666/pRL859 (lane 3), and pRL768/pRL859 (lane 4). Transformants were grown in liquid synthetic media devoid of
leucine and tryptophan to mid-log phase. The cells were subsequently harvested by centrifugation, and lysates prepared for immunoprecipitation of Myo4p-myc13. Immunoprecipitations were performed in the absence (lane 1) and presence (lanes 2-4) of anti-myc antibody. An aliquot of the lysate (total) and pellet fractions (IP) was analyzed by Western-blot for the presence of Myo4p-myc13 using anti-myc antibody and for the presence of She2p using a rabbit polyclonal She2p antiserum.

FIG. 5. mRNA tethered to She2p-MS2 is transported but not anchored in daughter cells. Yeast strain YLM1309 was transformed with the following combination of plasmids: pRL666 (YEplac181/She2p-MS2)/pRL855 (YEplac195/lacZ-ADHII), pRL666/pRL856 (YEplac195/lacZ-MS2-ADHII) and pRL666/pRL823 (YEplac195/lacZ-ASH1). Transformants were grown to mid-log phase in synthetic media containing 2% galactose but lacking leucine and uracil. The cells were subsequently fixed and processed for *in situ* hybridization using Cy3 conjugated complementary DNA probes for lacZ mRNA. Representative images for *in situ* hybridization, DAPI staining and Nomarski optics are shown. The top panel shows representative cells prior to anaphase, while the bottom panel shows representative anaphase cells. Prior to anaphase Cy3 images for She2p-MS2/lacZ-MS2 mRNA and She2p-MS2/lacZ-ASH1 mRNA are representative of localized mRNA while the image corresponding to She2p-MS2/lacZ mRNA is representative of unlocalized mRNA. At anaphase Cy3 images corresponding to She2p-
MS2/lacZ mRNA and She2p-MS2/lacZ-MS2 mRNA are representative of unlocalized mRNA while the Cy3 image for She2p-MS2/lacZ-ASH1 mRNA is representative of localized mRNA. Also indicated is the percentage of cells with asymmetric signal for lacZ mRNA (n=100 cells).

FIG. 6. Revised model for ASH1 mRNA localization. She2p binds ASH1 mRNA and subsequently associates with She3p/Myo4p forming the heterotrimeric complex. This complex then transports the mRNA cargo to daughter cells on the polarized actin cytoskeleton. Once delivered to the daughter cell, a molecular remodeling event occurs releasing She2p from the complex, enabling anchoring factor(s) (AC) to associate with ASH1 mRNA, She3p and Myo4p. Anchored ASH1 mRNA is translated in the daughter cell and newly synthesized Ash1p enters the daughter cell nucleus.
Table I

Yeast strains used in this study

| Strain          | Genotype                                           | Source |
|-----------------|----------------------------------------------------|--------|
| K699            | *Mata, ade2-1, his3-11, leu2-3,112, ura3, trp1-1, ho, can1-100* | (8)    |
| YLM1309<sup>b</sup> | *Mata, ade2-1, his3-11, leu2-3,112, trp1-1, ura3, Thi study ho, can1-100, she2Δ silent, MYO4-myc13:kanMX6* |        |
| YLM1320<sup>b</sup> | *Mata, ade2-1, his3-11, leu2-3,112, trp1-1, ura3, Thi study ho, can1-100, she2Δ silent, she3Δ silent, MYO4-3HA:kanMX6* |        |
| YLM1406<sup>b</sup> | *Mata, ade2-1, his3-11, leu2-3,112, trp1-1, ura3, Thi study ho, can1-100, she2Δ silent, she3Δ silent, MYO4-myc13:kanMX6* |        |
| W303-She2p-myc<sup>b</sup> | *Mata, ade2-1, his3-11, leu2-3,112, ura3, trp1-1, ho, can1-100, SHE2-myc13:kanMX6* | (26)   |
| W303-She3p-myc<sup>b</sup> | *Mata, ade2-1, his3-11, leu2-3,112, ura3, trp1-1, ho, can1-100, SHE3-myc13:kanMX6* | (26)   |
W303-Myo4p-myc<sup>b</sup> Mata, ade2-1, his3-11, leu2-3,112, ura3, trp1-1, ho, can1-100, MYO4-myc13:kanMX6

<sup>b</sup>K699 genetic background
Table II

Plasmids used in this study

| Plasmid           | Genotype                                      | Source       |
|-------------------|-----------------------------------------------|--------------|
| pESC-LEU          | yeast multicopy plasmid marked with *LEU2*   | Stratagene   |
|                   | for galactose regulated expression            |              |
| YCplac22          | yeast single copy shuttle plasmid marked with *TRP1* | (43)         |
| YCplac111         | yeast single copy shuttle plasmid marked with *LEU2* | (43)         |
| YEplac112         | yeast multicopy shuttle plasmid marked with *TRP1* | (43)         |
| YEplac195         | yeast multicopy shuttle plasmid marked with *URA3* | (43)         |
| YEplac181         | yeast multicopy shuttle plasmid marked with *LEU2* | (43)         |
| C3319             | YEplac181 vector containing *ASH1*            | (11)         |
| pRL171            | Yeplac181 vector expressing She2p             | This work.   |
| pRL198            | YCplac111 containing Myo4p-myc<sub>6</sub>      | This work    |
| pRL199            | YCplac111 containing She2p-myc<sub>6</sub>     | This work    |
| pRL200            | YCplac111 containing She3p-myc<sub>6</sub>     | This work    |
| pRL453            | YCplac111 containing wild-type She2p          | This work    |
| pRL460            | YCplac22 containing She3p-myc<sub>6</sub>      | This work    |
| pRL666            | Yeplac181 vector expressing She2p-MS2         | This work    |
| pRL768            | YEplac181 expressing R63K-She2p-MS2           | This work    |
| pRL804            | pESC-LEU vector containing 4xFLAG-MS2 fusion  | This work    |
| Plasmid   | Description                  | Source     |
|-----------|------------------------------|------------|
| pRL823    | YEplac195 vector expressing *lacZ-ASH1* (full length) | This work  |
| pRL828    | YEplac112 vector expressing She2p-MS2 | This work  |
| pRL855    | YEplac195 vector expressing *lacZ-AdhII* | This work  |
| pRL856    | YEplac195 vector expressing *lacZ-MS2-AdhII* | This work  |
| pRL857    | YEplac195 vector expressing *lacZ-MS2-E3-AdhII* | This work  |
| pRL858    | YEplac112 vector expressing *ASH1* | This work  |
| pRL859    | YEplac112 vector expressing *ASH1* -MS2 | This work  |
Myo4p-myc$_{13}$

|        | +   | +   | +   |
|--------|-----|-----|-----|
| She3p  | +   | +   | -   |
| She2p  | +   | +   | +   |

**Total**

- Myo4p-myc$_{13}$
- She2p

**IP**

- Myo4p-myc$_{13}$
- She2p

FIG. 1B
| Myo4p-myc<sub>13</sub> | - | - | + |
|---------------------|---|---|---|
| She3p-myc<sub>13</sub> | + | + | - |

**Total**

- Myo4p-myc<sub>13</sub>
- She3p-myc<sub>13</sub>
- She2p

**IP**

- Myo4p-myc<sub>13</sub>
- She3p-myc<sub>13</sub>
- She2p
B. 

\[ \text{GAL1p} \rightarrow 4\times\text{FLAG-MS2} \]

\[ \text{GAL1p} \rightarrow \text{lacZ} \rightarrow \text{MS2} \rightarrow \text{ADHII 3'UTR} \]

\[ \text{GAL1p} \rightarrow \text{lacZ} \rightarrow \text{MS2} \rightarrow \text{E3} \rightarrow \text{ADHII 3'UTR} \]

C. 

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| **Total** | | | | | |
| **Myo4p-myc\textsubscript{13}** | | | | | |
| **She3p-myc\textsubscript{13}** | | | | | |
| **She2p-myc\textsubscript{13}** | | | | | |
| **4xFLAG-MS2** | | | | | |

| MS2 | MS2-E3 | MS2 | MS2-E3 |
|-----|--------|-----|--------|
| **Myo4p-myc\textsubscript{13}** | | | |
| **She3p-myc\textsubscript{13}** | | | |
| **She2p-myc\textsubscript{13}** | | | |
| **4xFLAG-MS2** | | | |

FIG. 3B, C
A.

|                | She2p | She2p-MS2 | She2p-R63K-MS2 |
|----------------|-------|-----------|---------------|
| ASH1-MS2       | +     | +         | +             |
| Cy3            |       |           |               |
| DAPI           |       |           |               |
| Nom.           |       |           |               |
| % Localized    | 60%   | 30%       | 3%            |

B.

| Myo4p-myc<sub>13</sub> | + | + | + | + |
|------------------------|---|---|---|---|
| She2p                  | + | + | - | - |
| She2p-MS2              | - | - | + | - |
| R63K-MS2               | - | - | - | + |

- Myo4p-myc<sub>13</sub>
- She2p-MS2/R63K-MS2
- She2p
- Myo4p-myc<sub>13</sub>
- She2p-MS2/R63K-MS2
- She2p

FIG. 4 A, B
ASH1 mRNA anchoring requires reorganization of the Myo4p/She3p/She2p transport complex
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