Role of Loc1p in assembly and reorganization of nuclear *ASH1* messenger ribonucleoprotein particles in yeast

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Directional transport of mRNA is a universal feature in eukaryotes, requiring the assembly of motor-dependent RNA-transport particles. The cytoplasmic transport of mRNAs is preceded by the nuclear assembly of pre-messenger ribonucleoprotein particles (mRNPs). In budding yeast, the asymmetric synthesis of HO 1 (*ASH1*) pre-mRNP originates already cotranscriptionally and passes through the nucleolus before its nuclear export. The nucleolar localization of *ASH1* mRNA protein 1 (Loc1p) is required for efficient *ASH1* mRNA localization. Immunoprecipitation experiments have revealed that Loc1p forms cocomplexes with other components of the *ASH1* transport complex. However, it remains unclear how Loc1p is recruited into this mRNP and why Loc1p is important for *ASH1* mRNA localization. Here we demonstrate that Loc1p undergoes a direct and specific interaction with the *ASH1* mRNA-binding Swi5p-dependent HO expression protein 2 (She2p). This cocomplex shows higher affinity and specificity for RNA bearing localization elements than the individual proteins. It also stabilizes the otherwise transient binding of She2p to *ASH1* mRNA, suggesting that cooperative mRNA binding of Loc1p with She2p is the required nuclear function of Loc1p for *ASH1* mRNA localization. After nuclear export, myosin-bound She3p joins the *ASH1* mRNP to form a highly specific cocomplex with She2p and *ASH1* mRNA. Because Loc1p is found only in the nucleus, it must be removed from the complex directly before or after export. In vitro and in vivo experiments indicate that the synergistic interaction of She2p and She3p displaces Loc1p from the *ASH1* complex, allowing free Loc1p to rapidly reenter the nucleolus. Together these findings suggest an ordered process of nuclear assembly and reorganization for the maturation of localizing *ASH1* mRNPs.

macromolecular complex | reconstitution experiments | Puf6p | cotranscriptional recruitment

Messenger RNA localization is a universal feature of eukaryotes (1–3). By complementing transcriptional control (4), it fulfills a variety of functions, including the establishment of cell polarity and specialization of subcellular regions. In recent years, the directional transport of asymmetric synthesis of HO 1 (*ASH1*) mRNA in budding yeast has emerged as a particularly well-suited model to study mechanistic principles of RNA localization. Here, comparably few proteins participate in the directional transport of *ASH1* mRNA and about 30 other transcripts (5, 6).

Chromatin-immunoprecipitation experiments revealed that the dedicated RNA-binding Swi5p-dependent HO expression protein 2 (She2p) binds already cotranscriptionally to nascent *ASH1* mRNA (7, 8). Two additional RNA-binding proteins, pumilio-homology domain family protein 6 (Puf6p) and heterogeneous nuclear RNP K-like protein 1 (Khd1p), are also present in the nucleus, bind to *ASH1* mRNA, and act in the cytoplasm as translational repressors during *ASH1* transport (9–12). A fourth nuclear factor, termed localization of *ASH1* mRNA protein 1 (Loc1p), has been implicated in the assembly of nuclear pre-messenger ribonucleoprotein particles (mRNPs). Like Puf6p,

Loc1p is a nuclear protein (13) with an enrichment in the nucleolus (14, 15) and participates in the assembly of the large ribosomal subunit (16–18).

The composition of the nuclear *ASH1* mRNP was previously analyzed by coimmunoprecipitation experiments. By using She2p as bait, Puf6p, Loc1p, and *ASH1* mRNA were copurified (11, 19). The translational inhibitor Khd1p, which interacts with the 5' region of *ASH1* mRNA (12), was absent from such purifications (11, 20). These data suggest the formation of a nuclear mRNP consisting of *ASH1* mRNA, She2p, Loc1p, and Puf6p.

Genomic deletion of *LOC1*, similar to *PUF6* (9, 11), leads to less efficient *ASH1* mRNA localization (13). Although Loc1p does not shuttle between nucleus and cytoplasm (13), *loc1Δ* cells show an up-regulation of cytoplasmic *ASH1* mRNA translation (13, 21), indicating that this protein is required for translational repression during localization. *ASH1* mRNA contains four *cis*-acting localization elements, which are also termed zip-code elements. These elements are specifically recognized by the transport complex. In the 5'–3' order of the *ASH1* mRNA the zip-code elements are termed E1, E2A, E2B, and E3. Puf6p- (11) and Loc1p-dependent translational repression (21) seems to be mediated only by the E3 zip-code element. Thus, Loc1p might be required to remodel the nuclear precomplex for the assembly of translational repressors at the E3 element.

Significance

Cytoplasmic mRNA localization is preceded by the formation of nuclear pre-mRNPs. To date the requirement of the nuclear mRNP assembly is not well understood. We used *ASH1* mRNA localization from budding yeast to understand the mechanisms of nuclear priming for cytoplasmic mRNA transport. We found that the nuclear factor Loc1p is required to stably and specifically tether the bona fide *ASH1* RNA-binding protein She2p to RNA in the nucleus, offering an explanation for the requirement of Loc1p for *ASH1* mRNA localization. Because Loc1p is not part of the cytoplasmic *ASH1* mRNP, it must be removed before mRNA transport. We show that the cytoplasmic transport factor She3p displaces Loc1p from the *ASH1* mRNP, allowing for the maturation of the localizing *ASH1* mRNP.

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Next, we asked whether the protein interaction between Loc1p and She2p is altered by binding to zip-code RNA. For better purification of large amounts of zip-code RNA, we used the tRNA-scaffold technique (29). We fused the 118-bases-long *ASH1* E3 element to a tRNA (*ASH1* E3-118 tRNA), expressed it in bacteria, and purified it. Using amylose resin and MBP-Loc1p, we pulled down She2p as well as *ASH1*-E3-118 tRNA (Fig. 1D), suggesting the formation of a ternary complex of these components.

Cooperative Binding of She2p and Loc1p to *ASH1* mRNA. The pull-down of She2p with RNA and Loc1p indicates a joint complex, but fails to yield direct information about their RNA binding within the complex. Furthermore, pull-down experiments are performed at micromolar concentrations that greatly exceed the nanomolar concentrations of these proteins in the cell. To directly assess RNA binding under more physiological conditions, we performed UV cross-linking experiments with radioactively labeled *ASH1* E3 RNA. Because UV cross-linking efficiency occurs preferentially with certain amino acids, such experiments yield semiquantitative information on affinities. As demonstrated before (8), at a concentration of 250 nM, She2p alone showed a well-detectable cross-linking product (Fig. 2A, lane 3). Also Loc1p alone could be cross-linked to the RNA (Fig. 2A, lanes 4–6). However, this cross-linking gave a clearly detectable signal only at 1 μ M protein concentration. The combination of Loc1p and She2p led to a strong increase of the Loc1p–RNA

cross-link (Fig. 2A, lanes 7–9). This observation indicates that She2p is required for efficient recruitment of Loc1p to *ASH1* mRNA. Because the She2p cross-link also showed an increased intensity in the presence of Loc1p, these results suggest cooperativity in RNA binding.

To validate these observations with a more quantitative assay, we performed EMSAs with constant, low concentrations of MBP-Loc1p and increasing concentrations of She2p (Fig. 2B). As previously shown (8, 30), in our EMSAs She2p alone does not form a protein–RNA complex stable enough to yield a significant mobility shift even at 1 μ M She2p concentration (Fig. 2B). In contrast, Loc1p alone gives rise to a distinct band shift at a concentration of 125 nM. When both proteins are combined, a much stronger band shift was observed (Fig. 2B). Supershift experiments with increasing antibody concentrations further confirmed that She2p was indeed present in this shifted band (Fig. S2A, Left). Mobility shift assays with gradient gels that better separate larger complexes show the additional slow-migrating band expected for the ternary complex of She2p, Loc1p, and RNA (Fig. S2A, Right). These findings, together with the UV cross-linking experiments, indicate a cooperative recruitment of She2p and Loc1p onto the *ASH1* mRNA and a stabilization of She2p on the RNA by Loc1p.

Coccomplex of Loc1p and She2p Forms Only with Zip-Code RNA. Next, we wanted to assess the specificity of the ternary complex formed by She2p, Loc1p, and RNA for the *ASH1* E3 zip-code element. First, we performed pull-down experiments with glutathione resin and GST-tagged She2p as bait. In these experiments with *ASH1* E3-51 RNA and tRNA competitor but in the absence of Loc1p, we observed copurification of *ASH1* E3-51 RNA but not of the tRNA competitor (Fig. 3A). Addition of Loc1p resulted in the pull-down of *ASH1* E3-51 RNA and Loc1p, but not of tRNA competitor. Interestingly, in the experiment with She2p and Loc1p, a higher molecular weight band was observed in the urea PAGE (Fig. 3A, asterisk). Because this band disappeared after phenol-chloroform extraction (Fig. 3A, Right), the ternary complex of She2p, Loc1p, and *ASH1* E3-51 RNA was likely not completely disrupted by the presence of urea in the gel. Such a high molecular weight band was not observed in absence of Loc1p (Fig. 3A, Left).

When we repeated these pull-down experiments with the HIV TAR-57 tRNA as an unspecific, zip-code-lacking control instead of an *ASH1* zip-code RNA, we failed to observe copurification of Loc1p or RNA (Fig. 3B). Because She2p and Loc1p undergo a direct protein–protein interaction in the absence of RNA (Fig. 1A), this result suggests different binding modes of this complex for specific and unspecific RNAs. The disruption of the protein–protein interaction between She2p and Loc1p in the presence of HIV TAR-57 RNA (Fig. 3B) suggests competitive binding events, instead of the previously observed cooperative binding of She2p and Loc1p to zip-code RNA (Figs. 1D and 2A and B). Consistent with this interpretation is the observation that in an amylose-resin pull-down, MBP-Loc1p copurified She2p only in the presence of zip-code-containing RNAs (Fig. S2B).

To confirm this difference in specific and unspecific RNA-binding modes by a different approach, we performed again UV cross-linking experiments. Radioactively labeled *ASH1* E3-51 tRNA efficiently cross-linked with She2p, Loc1p, and as a positive control She3p (Fig. 3C). Also in the cocomplex, Loc1p and She2p both efficiently cross-linked with the zip-code-containing RNA, as observed before (Fig. 2A). In contrast, the control HIV TAR-57 tRNA failed to show major cross-linking bands with She2p, Loc1p, or their cocomplex. Only She3p, which served as a control for unspecific binding (8), showed a clear cross-link (Fig. 3C). Also in competition experiments we observed a preference for *ASH1* E3 zip-code element (Fig. S2C). In summary,

Fig. 2. She2p and Loc1p recruit each other to the *ASH1* mRNA. (A) UV cross-linking experiments with MBP-Loc1p, She2p, and radioactively labeled *ASH1* E3-51 RNA. In the presence of She2p and Loc1p, cross-linking of both proteins to the *ASH1* mRNA is increased (lanes 7–9). Note that in UV cross-linking experiments the used concentrations better reflect the physiological range than in pull-down experiments. (B) EMSA with She2p, MBP-Loc1p, and radioactively labeled *ASH1* E3-51 RNA. As observed before (8, 30), nanomolar concentrations of She2p do not lead to the formation of a significant band shift. This and complementing observations suggested a rather transient binding mode of She2p to the *ASH1* E3 RNA. In contrast, Loc1p shows a weak but detectable band shift at 125 nM concentration. When She2p is added with increasing concentrations, the observed band shift is considerably strengthened, indicating a cooperative binding event.

Fig. 3. The cocomplex of Loc1p and She2p mediates specific and stable binding to zip-code RNA. (A) In pull-down assays with glutathione Sepharose, GST-She2p copurifies *ASH1* E3-51 tRNA and H6-Loc1p. *Upper* gel shows PAGE and *Lower* gel shows an urea PAGE. The pull-down of *ASH1* E3-51 tRNA with GST-She2p and Loc1p showed an RNA species migrating at higher molecular weight (open asterisk). This species disappeared only after phenol-chloroform extraction (*Lower Right* gel), indicating the presence of stable ternary complexes in the urea gel. (B) Pull-down assays as in A but with the control HIV TAR-57 tRNA showed no copurification of Loc1p or RNA. In contrast to *ASH1* E3-51 tRNA, no ternary complex is formed and Loc1p appears to outcompete the unspecific RNA binding of She2p. Pull-down experiments with Loc1p as bait gave similar results (Fig. S2B). (C) UV cross-linking experiments with radioactively labeled *ASH1* E3-51 tRNA (*Upper*) or HIV TAR-57 tRNA (*Lower*). Whereas *ASH1* E3-51 tRNA efficiently cross-linked with She2p, Loc1p, She3p, and the She2p-Loc1p cocomplex, HIV TAR-57 tRNA cross-linked only with She3p. (D) Representative images of nitrocellulose (*Left*, RNA-protein complexes) and nylon (*Right*, free RNA) membranes from filter-binding assays with *ASH1* E3-51 tRNA or HIV TAR-57 tRNA. (E) Table summarizing the equilibrium dissociation constants (K_d) of three independent filter-binding experiments (D). The determined K_d value of Loc1p is in good agreement with EMSA in Fig. 1C and the K_d s for She2p with *ASH1* E3-51 and HIV TAR-57 RNA are consistent with previous quantifications (8, 30, 37). (F) Table showing the percentage of radioactively labeled RNA bound to the nylon membrane at saturated protein concentrations (marked with parentheses on nylon membrane in D). RNA bound to the nylon membrane represents the fraction of RNA that was not bound by protein and therefore was not retained on the nitrocellulose membrane. Because the She2p-Loc1p cocomplex interacts very stably with RNA and yields almost no signal on the nylon membrane, we defined these conditions as 0% retention. A stronger retention of RNA on the nylon membrane with She2p confirms its previously reported low complex stability. Values with $\pm$ show SD from three independent experiments.

these experiments suggest a clear preference in binding for the *ASH1* E3-51 RNA over the control RNA.

Cocomplex of She2p and Loc1p Shows Selectively Increased Affinity and Stability for Zip-Code RNA. For a quantitative assessment of the observed difference in RNA binding, we performed double-filter-binding experiments (31). In these experiments, a nitrocellulose membrane retains protein-bound radioactively labeled RNA, whereas a second nylon membrane retains free, unbound RNA. We used the radioactivity retained on the first membrane to determine equilibrium dissociation constants (K_d) and radioactivity on the second, nylon membrane to estimate the fraction of unbound RNA. Because filter-binding assays involve washing steps after initial binding, the latter can be used to assess complex stabilities.

Binding of the She2p-Loc1p cocomplex to *ASH1* E3-51 RNA showed an improved affinity ($K_d = 18$ nM) compared with that of the individual proteins (K_d for She2p = 82 nM and for

Loc1p = 52 nM; Fig. 3 *D* and *E* and Fig. S3). The cocomplex of She2p and Loc1p had an over 40-fold lower affinity for the HIV TAR-57 tRNA (K_d = 769 nM). Interestingly, the She2p-Loc1p cocomplex had a 5-fold lower K_d for the control RNA compared with Loc1p alone (769 nM vs. 152 nM; Fig. 3 *D* and *E* and Fig. S3). The opposite effect was observed in binding experiments with the *ASH1* RNA (18 nM vs. 52 nM; Fig. 3 *D* and *E* and Fig. S3), further supporting the notion that the modes of binding for zip-code-containing and unspecific RNAs might be different.

Also the amounts of protein-free RNA retained on the nylon membrane indicated a difference in binding. The cocomplex of Loc1p and She2p as well as Loc1p alone retained almost no free RNA on the nylon membrane, indicating that the vast majority of RNA was stably associated with the protein-bound fraction at the nitrocellulose membrane (Fig. 3D). In contrast, She2p alone was unable to retain a comparable amount of RNA on the first membrane (Fig. 3D). These results indicate that the interaction of She2p with

RNA is stabilized by Loc1p. This stabilization effect was quantified by comparing signal intensities on the nylon membrane with the stable ternary complex of Loc1p, She2p, and RNA defined as 0% retention. Whereas we did not observe a difference in complex stability between Loc1p alone and the ternary complex, She2p showed a larger fraction of free RNA bound to the nylon membrane (Fig. 3F). The latter confirms the previously described (8, 30) low complex stability of the She2p–RNA interaction.

In Pull-Down Experiments Loc1p and She2p but Not Loc1p and Puf6p Form a Cocomplex with RNA. Like Loc1p, Puf6p coimmunoprecipitates with She2p and *ASH1* mRNA from yeast extracts (19) and localizes to the nucleolus (14, 15). However, in pull-down experiments Loc1p and Puf6p fail to interact (Fig. 1B). To study Puf6p interactions in the presence of RNA we used the E3-118 zip-code element, which contains two conserved Puf6p binding sites (see Table S3) (11). Initially we tested whether Puf6p can be pulled down by MBP-Loc1p or H6-Loc1p in the presence of *ASH1* E3-118 RNA. As before, we ensured that both proteins reproducibly showed monodisperse behavior in size-exclusion chromatography and normal RNA-binding capacity (Fig. 1C and Fig. S1B and C). Puf6p was not retained by MBP-Loc1p on amylose beads (Fig. 4A), although Puf6p alone efficiently binds to E3 RNA (Fig. S1C) (8). Even by using pull-down conditions more closely resembling a physiological situation (250 nM protein) and different Loc1p and Puf6p tags, we were not able to detect an interaction between Loc1p and Puf6p (Fig. S4A and B). Amylose pull-down experiments with MBP-Loc1p, She2p, Puf6p, and *ASH1* E3 mRNA showed

similar results. All factors were retained on amylose resin, except for Puf6p (Fig. 4B). These data indicate that the proteins Loc1p and She2p form a stable cocomplex with *ASH1* mRNA, in which Puf6p does not participate.

UV Cross-Linking Reveals That Puf6p Is Not Recruited by Other Factors to the *ASH1* E3 Zip-Code RNA. To directly assess RNA-binding events at near-physiologic concentrations, we performed UV cross-linking experiments with radioactively labeled *ASH1* E3-118 RNA. Puf6p alone showed only a faint RNA cross-link (Fig. 4C, lane 4; for a longer exposure of this UV cross-link, see Fig. S4D). Also the presence of other proteins, such as She2p (Fig. S4C, lanes 7 and 8), She2p and She3p (Fig. 4C, lanes 6 and 7, and Fig. S4D), or She2p and Loc1p (Fig. 4C, lanes 8 and 9, and Fig. S4D) did not enhance the intensity of the Puf6p RNA cross-link. Vice versa, Puf6p did not alter the cross-linking of Loc1p, She2p, and She3p or combinations thereof. This indicates that Puf6p is not recruited by any of these factors to the *ASH1* E3 zip-code RNA.

The Cocomplex of She2p and She3p Displaces Loc1p from the RNA. She2p and *ASH1* mRNA, but not Loc1p mRNA, is exported into the cytoplasm (13, 22). Therefore, a mechanism must be in place that allows Loc1p to be removed from the mRNA before or during its export into the cytoplasm. Because Loc1p contains multiple potential nuclear-localization signals (Fig. S5), one mechanism could be that a strong nuclear reimport of Loc1p is sufficient to strip off this protein from the RNA. Alternatively, Loc1p could be actively displaced from the RNA by another component of the transport complex that joins the mRNP during or after nuclear export. A potential candidate for such

Fig. 4. Puf6p does not join the ternary complex of Loc1p, She2p, and RNA. (A) Pull-down experiment with amylose resin and MBP-Loc1p shows that Puf6p does not form a cocomplex with Loc1p and RNA. (B) Pull-down experiment with MBP-Loc1p, She2p, Puf6p, and the *ASH1* E3 zip-code RNA. All components except for Puf6p can be pulled down as a cocomplex. Experimental setup is as shown in Fig. 1. (C) UV cross-linking experiments with radioactively labeled *ASH1* E3-118 RNA and She2p, MBP-Loc1p, She3p-H6 and Puf6p. The presence of She2p and She3p-H6 (lanes 6 and 7) or She2p and MBP-Loc1p (lanes 8 and 9) does not increase the intensity of the Puf6p–RNA cross-link, indicating the absence of a recruitment mechanism for Puf6p. A longer exposure of this cross-link is depicted in Fig. S4D.

an active displacement is the myosin adapter and RNA-binding protein She3p. In the cytoplasm, it forms a high-affinity cocomplex with She2p and *ASH1* mRNA. Also Loc1p binds to both She2p (Fig. 1A) and *ASH1* mRNA (Figs. 1C and D and 2). Thus, for an active displacement, She3p would need to out-compete Loc1p from both binding partners.

We first tested whether the addition of She3p to Loc1p, She2p, and *ASH1* E3 element changes the association of Loc1p with the RNA. To detect RNA-binding events at near-physiologic concentrations, we used again UV cross-linking of radioactively labeled *ASH1* E3 zip-code RNA. Whereas we recapitulated the Loc1p recruitment to the *ASH1* RNA by She2p (Fig. 5A, compare lanes 3–5 with lanes 6 and 7), the combined presence of She2p and She3p almost completely abolished RNA cross-linking by Loc1p (Fig. 5A, lanes 8 and 9). This finding indicates that at

nanomolar, near-physiological concentrations the She2p-She3p complex displaces Loc1p from zip-code-containing RNA.

Formation of the Ternary Complex of She2p, She3p, and *ASH1* RNA Disrupts the Interaction between She2p and Loc1p. This UV cross-linking experiment does not rule out the possibility that Loc1p remains directly bound to She2p, even when it is displaced from the RNA. Hence, we tested in pull-down experiments whether She3p alters the interaction of She2p and Loc1p in the absence and presence of RNA. Indeed, we found that MBP-Loc1p fails to interact with She2p and She3p in the absence of the E3 element (Fig. 5B). We also performed these experiments with the *ASH1* E3 element and an unspecific tRNA competitor. Also under these experimental conditions Loc1p was not able to pull down She2p or She3p (Fig. 5C). We conclude that She3p also outcompetes the protein interaction of Loc1p with She2p.

Fig. 5. She3p outcompetes Loc1p from the mRNP. (A) UV cross-linking experiment with She2p, She3p-H6, and MBP-Loc1p shows that in the presence of She2p and She3p Loc1p-RNA cross-linking is almost abolished. This finding suggests that She3p is able to outcompete Loc1p from its cocomplex with She2p and RNA, resulting in the synergistic cocomplex of She2p, She3p, and RNA (8), as well as free Loc1p. (B) Pull-down experiment with amylose resin and MBP-Loc1p, She2p, and She3p. None of the components can be pulled down efficiently with MBP-Loc1p, indicating that in the presence of She3p the protein interaction between Loc1p and She2p is disrupted. (C) Pull-down experiment with MBP-Loc1p, She2p, She3p-H6, *ASH1* E3-51 RNA, and tRNA competitor. Also in the presence of RNA, Loc1p is not able to form a cocomplex with any of these components.

Mutational Studies with She2p Indicate Overlapping Binding Sites of She3p and Loc1p. Because we observed an outcompetition of Loc1p by She3p, these proteins most likely share overlapping binding sites on She2p. To test this assumption we performed pull-down experiments with mutant versions of She2p. It was previously shown that the She2p mutation of leucine 130 into tyrosine [She2p (L130Y)] impairs its oligomeric state and its in vivo interaction with She3p (30). A recent study further showed that the deletion of the very C terminus of She2p [She2p (Δ C)] has no strong effect on in vitro binding to She3p, whereas the deletion of its protruding helix [She2p (Δ hE)] abolishes the She3p interaction (8). We first recapitulated these results (Fig. S6A) and then tested these mutant versions of She2p for Loc1p binding. We found that Loc1p shows reduced binding to She2p (L130Y), whereas She2p (Δ C) is bound like wild-type She2p (Fig. S6B). The observation that mutations in She2p affect Loc1p and She3p binding is consistent with the assumption that both proteins share an overlapping interaction surface on She2p.

Deletion of Cytoplasmic She3p Alters Nuclear Distribution of Loc1p.

The observed displacement of Loc1p from the complex with She2p and *ASH1* E3 RNA by She3p (Fig. 5) suggests that this mechanism might also contribute in vivo to the removal of Loc1p from the *ASH1* mRNP. She3p has been previously shown to be exclusively cytoplasmic (8). First, we wanted to elucidate whether Loc1p is found at or close to the nuclear periphery, where an interaction with She3p could potentially occur. We performed double staining of GFP-tagged Loc1p and mCherry-tagged nuclear pore marker Nup120. As previously shown by Du et al. (14), Loc1p is clearly found in the nucleolus (Fig. 6A). The overlay with Nup120-mCherry shows that this nucleolar localization of Loc1p also reaches parts of the nuclear periphery.

If She3p is indeed necessary for removing Loc1p from *ASH1* mRNA during nuclear export, the Loc1p distribution should be altered in a *she3 Δ* strain. Because the majority of Loc1p is likely involved in ribosome biogenesis inside the nucleolus, alterations in Loc1p distribution are expected to be relatively mild. We therefore overexpressed *ASH1* mRNA from a 2 μ plasmid and

Fig. 6. (A and B) Localization of Loc1p in dividing wild-type (A) and *she3 Δ* cells (B). Loc1p is visualized via GFP tag (green) and the nuclear envelope by Nup120-mCherry (red). (A and B, Middle) Overlays of Nup120-mCherry and GFP-Loc1p with magnifications of single cells. (Scale bars, 1 μ m.) In wild-type cells (A), GFP-Loc1p shows a distinct localization in the nucleolus, as previously described (14, 15). In the *she3 Δ* strain (B), part of the nucleolar Loc1p becomes dispersed in the nucleoplasm, demonstrating that cytoplasmic She3p influences the nuclear distribution of Loc1p. Occasionally, we also observed GFP-Loc1p signal in the cytoplasm of *she3 Δ* cells. (Bottom) Cartoons illustrate the differences in Loc1p distribution.

focused on cells in anaphase when *ASH1* expression peaks (32). In these cells with wild-type She3p expression, GFP-Loc1p distribution is clearly limited to the nuclear crescent that is typical for the yeast nucleolus (Fig. 6A) (14). In contrast, cells that are deficient for She3p show a dispersed GFP-Loc1p staining in the nucleoplasm (Fig. 6B). This observation indicates that cytoplasmic She3p indeed affects Loc1p distribution in the nucleolus. Occasionally, we also observed a weak cytoplasmic staining of GFP-Loc1p in *she3Δ* cells. However, this is an infrequent event and weaker than the nucleoplasmic staining in mutant cells.

Discussion

To date little is known about the molecular mechanisms involved in the early nuclear stages of localizing mRNPs and how nucleolar transitions are organized. Nucleolar trespassing of pre-mRNPs before cytoplasmic mRNA localization is not limited to the SHE complex in yeast. For instance, mammalian Staufen2 also passes through the nucleolus before its nuclear export (33). Here we present a study in which we systematically assessed the interplay between transport factors that associate with *ASH1* mRNA already in the nucleus. This allows us to provide a mechanistic understanding of these early events.

It was recently shown that the interaction of Loc1p and Puf6p with *ASH1* mRNA is impaired when She2p is prevented from entering the nucleus (19). This suggests interdependence between these protein–RNA interactions. Furthermore, Loc1p remains in the nucleus but is nevertheless important for cytoplasmic mRNA localization. Together, these findings indicate that the nuclear assembly of the pre-mRNP is important for cytoplasmic mRNA localization and translational repression.

A puzzling observation of previous studies was that the *in vitro* interaction between She2p and *ASH1* mRNA is very transient (8, 30). However, because She2p is cotranscriptionally recruited to *ASH1* mRNA (7, 8) and exported in an RNA-dependent manner to the cytoplasm (22), the formation of a stable complex in the nucleus is expected to occur. In the cytoplasm the interaction of *ASH1* mRNA and She2p is stabilized by the formation of a highly specific cocomplex with cytoplasmic She3p (8). An unresolved question is therefore whether and how She2p binding is modulated to form a stable complex with *ASH1* mRNA in the nucleus and whether such a nuclear complex would be specific for *ASH1* mRNA.

We found that She2p specifically binds to Loc1p even in the absence of RNA. We further observed a Loc1p-dependent stabilization of She2p binding to RNA. Loc1p alone shows only a modest preference for *ASH1* E3 RNA over HIV TAR-57 RNA (about 3-fold stronger K_d ; Fig. 3E). However, Loc1p in cocomplex with She2p shows an about 40-fold better K_d for *ASH1* E3 RNA over HIV TAR-57 RNA, clearly indicating a specific selection of zip-code RNA.

Our pull-down experiments further indicated that zip-code-containing RNA is bound in a different mode by She2p and Loc1p than a control RNA (Fig. 3A and B and Fig. S2B). In fact, ternary complexes formed only with zip-code RNA. Together with UV cross-linking and filter-binding assays, these findings suggest that Loc1p is required to stably and specifically associate She2p with nuclear *ASH1* mRNA. The cooperative, mutual recruitment and stabilization of Loc1p and She2p on the RNA are in agreement with the previous observation that in a nuclear localization-deficient She2p strain almost no *ASH1* mRNA was immunoprecipitated with TAP-tagged Loc1p (19).

An interesting question arising from these insights is where this stable ternary complex forms in the nucleus. For She2p a cotranscriptional recruitment to the *ASH1* mRNA has already been described (Fig. 7, Upper, A), whereas the exact location of Loc1p joining the early mRNP is unknown. One possible scenario is that Loc1p encounters She2p at or in the nucleolus, where Loc1p is enriched. Loc1p has a theoretical isoelectric point of 10.3, which is very similar to that of the vast majority of

ribosomal proteins. Although nucleolar localization signals are difficult to predict (34), the considerable number of putative signals in Loc1p (Fig. S5) indicates that this protein is very likely localizing directly to the nucleolus without the need of a nucleolar hub protein. In contrast, She2p does not possess detectable nucleolar localization signals. Therefore, the interaction of Loc1p might not only stabilize She2p on the *ASH1* mRNA, but also piggyback She2p into the nucleolus (Fig. 7, Upper, A and B). Such indirect nucleolar recruitment mechanisms have been reported already for other proteins without intrinsic nucleolar localization signal, such as Nucleolin and Nucleophosmin (35).

Because She2p associates with *ASH1* mRNA already cotranscriptionally, it is also tempting to speculate that Loc1p binds to She2p already after or even before its association with the *ASH1* transcription complex in the nucleoplasm (Fig. 7, Upper, A). The recruitment of Loc1p could help to stably tether She2p to the *ASH1* mRNA already at the onset of mRNP biogenesis. This complex would remain associated until joining of She3p and formation of the She2p–She3p complex in the cytoplasm displaces Loc1p from the mRNP (see below).

In a mutant yeast strain with cytoplasmically trapped She2p, the nuclear interaction between Puf6p and *ASH1* mRNA is strongly impaired (19). In our *in vitro* assays we failed to observe a direct interaction of Puf6p with She2p or Loc1p or a recruitment of Puf6p by these factors to the *ASH1* mRNA. Hence, there must be another explanation for this dependence of Puf6p on She2p. Like for *puf6Δ*, the *LOC1* deletion strain exhibits defects in *ASH1* translational repression (13, 14). Furthermore, Puf6p shows a strong nucleolar enrichment (15) similar to that of Loc1p. Therefore, Loc1p-dependent piggybacking of She2p and *ASH1* mRNA into the nucleolus would be an obvious mechanism to bring the mRNP in the vicinity of Puf6p and to allow its loading into the complex (Fig. 7, Upper, C). Such a scenario would be consistent not only with our *in vitro* observations but also with the dependence of Puf6p binding to *ASH1* mRNA on She2p *in vivo* (19). Interestingly, Loc1p- as well as Puf6p-dependent translational repression is mediated by the E3 zip-code element (11, 21), indicating that this element fulfills a special function in *ASH1* mRNA.

After nucleolar trespassing, the *ASH1* mRNP is exported into the cytoplasm and Loc1p has to be removed from the *ASH1* mRNP. We observed that the cocomplex of She2p and She3p is able to displace Loc1p from the *ASH1* mRNP. She3p is strictly cytoplasmic and constitutively tethered to the cytoskeleton (8). Furthermore, with a molecular weight of 47 kDa She3p is too large to freely diffuse through the nuclear pore (36). Also the nuclear protein Loc1p was reported not to shuttle between nucleus and cytoplasm, as judged by shuttling and heterokaryon assays (13). Because these techniques are not very sensitive, minor subfractions might have remained undetected. Based on the observation that the deletion of *SHE3* does affect the nuclear localization and occasionally leads to cytoplasmic accumulation of Loc1p, it is conceivable that an outcompetition of Loc1p by the She2p–She3p cocomplex indeed occurs at or close to the nuclear pore (Fig. 7, Upper, D).

From the data presented here, we conclude that a stable and specific ternary complex is formed within the nucleus consisting of She2p, Loc1p, and *ASH1* mRNA. This pre-mRNP trespasses the nucleolus in a Loc1p-dependent manner. Because we do not observe a direct interaction of Puf6p with the pre-mRNP constituents or recruitment to the *ASH1* mRNA, it seems likely that Puf6p joins the mRNP independently, probably within the nucleolus. After nucleolar trespassing, the mRNP is exported into the cytoplasm, where the mature transport complex together with She3p and Myo4p is formed. Our data implicate that She3p outcompetes Loc1p for the interaction with She2p and *ASH1* mRNA during or shortly after nuclear export. The reported assembly line of the nuclear mRNP ensures that all required factors associate and dissociate at the right time to

Fig. 7. Model for sequential binding and release of Loc1p to the *ASH1* mRNA. (Upper) She2p enters the nucleus and binds cotranscriptionally to the *ASH1* mRNA (A). Loc1p joins this subcomplex, which leads to stabilization of She2p on the *ASH1* mRNA. Loc1p contains nucleolar localization signals and is strongly enriched in the nucleolus, suggesting that it recruits She2p into the nucleolus through their direct interaction (B). No direct interaction between Puf6p and other core factors could be detected in this study. Nevertheless, *in vivo* the *ASH1* mRNA association of Puf6p depends on She2p. It is likely that Loc1p and She2p are required to recruit *ASH1* mRNA into the nucleolus, where Puf6p can interact with the transcript (C). Because Loc1p is not part of the cytoplasmic transport complex, it has to be removed from the complex before or during nuclear export (D). The synergistic cocomplex of She2p and She3p outcompetes Loc1p from the mRNP and ensures that Loc1p is absent from the cytoplasmic *ASH1* transport complex. (Lower) Schematic drawing of the changes of RNA-binding properties during the stepwise assembly of the *ASH1* mRNP. In particular, the affinity and specificity, but also complex stability, improve from the initial nuclear binding events to the assembly of the mature cytoplasmic transport complex. This gradient in binding properties is suitable to explain the directionality of complex maturation. Asterisk indicates that this K_d has been estimated from figure 3C of ref. 8.

escort the *ASH1* mRNA from its site of transcription all of the way to its localization at the bud tip.

Materials and Methods

Protein Expression and Purification. Full-length Loc1p was expressed with an N-terminal MBP-tag or H6-tag in *Escherichia coli* strain BL21 (DE3). After induction with 0.25 mM isopropyl β -D-1-thiogalactopyranoside in the logarithmic growth phase cells were cultured for 16 h at 18 °C and harvested. Cells were sonicated at 4 °C in lysis buffer (20 mM Hepes, pH 7.4, 500 mM KCl, 0.5 mM EDTA, complete protease inhibitor or 20 mM K-Phosphate, pH 7.5, 500 mM KCl, 50 mM Imidazole, and complete protease inhibitor). After centrifugation the clarified supernatant was applied to an amylose/Ni Sepharose resin column and washed with high-salt buffer (1 M KCl) to remove nucleic acids and subsequently with low-salt buffer (150 mM KCl). The protein was eluted with 20 mM maltose or 750 mM imidazole in low-salt buffer and further purified using ion exchange chromatography (SP FF; GE Healthcare), heparin affinity chromatography, and size exclusion chromatography (e.g., Superdex S200; GE Healthcare).

Full-length She2p and Puf6p were expressed and purified as GST-fusion proteins in *E. coli* strain BL21 (DE3) and purified as previously described (8, 37).

His₆-tagged She3p was coexpressed with She2p in insect cells (High Five) using the bac-to-bac system (Invitrogen) and purified with affinity, ion exchange, and size exclusion chromatography as previously described (8). Plasmids and primers used in this study are listed in Tables S1 and S2.

To remove RNA contaminations from each of the above-mentioned proteins, a high-salt washing step was carried out during the affinity chromatography and validated via measurement of the OD₂₅₄ to OD₂₈₀ ratio. Proteins with an OD₂₅₄ to OD₂₈₀ ratio below 0.6 were specified as RNA/DNA free.

RNA Preparation. RNA was produced by *in vitro* transcription (E3-118), tRNA scaffold expression, and purification (E3-51 tRNA, E3-118 tRNA, and TAR-57 tRNA) or total chemical synthesis (E3-51; Dharmacon) (38). For *in vitro* transcription the 118-nt DNA fragment of the *ASH1* gene (plasmid pRS405-*ASH1* in Table S1) was PCR amplified using primers with the T7 promoter sequence at the 5' end. This fragment was used as a template for *in vitro* transcription (MEGAscript Kit; Ambion). The transcribed RNA was purified as described in the manufacturer's instructions, using DNase digestion,

phenolic extraction, and ethanol precipitation. The integrity of the RNA was confirmed by agarose gel electrophoresis.

tRNA_{Met} fusion constructs (E3-51 tRNA, E3-118 tRNA, and TAR-57 tRNA) were overexpressed in *E. coli* JM 101 cells, using 2x typtone-yeast media. The RNA was phenol-chloroform extracted, ethanol precipitated, and further purified by ion exchange chromatography (DAEA and MonoQ columns), as described in ref. 29. Pure fractions were identified by 8% (vol/vol) Tris/borate/EDTA (TBE) Urea PAGE, pooled, and ethanol precipitated.

For UV cross-linking and EMSA experiments *in vitro*-transcribed E3-118 zip-code RNA, synthesized E3-51, or recombinant E3-51 tRNA, E3-118 tRNA, or HIV TAR-57 tRNA constructs were radioactively labeled using [γ -³²P]ATP and T4 polynucleotide kinase. Free nucleotides were separated from the RNA, using either Sepharose spin columns (NucAway; Ambion, E3-51 element) or phenol-chloroform extraction (E3-118, E3-51 tRNA, E3-118 tRNA, and HIV TAR-57 tRNA). For interaction studies with Puf6p the E3-118 or E3-118 tRNA zip-code RNA was used, because it includes the corresponding Puf6p binding element (11). For interaction studies without Puf6p the E3-51 zip-code element was used because of better recovery rates after isotope labeling. The sequences of RNAs used in this study are listed in Table S3.

EMSA. In a total volume of 20 μ L 5 nM radioactively labeled RNA, 100 μ g/mL tRNA competitor, and the indicated protein concentration were mixed in HNMD buffer (20 mM Hepes, pH 7.8, 200 mM NaCl, 2 mM MgCl₂, 2 mM DTT) with 4% glycerol. After 25 min incubation at 25 °C the RNA protein complexes were resolved by native TBE PAGE (6% polyacrylamide, 1 $\times$ TBE running buffer). Gels were incubated for 15 min in fixing solution [10% (vol/vol) acetic acid, 30% (vol/vol) methanol] and vacuum dried. Gels were analyzed with radiograph films. For supershift EMSAs monoclonal She2p antibody (30) was included in the reaction. DTT was omitted from the HNMD reaction buffer for supershift EMSAs. For detection of cocomplexes 4–20% TBE gradient gels were used.

UV Cross-Linking Experiments. In a total volume of 20 μ L 5 nM radiolabeled RNA, 100 μ g/mL tRNA, and the indicated protein concentrations were mixed in HNMD buffer. After 25 min incubation at 25 °C the samples were subjected to 2.25 J UV radiation over 15 min on ice. The distance between the samples and the UV lamp was ~8 cm. After adding SDS loading dye, samples were incubated for 5 min at 90 °C and separated on 10% SDS/PAGE. Gels were fixed, dried, and analyzed with radiograph films.

In Vitro Pull-Down Experiments. In a total volume of 100 μ L 7.5 or 10 μ M protein, 4 μ M E3-51/E3-118 tRNA, and 5 mg/mL yeast tRNA competitor (unless stated otherwise) as indicated were mixed in appropriate pull-down buffer (20 mM Hepes, pH 7.8, 150 mM or 200 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT or 20 mM K phosphate, pH 7.5, 150 mM or 200 mM KCl, 2 mM $MgCl_2$, and 30 mM imidazole). Samples were centrifuged for 10 min and supernatant was incubated for 30 min at 4 °C on a rotating wheel with 50 μ L amylose resin (MBP-Loc1p), GST-Sepharose (GST-Puf6p), or Ni-NTA (She3p-H6). Binding reactions were washed four times with 200 μ L and once with 50 μ L pull-down buffer. Bound proteins were eluted with maltose (amylose resin), glutathione (GST-Sepharose), or imidazole (Ni-NTA). On SDS/PAGE 10% of the input, 20% of the last wash step, and 20% of the elution were analyzed by Coomassie blue staining. RNA was analyzed on a 1.5% agarose gel or an urea PAGE with GelRed DNA stain, according to ref. 38.

Filter-Binding Assays. In a total volume of 80 μ L the indicated protein concentrations and 0.5 nM of radiolabeled RNA (*ASH1* E3-51 tRNA, HIV TAR-57 tRNA) were incubated for 25 min in filter-binding buffer (10 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM DTT, and 5 mM $MgCl_2$) supplemented with 30 μ g/mL yeast tRNA competitor. Samples were applied to nitrocellulose and nylon membranes, using a Dot Blot Apparatus (BioRad), and washed twice with filter-binding buffer. Membranes were air dried and analyzed by phosphoimaging. Quantification of signal intensities was carried out using the Dot Blot Analyzer macro within the ImageJ program and further analyzed with the program Origin 8.6.

Generation of Yeast Strains. Strain RJY4358 (LOC1-GFP::HIS3MX6) was generated by one-step tagging of W303a according to ref. 39. To construct strain RJY4359 (LOC1-GFP::HIS3MX6 *she3 Δ ::URA3*), RJY4358 was transformed with

a DNA construct containing the URA3 gene flanked by sequences of the 5' and 3' regions of SHE3, which was obtained by PCR amplification from the *she3 Δ ::URA3* locus of strain RJY90 (40). Before imaging, both strains were transformed with plasmid pRJ88 (YEplac181-ASH1), which allows overexpression of *ASH1* mRNA during mitosis, and plasmid pRS314-Nup120-mCherry, which allows detection of nuclear pores. Genotypes of used yeast strains as well as plasmids for transformation are listed in Tables S1 and S4.

In Vivo Imaging. For imaging, a single yeast colony from a fresh plate grown at 30 °C was inoculated in 1 mL of synthetic complete (SC) medium with 2% glucose but lacking leucine and tryptophane. Cells were grown at 30 °C for 4–4.5 h to enrich for mitotic cells. Cells were collected by short spin and resuspended in 100 μ L of SC medium. One microliter of cell suspension was placed on a multiwell slide coated with agarose before microscopic observation. For each mitotic cell pair, Z-stacks containing 50 images at 200-nm distance were acquired for GFP, mCherry, and DIC. Due to the weaker signal of Nup120-mCherry, mCherry images were deconvoluted before mounting. Image processing and mounting were performed with AxioVision software version 4.8 (Zeiss). GFP and mCherry overlay pictures were generated using Adobe Photoshop CS3. Essentially the mCherry Layer was duplicated and inserted in the GFP file. By using exclusion in the layer option an overlay was generated and one representative cell was magnified.

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