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Strukturelle und funktionelle Charakterisierung von Loc1p und interagierenden Transkripten

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1 Introduction

1.1 mRNA localization in eukaryotes

Cellular asymmetry due to local accumulation of cell-fate determinants is necessary for biological processes as body axis determination, synaptic plasticity, and cell motility. One way to establish such intracellular asymmetry is the spatially and temporally restricted translation of a localized mRNAs (Figure 1-1). Since a single mRNA molecule can give rise to multiple protein molecules, this process leads to the formation of protein gradients required for the differentiation and polarization of cells. The prevalence of this process is underlined by genome wide studies revealing that 71 % of the expressed genes in *Drosophila melanogaster* show a subcellular localization pattern during early embryogenesis (Lecuyer et al., 2007). During *Drosophila* oocyte maturation about 22 % of the transcribed genes show a distinct localization pattern (Jambor et al., 2015). mRNA localization was even observed in prokaryotes whereat the molecular mechanisms are not yet completely understood (Kannaiah and Amster-Choder, 2014).

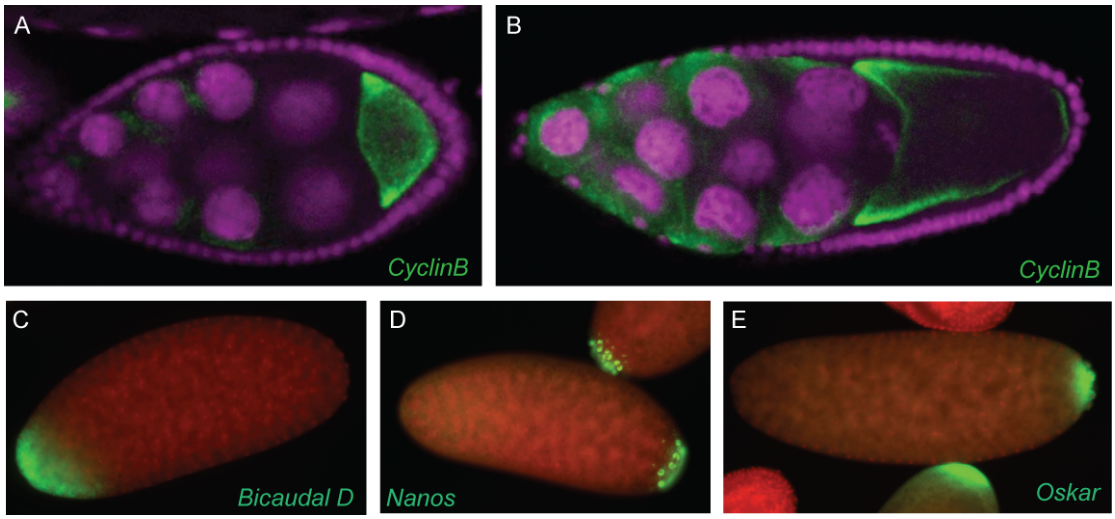

Figure 1-1 mRNA-localization patterns in *Drosophila melanogaster* during oogenesis and embryogenesis: Pictures are taken from the genome-wide study of mRNA localization during oogenesis (Jambor et al., 2015) and embryogenesis (Lecuyer et al., 2007) assessed by high-resolution fluorescence *in situ* hybridization (FISH). Pictures of oocytes (A and B) as well as embryos (C, D, and E) are aligned with their anterior part to the left and the posterior part to the right. mRNA is stained in green, whereas nuclei are stained in purple (A and B) or red (C, D, and E). **A and B** *CyclinB* mRNA is uniformly distributed within the oocyte at stage 8(A) and shows a complex localization pattern during stage 9 (B) at the anterior and posterior pole of the oocyte. **C** *Bicaudal D* mRNA localizes to the anterior part of the embryo during the stages 1-3 of embryogenesis. **D and E** *Nanos* and *oskar* mRNAs localize to the posterior pole during the stages 1-3 of embryogenesis. The localization pattern of *nanos* mRNA resembles a granular-like assembly, whereas *oskar* mRNA shows a crescent-shaped accumulation.

1.1.1 Local transcription

There are different approaches to obtain mRNA localization. One of the easiest but rarely observed mechanisms is the local transcription of an mRNA. A well-known example is the transcription of acetylcholine receptor (AChR) d- and e-subunits in vicinity of synaptic myonuclei (Brenner et al., 1990; Simon et al., 1992). Vertebrate muscle fibers form a syncytium that allows for local transcription of those synaptic mRNAs in a subset of nuclei. Agrin, a pre-synaptic signal-transduction molecule, activates together with neuregulin the synaptic expression of AChR subunits within synapse-proximal myonuclei (Fromm and Rhode, 2004; Glass et al., 1996).

1.1.2 Degradation and stabilization

Another approach to obtain mRNA localization is the degradation of mRNA in large parts of the cell and its specific stabilization at a distinct subcellular area. In *D. melanogaster* embryos *nanos* (*nos*) mRNA, which organizes the abdominal segmentation of the embryo, is localized at the posterior pole (Figure 1-1) by this mechanism. Translational repression and degradation of *nos* is mediated by Smaug protein. Smaug binds to the smaug response element (SRE) in the 3' untranslated region (3' UTR) of *nos* and recruits the Cup protein that interacts with eIF4E. The complex formed by Smaug, Cup, and eIF4E blocks the recruitment of the 40S ribosomal subunit, thus repressing the translational initiation (Jeske et al., 2011; Nelson et al., 2004). In addition, Smaug recruits the CCR4-NOT complex to the *nos* mRNA which results in the removal of the poly(A) tail (Jeske et al., 2006). Both mechanisms lead to translational repression and decay in the bulk embryo. The Oskar protein localized at the posterior pole of the embryo prevents Smaug binding to the SRE of *nos* in this subregion thereby allowing for local *nos* translation (Zaessinger et al., 2006). Only 4 % of the *nos* mRNA is localized at the posterior pole of the embryo and thus protected from degradation (Bergsten and Gavis, 1999).

1.1.3 Diffusion and anchoring

mRNA localization is alternatively achieved by the diffusion of mRNA within the cytoplasm and trapping at a specific site of the cell. During the late oogenesis of *D. melanogaster* several RNAs such as *nos*, *cyclinB* (*cycB*; Figure 1-1), *germ cell-less*, and *polar granule component* (*pgc*) are anchored at the posterior pole of the oocyte

(Lasko, 2012; Nakamura et al., 1996; Santos and Lehmann, 2004). *Nos* mRNA is synthesized by nurse cells and released into the oocyte where cytoplasmic streaming drives its diffusion (Forrest and Gavis, 2003). At the posterior pole, it becomes trapped in granules in an actin-dependent way (Forrest and Gavis, 2003; Nakamura et al., 1996). Recently, a mechanism has been proposed for the anchoring of *nos* mRNA. Upon localization at the posterior pole, *nos* accumulates in granules and during maturation both the number of granules as well as mRNA content per granule increases. Furthermore, these granules are heterogeneous and contain, besides *nos, pgc* and *cycB* indicating a co-assembly at the posterior cortex (Little et al., 2015).

1.1.4 Active transport

Among the various mRNA localization methods, active transport along the cytoskeleton is probably the most common mechanism. The specific localization requires so-called localization elements (LEs) or zipcodes within the mRNA that are recognized by trans-acting factors to form messenger ribonucleoprotein (mRNP) complexes. LEs are frequently found in the 3´ UTR of localizing mRNA and are often predicted to adopt stem-loop secondary structures. mRNP formation often starts in the nucleus via the interaction of dedicated nuclear factors and mRNA. These early nuclear events have been shown to be a prerequisite for correct cytoplasmic localization.

Nuclear priming

A particularly well-studied example for nuclear RNP assembly is the *oskar* (*osk*) mRNA localization in oocytes of *D. melanogaster* to the posterior pole (Figure 1-1). Two structural elements within the *osk* mRNA are required for correct localization at the pole: the spliced *oskar* localization element (SOLE; Ghosh et al., 2012) and the *osk* 3' UTR containing the dimerization element (Jambor et al., 2011). The bipartite SOLE element is separated by an intron and requires exon-exon joining upon splicing for its formation. Furthermore, the correct positioning of the exon junction complex (EJC) upstream of the SOLE contributes to the localization of this mRNA which indicates the importance of nuclear events for correct cytoplasmic localization (Ghosh et al., 2012).

Another example for nuclear priming is the localization of β-*actin* mRNA. Correct localization of this mRNA to the growth cones of neurons and to the leading edge of fibroblasts facilitates neurite outgrowth and fibroblast migration in *Mus musculus* and *Gallus gallus* (Eom et al., 2003; Kislauskis et al., 1994; Zhang et al., 2001). The trans-acting factor Zipcode binding protein 1 (ZBP1) binds to a bipartite LE within the 3' UTR of β-*actin* mRNA (Chao et al., 2010). However, accurate localization of β-*actin* mRNA requires a cotranscriptional binding of ZBP2 and ZBP2-mediated recruitment of ZBP1 to the site of transcription (Gu et al., 2002; Pan et al., 2007).

A third example that shows the importance of nuclear mRNP formation is the transport of *Vg1* and *VegT* RNAs to the vegetal pole of *Xenopus laevis* oocytes. *Vg1* encodes a member of the transforming growth factor β (TGFβ) that induces mesoderm activity (Dale et al., 1993). *VegT*, in turn, encodes a T-Box transcription factor necessary for mesoderm and endoderm development (Kofron et al., 1999). Already within the nucleus do the RNA-binding proteins hnRNP1 and Vg1RBP/Vera form an mRNP with *VegT* and *Vg1* mRNA (Kress et al., 2004; Lewis et al., 2008). Simultaneously, the protein 40LoVe is recruited by hnRNP1 to the *Vg1* RNA also within the nucleus (Czaplinski et al., 2005; Czaplinski and Mattaj, 2006; Kroll et al., 2009). Upon nuclear export, the RNP is remodeled and the cytoplasmic factors Proline rich RNA-binding protein and Staufen join the complex (Kress et al., 2004).

Motor-dependent transport

After nuclear export, motor proteins join the mRNP and enable the processive movement along the cytoskeleton. Motor proteins are distinguished into three classes: kinesin, dynein, and myosin. Kinesin mediates the plus-end directed transport along microtubules, whereas dynein transports its cargo to the minus end of microtubules. Myosins usually move towards the plus end of actin cables. Motor activity depends on ATP hydrolysis that generates the force of movement through conformational changes. Almost all motors require dimerization of coiled-coiled segments to enable processive movement (Vale, 2003).

The combination of different localization elements, trans-acting factors, and motor proteins creates multiple prospects to deliver mRNA. *D. melanogaster osk* mRNA is an exciting example where multiple transport complexes are both temporally and

spatially controlled in order to generate a distinct localization pattern from oogenesis to embryogenesis and larval development. During mid oogenesis, *osk* mRNA localizes kinesin-dependently to the posterior pole of the oocyte. Although the essential LEs for this transport are the SOLE and a dimerization element (Ghosh et al., 2012; Jambor et al., 2011), another transport process precedes: *osk* mRNA is transcribed within nurse cells and due to the oocyte entry signal (OES) is transported by dynein through the ring canals into the oocyte (Jambor et al., 2014). During larval development this OES signal is re-used to guide *osk* mRNA into salvary glands (Jambor et al., 2014). The temporally and spatially restricted use of several LEs within one mRNA implies the presence of tight regulatory mechanisms that precisely activate or repress localization signals.

Translational repression

Local activation of translation is thought to be preceded by translational silencing during the transport of an mRNA.

The molecular mechanism of *nos* mRNA repression in *D. melanogaster* has been studied in detail. Smaug protein binds to the SRE of *nos* mRNA and interacts with the Cup protein. Cup, in turn, binds eIF4E that prevents 40S subunit recruitment, thus translationally represses *nos* mRNA (Jeske et al., 2011; Nelson et al., 2004). Moreover, *osk* mRNA is translational repressed during transport to the posterior pole of the oocyte. The balance of adenylation and deadenylation mediated by the GLD-2 and CCR4-NOT complexes as well as the presence of the translational repressors Cup and Bruno restricts *osk* expression to the posterior pole of the oocyte (Barckmann and Simonelig, 2013). Another example for translational repression during transport is the β-*actin* mRNA. ZBP1 binds already cotranscriptionally to the LE of β-*actin* mRNA and impairs the formation of 80S ribosomal complexes. This blocking of translational initiation is released by the Src-dependent phosphorylation of ZBP1 which decreases ZBP1 binding to β-*actin* mRNA and abolishes the translational repression (Hüttelmaier et al., 2005).

Anchoring of mRNPs

Localized transcripts are often anchored at the particular site via interactions with the cytoskeleton. Especially diffusion and anchoring based localization mechanisms depend on efficient capturing of the mRNA at the target site (Paragraph 1.1.3).

However, also various examples of actin-dependent anchoring of actively transported mRNAs were observed in different organisms. One example is the anchoring of β-*actin* mRNA to protrusions of *G. gallus* embryo fibroblasts. The elongation factor 1 alpha (EF1α) binds β-*actin* mRNA as well as F-actin, thereby linking the mRNA to the cytoskeleton of crawling cell protrusions (Liu et al., 2002). At the target site, β-*actin* mRNA is translational activated by the Src-dependent phosphorylation of ZBP1, which decreases the binding of this translational repressor to the mRNA (Hüttelmaier et al., 2005). Another example is the anchoring of *Vg1* mRNA to the vegetal pole of *Xenopus* oocytes. Here it was shown that cytochalasin B treatment, which binds F-actin filaments and inhibits actin polymerization, leads to the diffusion of *Vg1* mRNA in the vegetal cytoplasm. Therefore the actin cytoskeleton is required for anchoring *Vg1* mRNA to the vegetal pole (Yisraeli et al., 1990). Also *D. melanolgaster osk* mRNA is anchored via actin to the posterior pole of the oocyte. A clear evidence for anchoring is that a mutation of moesin, which is a linker between actin and the cell membrane, results in *osk* mRNA de-localization (Jankovics et al., 2002).

1.2 mRNA localization in budding yeast

A well-studied example is the *ASH1* mRNA localization in the budding yeast *Saccharomyces cerevisiae* that is required to control mating-type switching. Haploid *S. cerevisiae* cells have either the mating type MATa or MATα. Upon budding, mother cells switch their mating type from a to α and *vice versa* (Strathern and Herskowitz, 1979). The molecular basis of the switch is a homologous recombination of the MAT gene locus initiated by a double strand cleavage of HO endonuclease (Haber, 1998). Only the daughter cell contains Ash1p protein that represses HO endonuclease and thereby restricts mating-type switching to the mother cell (Bobola et al., 1996; Sil and Herskowitz, 1996). The selective expression of Ash1p in the daughter cell is achieved by the motor-dependent transport of its transcript into the daughter cell (Long et al., 1997; Takizawa et al., 1997). *ASH1* mRNA contains 4 LEs (E1, E2A, E2B, and E3). Except for the E3 LE, all other LEs are located in the coding region (Figure 1-2). The E3 element resides in the untranslated region directly 3´ to the stop codon (Chartrand et al., 1999; Gonzalez et al., 1999). Secondary structure predictions of the four LEs show large

diversity; stem loops of various sizes and a few conserved nucleotides being the common paradigm (Jambhekar et al., 2005; Olivier et al., 2005). Each of the LEs is able to promote localization of a reporter construct to the recipient bud of *S. cerevisiae* (Olivier et al., 2005), whereas for localization of *ASH1* mRNA a single LE is not sufficient (Chartrand et al., 2002). Besides *ASH1* mRNA, approximately 30 other mRNAs are transported by the same motor complex to the bud of dividing yeast cells (Aronov et al., 2007; Shepard et al., 2003; Takizawa et al., 2000).

A screen for mutants defective in HO endonuclease expression identified five genes called SHE1-5 (Swi5p dependent HO expression; Jansen et al., 1996) which are necessary for Ash1p accumulation in daughter cell nuclei (Bobola et al., 1996). Subsequent studies confirmed that the asymmetric distribution of Ash1p is preceded by the motor-dependent transport of *ASH1* mRNA to the daughter cell tip and that deletion of a single SHE gene abolishes the localization (Long et al., 1997; Takizawa et al., 1997).

The SHE genes form the core factors for the *ASH1*-mRNA localization to the daughter cell. She1p is identical to Myo4p, a monomeric type-V myosin (Dunn et al., 2007; Haarer et al., 1994; Heuck et al., 2007; Reck-Peterson et al., 2001) that requires incorporation into the *ASH1* mRNP in order to move processively along actin filaments towards the plus end (Heym et al., 2013; Sladewski et al., 2013). Both She2p and She3p are RNA-binding proteins that form a synergistic complex with LEs of the *ASH1* mRNA (Müller et al., 2011). In addition, She3p also serves as an adaptor protein that links the mRNA to Myo4p motors. She4p, also known as Dim1p, interacts with the motor domain of Myo4p, thereby stabilizing the complex (Shi and Blobel, 2010; Toi et al., 2003). The cytokine She5p/Bni1p belongs to the family of formins and nucleates actin cables (Goode and Eck, 2007; Pruyne et al., 2002). Besides these core factors, three other proteins are involved in *ASH1*-mRNA localization, namely the accessory proteins Localization of *ASH1* mRNA protein 1 (Loc1p), Pumilio-homology domain family protein 6 (Puf6p), and heterogeneous nuclear RNP K-like protein 1 (Khd1p). Deletion of these accessory proteins does not completely abolish *ASH1*-mRNA localization to the daughter cell but decreases the efficiency of localization. Puf6p and Khd1p are translational repressors of the *ASH1* mRNA in the cytoplasm that bind already in the nucleus to its target (Deng et al., 2008; Gu et al., 2004; Irie et al., 2002; Paquin et al., 2007). Loc1p, however, is a

strictly nuclear protein that, besides its function in *ASH1*-mRNA localization (Du et al., 2008; Long et al., 2001), is involved in ribosome biogenesis (Harnpicharnchai et al., 2001; Urbinati et al., 2006).

The fact that only a limited number of factors are involved in *ASH1*-mRNA localization makes it an ideal model to investigate the mechanism underlying mRNA localization. The following paragraph describes the single steps of *ASH1*-mRNA localization as known at the beginning of my studies.

1.2.1 Nuclear mRNP assembly

The process of *ASH1*-mRNA localization already starts within the nucleus, where the RNA-binding protein She2p interacts cotranscriptionally with the LEs of the nascent *ASH1* mRNA (Figure 1-2; Müller et al., 2011; Shen et al., 2010)

The RNA-binding proteins Puf6p and Khd1p also interact with the *ASH1* mRNA within the nucleus (Figure 1-2). After nuclear export, they serve as translational repressors. Whereas Khd1p associates with the first LE (E1) of the *ASH1* mRNA (Irie et al., 2002), Puf6p binds in proximity of the E3-LE to the *ASH1* mRNA for repression (Gu et al., 2004). The fourth nuclear factor Loc1p is also implicated in translational repression of *ASH1* mRNA during its localization. However, in contrast to the other factors, it does not leave the nucleus. Nevertheless, genomic deletion of *LOC1* results in mis-localization and premature translation of Ash1p (Long et al., 2001). Loc1p, just as Puf6p, shows nucleolar enrichment (Huh et al., 2003) and has a second function in ribosome biogenesis (De Marchis et al., 2005; Harnpicharnchai et al., 2001; Ho et al., 2002; Urbinati et al., 2006). Initial experiments determined the composition of the nuclear mRNP by co-immunoprecipitations using She2p as bait. Loc1p and Puf6p as well as *ASH1* mRNA were co-purified, whereas Khd1p was absent from such purifications (Gu et al., 2004; Shen et al., 2009). She2p seems to play an integral part in the nuclear mRNP assembly, since a point-mutated She2p variant, which is unable to enter the nucleus, leads to the loss of Puf6p from the mRNP and additionally decreases the Loc1p association with *ASH1* mRNA (Shen et al., 2009). Furthermore, by using a temperature-sensitive RNA-export deficient strain, She2p not only became trapped in the nucleus but also accumulated with *ASH1* mRNA within the nucleolus (Du et al., 2008). All together, these studies suggest that a nucle(ol)ar transit of the mRNP is necessary for its maturation

(Figure 1-2). The fact that the strictly nuclear protein Loc1p affects translation in the cytoplasm implies a role in nuclear recruitment of translational repressors to the *ASH1* mRNA.

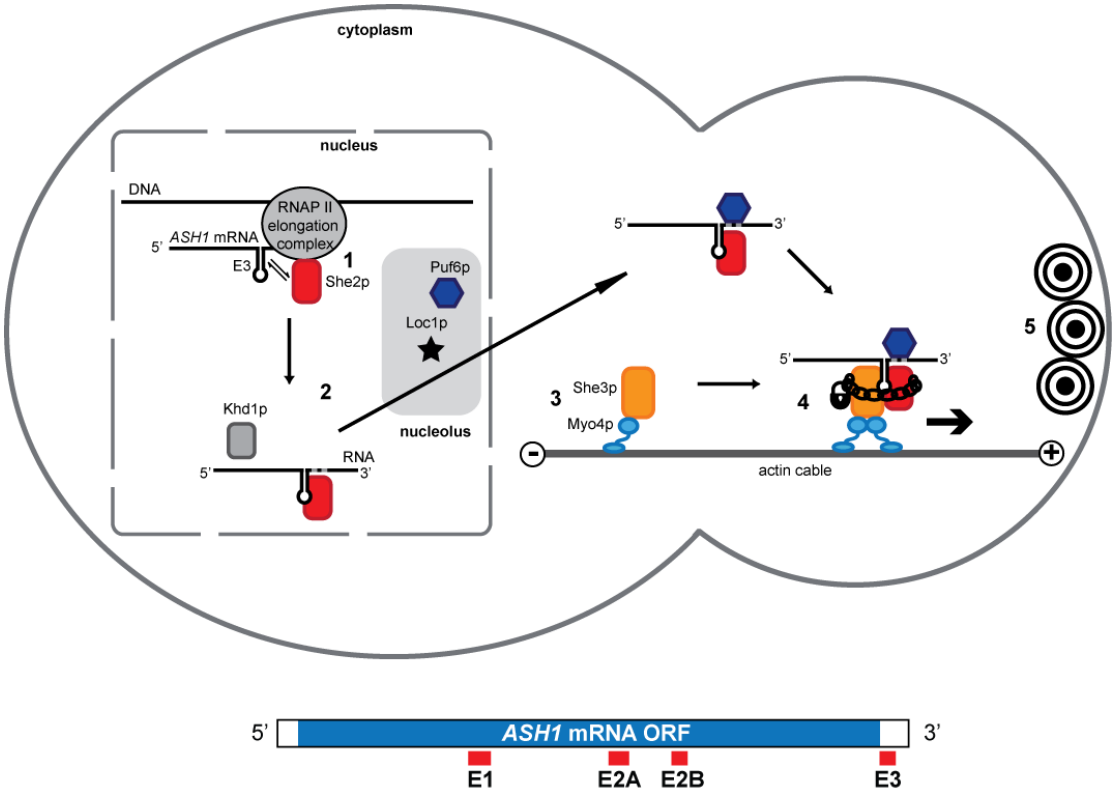

Figure 1-2 Model of *ASH1*-mRNA localization in *Saccharomyces cerevisiae*: Upper part Schematic drawing of mRNP assembly steps in a budding cell. The mother cell is depicted on the left site and the incipient bud on the right site. (1) *ASH1*-mRNA localization initiates already cotranscriptionally with the loading of She2p onto the nascent mRNA. (2) Within the nucleus the nucleolar factors Puf6p and Loc1p, as well as Khd1p associate with the *ASH1* mRNP. (3) In the cytoplasm the RNA-binding protein She3p that is bound by Myo4p (4) interacts with the mRNP exported from the nucleus to form the mature mRNP. This mature mRNP moves processivly along actin cables towards their plus ends. (5) *ASH1* mRNA localizes to the bud tip, is anchored, and translated resulting the local accumulation of Ash1p. **Lower part** Schematic drawing of the *ASH1* mRNA with 5' UTR and 3' UTR. The position of the LEs within the open reading frame (E1, E2A, and E2B) and the 3´untranslated region (E3) are depicted in red.

Cytoplasmic transport-complex assembly

The *ASH1* mRNA is exported into the cytoplasm with She2p and Puf6p, whereas Loc1p resides in the nucleus (Du et al., 2008; Kruse et al., 2002; Long et al., 2001). Within the cytoplasm, a pre-complex formed by both the myosin adaptor and RNA-binding protein She3p and the motor protein Myo4p joins the mRNP, thus forming the mature *ASH1* mRNP (Figure 1-2). At this point, She2p and She3p interact synergistically with the LEs of the *ASH1* mRNA (Bohl et al., 2000; Heuck et al., 2007; Müller et al., 2011). Mechanistically, this step is crucial, since Myo4p is a monomer in solution (Heuck et al., 2007) and thus non-processive (Dunn et al., 2007; Reck-Peterson et al., 2001). Moreover, the cytoplasmic complex formed by She3p and

Myo4p does not dimerize the motor (Heym et al., 2013; Hodges et al., 2008). Consequently, the formation of the mature transport complex has to induce a dimerization of the motor, which allows for processive movement along actin cables (Figure 1-2). The relevance of the RNA as an integral constituent for motor dimerization has been discussed controversially. Although there are *in vitro* studies showing that RNA is an inevitable component of a motile mRNP (Sladewski et al., 2013), other studies suggest that RNA is negligible for motor activation (Heym et al., 2013; Krementsova et al., 2011).

Translational repression and anchoring

The correct localization of *ASH1* mRNA and Ash1p requires the presence of an ATG codon within the mRNA sequence which implies the necessity of active translation for anchoring (Gonzalez et al., 1999; Irie et al., 2002). In line with these findings, Khd1p and Puf6p interact with the translation initiation factors eIF4G1 and eIF5B (Deng et al., 2008; Paquin et al., 2007), resulting in the translational repression of *ASH1* mRNA. At the bud tip Khd1p and Puf6p become phosphorylated which decreases their affinities to ASH1 mRNA-binding sites and thus results in translational activation (Deng et al., 2008; Paquin et al., 2007).

1.3 Ribosome biogenesis in budding yeast

The biogenesis of ribosomes includes the assembly of four ribosomal RNAs (5S, 5.8S, 25S, and 18S) and 79 ribosomal proteins (r-proteins). In addition, the efficient ribosome maturation requires 76 snoRNAs and more than 200 different assembly factors. R-proteins fulfill various molecular functions, such as the stabilization of folded rRNA as well as acting as chaperones to preserve the correct rRNA fold or to change the fold in order to establish binding sites for other r-proteins (Kressler et al., 2010; Woolford and Baserga, 2013).

Although assembly factors are a non-integral part of the ribosome, they associate with the ribosome during biogenesis. They often share properties with r-proteins, such as unusually high isoelectric points, and show high homology with r-proteins. However, assembly factors are much more diverse in their functionality since they not only include RNA-binding proteins but also RNA-modifying enzymes, endo- and exonucleases, kinases and phosphatases, RNA helicases as well as other ATPases, GTPases, and putative scaffolding proteins. The molecular function of assembly

factors often has been assigned to a particular step of processing, export, or maturation, depending on the depletion phenotypes observed (Kressler et al., 2010; Wilson and Doudna Cate, 2012; Woolford and Baserga, 2013).

Biogenesis of a ribosome starts with the transcription of 35S pre-rRNA by RNA polymerase I and of 5S RNA by polymerase III within the nucleolus of the cell (Figure 1-3). The 35S pre-rRNA is both processed and modified which can already occur already cotranscriptionally (Kos and Tollervey, 2010; Woolford and Baserga, 2013). It contains three rRNAs (18S, 5.8S, and 25S) that are separated by 3' and 5' external transcribed spacers (3' ETS and 5' ETS) along with two internal transcribed spacers (ITS1 and ITS2).

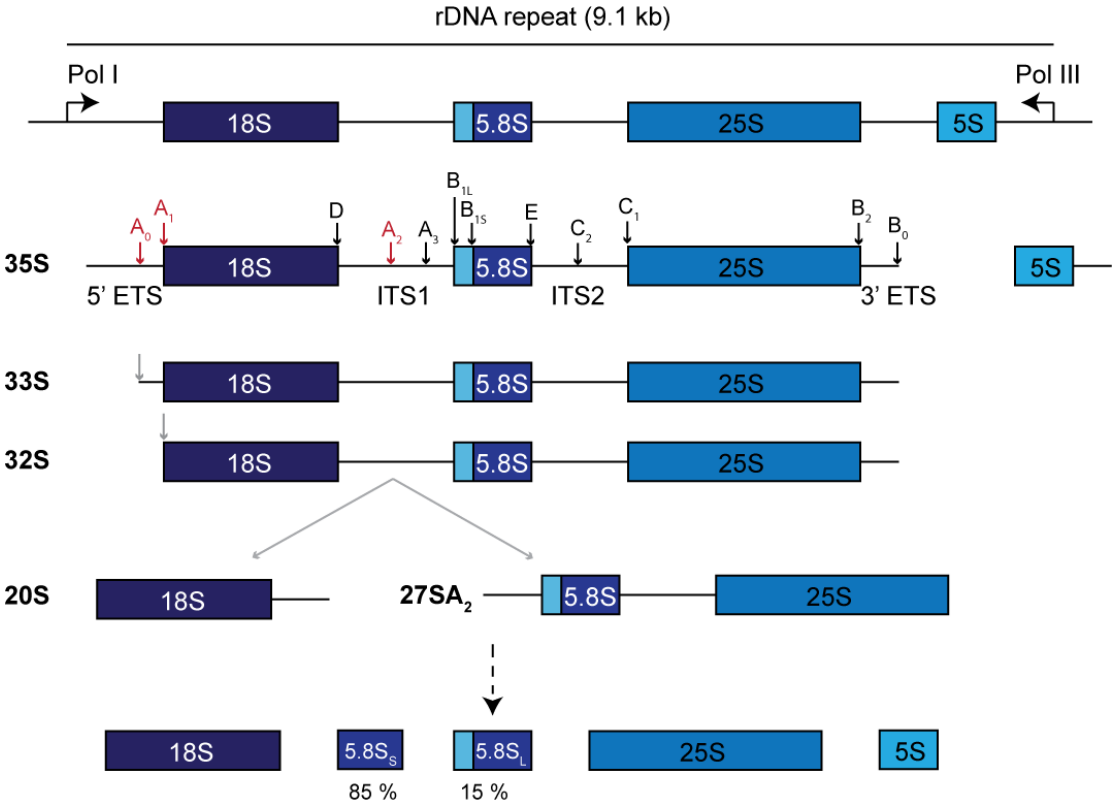

Figure 1-3: Nucleolar processing pathways of yeast pre-rRNA. rRNA is transcribed by Pol I (35 S pre-rRNA) and Pol III (5S pre-rRNA) from rDNA repeats. The 35S pre-RNA contains the sequences of 18S, 5.8S, and 25S rRNA, which are separated by external transcribed spacers (ETS) and internal transcribed spacers (ITS). The spacers are removed by ordered processing of pre-rRNA by endonucleases and exonucleases. Already during transcription, processing is initiated by the cleavage at sites A_0, A_1, and A_2 (depicted in red). The cleavage at A_0 gives rise to the 33S pre-rRNA that is further processed at site A_1 yielding 32S pre-rRNA. The subsequent cleavage at site A_2 separates the 20S pre-rRNA, which is needed for small subunit biogenesis, and the $27SA_2$ pre-rRNA. The $27SA_2$ processing is then divided into a major and minor pathway due to concurrent processing at sites A_3 (nucleolar), B_{1L}, and B_{1S} (both nucleoplasm). The processing is concluded in the nucleoplasm and cytoplasm, hence giving rise to the $5.8S_S$, $5,8S_L$, 25 S, and 5S rRNA.

During ribosome biogenesis endonucleolytic as well as exonucleolytic processing steps remove those spacers to ensure correct folding of the rRNA. In order to prevent misfolding of pre-rRNA, processing and folding is coordinated which is

supported by the presence of timers that prevent pre-mature folding. An example for such a timer is the coordinated folding and processing of the 18S rRNA. In this case, a conformational switch of the pre-rRNA directs the cleavage at the sites A_2 and D (Figure 1-3). The rRNA conformation *before* A_2 cleavage prevents the processing of Nob1 at the cleavage site D. In contrast, processing directly at site A_2 releases a conformational trigger which eventually allows for Nob1 processing at site D (Lamanna and Karbstein, 2011). Another interesting example of such a conformational switch is the assembly of the mature 5S rRNP within the 60S subunit. The mature 5S rRNP consists of the 5S rRNA as well as RpL5 and RpL11. Within the mature 60S subunit, RpL11 interacts with helix 84 (H84) of the 25S rRNA. Structural studies by Calvino and colleagues revealed that Symportin1 acts as a chaperone that occupies the RpL11 – H84 interaction site with a α-Solenoid. A trimeric complex formed by RpL11, RpL5, and Symportin, which also serves as a karyopherin, is imported into the nucleus, where the 5S rRNA is integrated into the complex. The binding of H84 of the pre-60S particle induces a complex reassembly and the binding of RpL11 to H84 omits Symportin1 (Calvino et al., 2015).

1.3.1 RNA chaperones in ribosome biogenesis

As mentioned before, both the ordered folding and processing of rRNA and the assembly into ribosomes require the presence of assembly factors acting as RNA chaperones. Those chaperones either induce favored RNA structures or prevent the formation of misfolded RNA. Although those RNA chaperones are of significant importance, for their majority a molecular mechanism in rRNA folding is not yet understood.

The RNA chaperones Imp3p and Imp4p are an exception since their function in 35S rRNA and U3 snoRNA annealing has been studied in detail. Imp3p and Imp4p aid the formation of a helix between the 18S site of the rRNA and the Box A' and Box A of the U3 snoRNA. This annealing activity supports the cleavage at sites A_0 and A_1, which is required for 18S rRNA maturation (Marmier-Gourrier et al., 2011; Shah et al., 2013).

1.3.2 Role of Loc1p in ribosome biogenesis

The first report that proved a connection between Loc1p and ribosome biogenesis was published by Harnpicharnchai and colleagues (Harnpicharnchai et al., 2001).

Co-immunoprecipitaitons using TAP-tagged Nop7, which is a constituent in 90S and 66S ribosome biogenesis, co-purified Loc1p. Reduced 60S subunit levels in a *loc1Δ* strain as well as the presence of "half-mer" polyribosomes in sucrose gradients further indicated a role of Loc1p in 60S subunit maturation (Harnpicharnchai et al., 2001). Subsequent co-immunoprecipitations of pre-60S particles confirmed Loc1p as a constituent thereof (De Marchis et al., 2005; Horsey et al., 2004; Saveanu et al., 2003).

Northern-blot analysis of pre-rRNA processing intermediates showed that genomic deletion of LOC1 results in impaired cleavage at sites A_0, A_1, and A_2 of the 35S pre-rRNA. The presence of the aberrant 23S rRNA product in the *loc1Δ* strain further indicates that cleavage at site A_3, in contrast to cleavage at the sites A_0, A_1, and A_2, is not affected. Furthermore, the export of 60S ribosomal subunits into the cytoplasm is reduced in the knock out strain (Urbinati et al., 2006). Loc1p has also been suggested to be necessary for the generation of different classes of ribosomes that contain distinct subsets of ribosomal protein paralogs. The fact that only one paralog of the duplicated ribosomal protein pair affects ASH1-mRNA localization, similar to the *loc1Δ* strain, indicates a distinct functionality of single paralogs (Komili et al., 2007).

1.4 Objectives

The localization of an mRNA frequently starts within the nucleus were trans-acting factors bind to the mRNA and form an initial nuclear mRNP. This nuclear priming process is often found to be a prerequisite for a correct cytoplasmic localization of the mRNA. Also the unicellular eukaryote *S. cerevisiae* engages nuclear mRNP formation to ensure the correct localization of the *ASH1* mRNA to the daughter cell tip. This nuclear mRNP contains just a few proteins (She2p, Loc1p, Puf6p, and Khd1p; Gu et al., 2004; Gu et al., 2002; Irie et al., 2002; Shen et al., 2009). It is a puzzling observation that the strictly nuclear protein Loc1p affects the cytoplasmic fate of the *ASH1* mRNA (Du et al., 2008; Huh et al., 2003; Long et al., 2001). The *ASH1* mRNP is exported into the cytoplasm where the RNA-binding protein She3p and the molecular motor Myo4p join the complex to form the mature transport mRNP (Müller et al., 2011). Mechanistically, this is an interesting process, since

formation of the mature complex needs to induce motor dimerization in order to gain processive movement along actin cables (Hodges et al., 2008).

The first aim of my studies was to understand the molecular mechanisms of nuclear *ASH1*-mRNP formation, especially the function of Loc1p. Furthermore, I wanted to understand the process of complex reorganization during the transition from a nuclear to a cytoplasmic mRNP. Another aim of my studies is to elucidate whether LEs of the *ASH1* mRNA are required for Myo4p dimerization during *ASH1*-mRNP assembly. To investigate this mRNP assembly, I characterized *in vitro* reconstituted complexes with different biochemical approaches. With the step-wise assembly and reassembly as well as biochemical characterization of assembly intermediates the nuclear (Publication 1) and the cytoplasmic complexes (Publication 2) were studied.

Loc1p fulfills a dual functionality in *S.cerevisiae.* On the one hand, it is involved in *ASH1*-mRNA localization, on the other hand it, is a constituent in the biogenesis of the large ribosomal subunit. Genomic deletion of *LOC1* leads to defects in the early processing events of pre-rRNA and accumulation of half-mer ribosomes. Loc1p was also implicated in the assembly of specialized ribosomes that contain a distinct set of r-protein paralogs. During my studies, I aimed to assess what molecular function Loc1p fulfills in ribosome biogenesis. To address this question, I used a combination of biochemical, structural, and *in vivo* yeast techniques. The result of my study on Loc1p function in ribosome biogenesis is being prepared for as a manuscript for submission to a peer-reviewed journal (Manuscript).

2 Methods

2.1 Recombinant expression and purification of proteins and RNA

Proteins and peptides were recombinantly expressed in *E. coli*, except for She3p FL that was expressed in insect cells using the bac-to-bac expression system (Invitrogen). Expression and purification of She2p, She3p, Puf6p, Myo4p and mutants thereof were performed essentially as previously described (Heuck et al., 2007; Müller et al., 2011; Niessing et al., 2004). The expression and purification procedures for Loc1p, Imp4p, and Hfq and variants thereof were established during this study and are described in the method section of publication 1 as well as in the manuscript. Since all proteins were used for RNP-reconstitution experiments precautions had to be taken to avoid RNA and RNAse contaminations. All proteins were purified using at least three different chromatographic approaches. During the initial affinity chromatography a high salt wash with up to 1 M NaCl/KCl was applied to remove nucleic acids. After ion-exchange chromatography and size-exclusion chromatography, proteins of high purity (95 %) were obtained, as assessed by SDS-PAGE. To verify the absence of nucleic acids from the purified proteins, the absorbance at the wavelength 260 nm and 280 nm was measured. Only proteins with an A_{260} to A_{280} ratio of 0.5 – 0.6, which indicated the absence of nucleic acids, were used for reconstitution experiments as described by Edelmann and colleagues (Edelmann et al., 2014).

For the recombinant expression and purification of RNA, the tRNA scaffold technology (Ponchon et al., 2009; Ponchon and Dardel, 2007) was used. TAR and the *ASH1* E3 LE were fused to the anticodon loop of the tRNA and expressed in the *E. coli* strain JM101. The purification was performed as described by Ponchon and colleagues (Ponchon et al., 2009), except for the RNAse H digestion, which was omitted here.

2.2 *In vitro* transcription of RNA

For small-scale *in vitro* transcription of *ASH1* E3 LE the MEGAshortscript T7 kit (life technologies) was used according to the manufacturers instructions. Synthetic DNA oligonucleotides containing a T7 promoter and the sequence of *ASH1* E3 LE were used as a template. After the *in vitro* transcription, the template was DNAse digested and RNA was extracted using phenol:chloroform:isoamylalcohol (25:24:1). Molecular and conformational homogeneity of RNA was assessed by native and denaturing TBE agarose-gel electrophoresis.

2.3 Biochemical characterization of mRNPs

2.3.1 Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were used to study the RNA-binding properties of proteins and to assess their specificities and affinities (Gagnon and Maxwell, 2011; Ryder et al., 2008). Briefly, in a total volume of 20 µL 5 nM radioactively labeled RNA, 100 µg/mL yeast tRNA competitor, and the respective protein concentration were mixed in binding buffer supplemented with 4 % (v/v) glycerol. After 25 min incubation at 25 °C, the RNA protein complexes were resolved by native TBE PAGE (6 % polyacrylamide). Gels were incubated for 15 min in fixing solution (10 % (v/v) acetic acid, 30 % (v/v) methanol) and vacuum dried. Gels were analyzed with radiograph films or by using Phosphorimager (Fujifilm FLA 5100).

Competitive EMSAs were used for the detection of binding preferences. Thereby, the interaction of the protein with a radioactively labeled RNA was competed for by the addition of increasing amounts of unlabeled RNA. In competitive EMSA experiments tRNA competitor was omitted.

To verify the presence of a distinct protein in a multi-protein RNP, a supershift EMSA (Smith, 1998) was performed. After RNP formation, monoclonal She2p antibody (Müller et al., 2009) was added to the reaction and incubated for another 10 min before the complexes were resolved by TBE PAGE (4-20 % polyacrylamide gradient gels).

2.3.2 *In vitro* pull-down experiments

In vitro pull-down experiments were used to detect RNA-dependent and RNA-independent protein interactions. Briefly, in a total volume of 100 µL 7.5 or 10 µM of indicated protein, 4 µM *ASH1* E3 LE and 5 mg/ml yeast tRNA competitor (unless stated otherwise) were mixed in appropriate pull-down buffer. The samples were centrifuged for 10 min and the supernatant was incubated for 30 min at 4 °C on a rotating wheel with 50 µL of the respective resin material. Binding reactions were washed four times with 200 µL and once with 50 µL pull-down buffer. Bound proteins were eluted and separated using SDS-PAGE and stained with Coomassie blue. RNA was analyzed on a 1.5 % agarose gel or an urea PAGE with GelRed DNA stain. For *in vitro* pull-down experiments that closely resemble physiological conditions, a larger reaction volume (1.5 ml) with a lower protein concentration (250 nM) was used.

2.3.3 UV-crosslinking

The approach of UV-crosslinking was used to determine which proteins of an mRNP directly contact the RNA (Reed and Chiara, 1999). Furthermore, this assay was used to determine changes in RNA-protein interaction upon reassembly of complexes. mRNPs were first assembled by combining the indicated protein concentrations with 5 nM of radioactively labeled RNA and 100 µg/ml yeast tRNA in a total volume of 20 µl. After 25 min incubation at room temperature the samples were UV-radiated with 2,25 J over 15 min. During UV radiation samples had a distance of 8 cm to the lamp and were kept on ice. Subsequently, SDS-loading dye was added and the samples were incubated at 95 °C for five minutes. The crosslinked complexes were resolved by 10 % SDS-PAGE and the gels were fixed in fixing solution (10 % (v/v) acetic acid; 30 % (v/v) methanol). After vacuum drying, the gels were analyzed with radiograph film or by using Phosphorimager (Fujifilm FLA 5100). Crosslinked RNA-protein complexes show a decreased mobility and are therefore shifted to higher molecular weight bands.

2.3.4 Filter-binding assay

Transient protein-RNA interactions (e.g. She2p - *ASH1* LE interaction) are often not detectable with an EMSA experiment, since the separation of complexes takes several minutes to hours. Therefore a filter-binding assay was applied for the

detection of these transient interactions (Niessing et al., 2004; Wong and Lohman, 1993). Briefly, the complexes were formed using the indicated protein concentration, 0.5 nM of radioactively labeled RNA, and 30 µg/µl yeast tRNA in a total volume of 80 µl. By using a Dot Blot apparatus (Bio-Rad), samples were directly applied to a stack of nitrocellulose, nylon, and several filter membranes. After two short washing steps, membranes were air dried and analyzed by Phosphorimaging (Fujifilm FLA 5100). Signal intensities were quantified with the Dot Blot Analyzer macro of the Image J program and K_d values were calculated with Origin 8.6.

2.3.5 Analytical size-exclusion chromatography

Size-exclusion chromatography of RNPs allows for the separation of bound and unbound RNA, since RNPs elute at a higher molecular weight than the free RNA. Furthermore, it is possible to analyze eluted fractions by PAGE which allows for determination of RNP composition. For unambiguous identification of RNP components, a column material appropriate for the RNP size has to be chosen and chromatography runs for the individual components have to be performed as reference. In this study size-exclusion chromatography was performed as described before (Müller et al., 2011). Briefly, complexes were assembled at stoichiometric ratios and applied to a Superose 6 10/300 GL column (GE Healthcare) with a flow-rate of 0.5 ml/min at 4 °C.

2.3.6 Static light scattering

Static light scattering in combination with size-exclusion chromatography allows for exact determination of molecular weight of the eluted RNP (Rambo and Tainer, 2010). In this study a Superose 6 10/300 GL (GE Healthcare) column was used with a flow rate of 0.1 ml/min at 4 °C. Static light scattering was measured with a 270 Dual Detector and a VE3580 RI Detector from Malvern. Before RNPs were applied the system was calibrated with 100 µl BSA at a concentration of 4 mg/ml. RNP complexes were assembled at stoichiometric ratios with sample concentrations in the range of 1.9 to 3.1 mg/ml in a total volume of 100 µl. Data analysis was performed using the Malvern OmniSEC 5.02 software. The molecular mass was determined by the mean of two independent experiments.

2.4 Ribosome purifications from yeast

Ribosome purification from *S. cerevisiae* was performed to get insights into ribosome biogenesis defects of the *loc1Δ* strain. The aim was to measure the total amount of ribosomes as well as the degree of rRNA modifications of the *loc1Δ* strain in comparison to a wild-type strain. All purification steps were done at 4 °C unless stated otherwise. Wild type yeast cells (S288c) and *loc1Δ* cells were cultured to an OD_{600} of 1.5 to 2.5, washed with water and 1 % KCl (w/v), and resuspended in buffer (100 mM TRIS pH 8; 10 mM DTT). After 15 min incubation at room temperature the cells were pelleted and resuspended in lysis-buffer (20 mM HEPES pH 7.5; 100 mM KOAc; 7.5 mM $Mg(OAc)_2$; 125 mM sucrose; 1 mM DTT; 0.5 mM PMSF; EDTA-free Complete protease inhibitors). The cells were disrupted in a microfluidizer (3 times; 20000 psi) and centrifuged for 15 min at 15500 rpm (SS34 rotor). The supernatant was again centrifuged for 30 min at 37 krpm (Ti-70 rotor) and only the clear fraction (S100) was further purified. Subsequently 15 ml S100 was layered over 4 ml 2M sucrose cushion and 4 ml 1.5M sucrose cushion (Sucrose cushion buffer: 1.5 or 2 M sucrose; 20 mM HEPES pH 7.5; 500 mM KOAc; 5 mM $Mg(OAc)_2$; 1mM DTT; 0.5 mM PMSF) and centrifuged in a Ti 70 rotor tube for 18 h at 53250 rpm. The ribosome pellet was resuspended in 500 µl water. For analysis of modified nucleosides by liquid chromatography-mass spectrometry (LC-MS) the rRNA was extracted by phenol:chloroform:isoamylalcohol (25:24:1) extraction, precipitated, and hydrolyzed with NaOH. LC-MS analysis of rRNA (Bruckl et al., 2009) was performed in collaboration with Caterina Brandmayr (group of Prof. Carell; LMU Munich).

2.5 tRNA purification from yeast

Since the transcription and initial processing steps of tRNAs take place in the nucleolus, where Loc1p is localized to, tRNAs from wild-type and *loc1Δ* strain were purified and analyzed for RNA modification by LC-MS.

Wild type (S288c) and *loc1Δ* cells were grown to the early log phase. The cells were harvested (10 min at 4500 rpm), resuspended in buffer (10 mM $Mg(OAc)_2$; 50 mM NaOAc; 150 mM NaCl; pH adjusted to 4.5), and again harvested (10 min at 4500 rpm) before flash freezing in liquid nitrogen. Subsequently cells were resuspended in buffer (10 mM $Mg(OAc)_2$; 50 mM TRIS; pH adjusted to 7.5) and

RNA was extracted twice with phenol. The RNA-containing supernatant was precipitated with 2 volumes of ethanol and 200 mM NaCl (final concentration) at -20°C over night. The pelleted RNA was resuspended in buffer A (40 mM Na_2HPO_4 pH 7) and bound to a MonoQ column (GE Healthcare). A linear gradient to 100 % buffer B (40 mM Na_2HPO_4 pH 7; 1 M NaCl) was applied and fractions containing pure tRNAs, as assessed by denaturting PAGE, were pooled and precipitated. For mass-spectrometric analysis pellets were resuspended in water and hydrolyzed with NaOH. LC-MS analysis of modified tRNAs (Bruckl et al., 2009) was performed in collaboration with Caterina Brandmayr (group of Prof. Carell; LMU Munich).

2.6 Fluorescence and radioactive chaperone assays

Fluorescence-based chaperone assays performed as previously described (Hopkins et al., 2009) using a FluoroMax-P fluorometer (Horiba). Experiments were performed using an initial RNA concentration of 100 nM in a total cuvette volume of 900 µl. When indicated 75 nM for the complementary unlabeled RNA were added to the reaction. MBP-tagged Loc1p and maltose-binding protein (MBP; as control) were added accordingly. The fluorescence of FAM was excited at the wavelength of 495 nm using a slit of 2 nm. The emission was detected at the wavelength of 517 nm using a slit of 3 nm for 0.5 s (integration time). Data analysis was performed using Origin 8.4.

2.7 Peptide array

In order to identify RNA-binding regions within the Loc1p sequence a Loc1p tiling array was purchased and tested for interaction with *ASH1* E3 LE. Peptides were N-terminally acetylated and shifted by three amino acids per peptide. The beta-alanine cellulose membrane with covalently spotted peptides was incubated with 0.003 nM radioactively labeled *ASH1* RNA in 10 ml buffer (20 mM Na_2HPO_4; 50 mM NaCl; pH 7) at 25 °C on a rotating wheel for 30 min. After washing the membrane 5 times with buffer for 10 min binding was analyzed using a Phosphorimager (Fujifilm FLA 5100). Signal intensities were analyzed using the Dot Blot Analyzer macro within the Image J software.

2.8 NMR experiments

NMR experiments were performed in collaboration with Janosch Henning (group of Prof. Sattler; TUM Munich). The aim of these experiments was to gain information on the molecular interactions between a single RNA-binding motif of Loc1p (amino acids 3 to 15) and dsRNA. To verify the specificity of the interaction control peptides (derived from the Loc1p sequence) that failed to bind RNA in the peptide array were included into the measurements. The interaction was investigated from the RNA point of view and the peptide point of view. For experiments intended to gain structural information of the peptide, ^{15}N- ^{13}C-labeled peptide was used. Before the experiments dsRNA was snap cooled to ensure that the energetically most favored conformation is formed. The RNA was kept on ice before measurements at 278 K were recorded, if not stated otherwise. Bruker Avance III spectrometers, equipped with a TCI cryogenic probe head at field strengths corresponding to 600 and 800 MHz proton Larmor frequency were used. For the assignment of imino signals a 2D NOESY was recorded. For titration experiments and thermal scans 1D-^{1}H-spectroscopy was applied. HNCACB and HCCH-TOCSY spectra were recorded for backbone and partial side chain assignment of ^{15}N, ^{13}C-labeled Loc1p (amino acids 3 to 15) For dsRNA-Loc1p (amino acids 3 to 15) binding studies, dsRNA was titrated into ^{15}N-, ^{13}C-labeled Loc1p (amino acids 3 to 15) and chemical shift perturbation were monitored by acquiring a ^{1}H-^{15}N HSQC spectrum for each titration point. Secondary structure prediction from secondary chemical shifts (C_α and C_β) was done according to Wishart and Sykes (Wishart and Sykes, 1994).

2.9 Crystallization

Loc1p (amino acids 3 to 15) and dsRNA in a molar ratio of three to one were mixed in water and incubated for 20 minutes at room temperature before centrifugation at 13000 g and 4 °C for at least 20 minutes. Initial crystallization screening was performed using a mosquito nanodrop-dispensing robot (ttplabtech) with commercial Natrix HT (Hampton Research) and Nucleix (Quiagen) screens in 96 well format. Crystallization was performed by sitting drop vapor diffusion using 100 nl sample and 100 nl precipitant at 20 °C. The final complex concentration was approximately 5 mg/ml. Refinement of particular crystallization condition was

performed by hanging drop vapor diffusion using 1 µl sample and 1 µl precipitant at 20 °C. The final complex concentration was 2.5 mg/ml. Crystals appeared after one day and were soaked with cryoprotectant (20 % (v/v) ethylenglycol or 40 % (v/v) PEG 400) before they were flash frozen in liquid nitrogen. Crystals were stored in liquid nitrogen and the X-ray diffraction image of crystals were collected at the synchrotron ESRF ID 29 (Grenoble, France) or at the synchrotron SLS X06DA (Villigen; Switzerland).

3 Publications

This work was submitted as a cumulative dissertation, containing two first author publications in peer-reviewed journals, one unpublished manuscript, and two review articles. The following table (Table 3-1) gives an overview of my contributions.

Contribution	Citation	Position
Original publication 1	Niedner, A.*, Müller, M.*, Moorthy, B.T., Jansen, R.P., and Niessing, D. (2013). Role of Loc1p in assembly and reorganization of nuclear *ASH1* messenger ribonucleoprotein particles in yeast. Proceedings of the National Academy of Sciences of the United States of America *110*, E5049-5058.	Page 26 ff
Original publication 2	Edelmann, F.T.*, Niedner, A.*, and Niessing, D. (2015). *ASH1* mRNP-core factors form stable complexes in absence of cargo RNA at physiological conditions. RNA Biology *12*, 233-237.	Page 27 ff
Original study, unpublished manuscript	Niedner, A., Hennig, J., Anasova, I., Müller, M., Brandmayr, C., Heym, R.G., Müller M., Gerber, A.P., Paillart, J.-C., Carell, T., Sattler, M., and Niessing, D. A conserved, intrinsically disordered RNA-binding motif operates as RNA-folding catalyst. (Manuscript in preparation)	Page 28 & External appendix
Review article 1	Edelmann, F.T.*, Niedner, A.*, and Niessing, D. (2014). Production of pure and functional RNA for in vitro reconstitution experiments. Methods *65*, 333-341.	External appendix
Review article 2	Niedner, A.*, Edelmann, F.T.*, and Niessing, D. (2014). Of social molecules: The interactive assembly of ASH1 mRNA-transport complexes in yeast. RNA biology *11*, 998-1009.	External appendix

Table 3-1: Table showing my contribution to published and unpublished articles and review articles. The asterisk indicates a shared first authorship.

3.1 Original Publication 1

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

The cytoplasmic localization of an mRNA is often preceded by formation of nuclear mRNPs. Also, *ASH1*-mRNA localization in budding yeast already starts within the nucleus where the RNA-binding protein She2p interacts cotranscriptionally with the mRNA. Two other factors, Loc1p and Puf6p, also interact with the *ASH1* mRNA in the nucleus, however, their recruitment to the mRNP remained not well understood.

This study offers new insights how the nuclear *ASH1* mRNP is formed. This work was originally started by Dr. Marisa Müller in the Niessing lab, who established protein-expressions, RNA-binding and pull-down experiments with Loc1p, and obtained a number of initial results. However, the majority of biochemical experiments important for the impact of this publication were performed by myself. *In vivo* data were contributed by the laboratory of Prof. Ralf-Peter Jansen (Eberhards Karls University Tübingen).

In this study I used pull-down experiments, EMSAs and supershift-EMSAs, UV-crosslinking and filter-binding assays to discover a cooperative binding of She2p and Loc1p to the *ASH1* mRNA. I also demonstrated that Loc1p interacts specifically with She2p and that this co-complex shows a higher specificity, stability, and affinity for *ASH1* mRNA than She2p alone. Furthermore, I identified that Puf6p, although it interacts with the *ASH1* mRNA, is not recruited to the mRNP. After nuclear export the myosin-bound She3p interacts with the complex to form the mature transport complex with an even higher affinity and specificity. Because Loc1p is only found in the nucle(ol)us of the cell, a reorganization of the complex upon export is necessary to remove Loc1p. *In vitro* experiments by myself and collaborative *in vivo* work indicated that the interaction between She2p and She3p displaces Loc1p from the *ASH1* mRNP. Based on these findings, we proposed a new model of ordered assembly and reorganization of the *ASH1* mRNP that ensures that all factors associate and dissociate at the right time escorting the *ASH1* mRNP from the site of transcription to the cytoplasm.

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

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Edited by James E. Dahlberg, University of Wisconsin Medical School, Madison, WI, and approved November 19, 2013 (received for review August 12, 2013)

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 nucle(ol)us. 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-immunoprecipitaton 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), *loc1*Δ 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.

Author contributions: A.N., R.-P.J., and D.N. designed research; A.N., M.M., and B.T.M. performed research; A.N., B.T.M., R.-P.J., and D.N. contributed new reagents/analytic tools; A.N., M.M., B.T.M., R.-P.J., and D.N. analyzed data; and A.N. and D.N. wrote the paper.

The authors declare no conflict of interest.

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In a point-mutated She2p that is unable to enter the nucleus, no Puf6p and very little Loc1p are found associated with *ASH1* mRNA (19). Thus, She2p appears to play an important role for the assembly and/or recruitment of the nuclear precomplex including Puf6p and Loc1p. Vice versa, preventing nuclear export of She2p with a temperature-sensitive *mex67-5* mutant results in accumulation of She2p and *ASH1* mRNA in the nucleolus (14). Together, these observations indicate that the nucle(ol)ar fate is an essential step for the assembly of mature, translationally silent *ASH1* mRNA-transport complexes.

ASH1 mRNA is exported into the cytoplasm along with She2p and Puf6p, whereas Loc1p remains located in the nucleolus (13, 14, 22). Once in the cytoplasm, the *ASH1* precomplex interacts with another subcomplex consisting of the type V myosin motor myosin 4 (Myo4p) and the RNA-binding protein She3p. The nuclear and cytoplasmic precomplexes form the mature transport complex via the direct interaction between She2p and She3p and their synergistic binding to the zip-code elements of the *ASH1* mRNA (8, 23–28).

Although Puf6p is associated with the localizing *ASH1* mRNP, in vitro binding studies suggested that Puf6p and She2p interact only indirectly via their joint binding to the *ASH1* mRNA (8). For Loc1p and She2p or for Loc1p and Puf6p no such information is available. It also remains unclear whether these factors depend on each other for RNA binding, whether they compete for *ASH1* mRNA interaction, and whether they all participate in the same mRNP or form different subcomplexes. Furthermore, no mechanism has been described that explains how Loc1p is removed from the complex prior to or during nuclear export.

Here we report the characterization of the nuclear *ASH1* mRNP assembly. We found that Loc1p and She2p directly and specifically interact with each other. This cocomplex of She2p and Loc1p shows a significantly better affinity, stability, and specificity toward *ASH1* mRNA. Because the RNA binding of She2p alone is very transient, this cooperative interaction of Loc1p and She2p with *ASH1* mRNA appears to be essential for stably tethering She2p to nuclear *ASH1* mRNA. We do not observe a similar recruitment of Puf6p to the mRNP by Loc1p, She2p, or She3p. Thus, the importance of both factors for Puf6p binding to *ASH1* mRNA is likely more indirect. Because Loc1p is absent in the cytoplasm, it has to be removed from the mRNP during complex maturation. We found that She3p outcompetes Loc1p from its cocomplex with She2p and RNA, which results in the synergistic cocomplex of She2p, She3p, and *ASH1* mRNA as well as free Loc1p. This outcompetition offers a mechanistic explanation for the removal of Loc1p from the mRNP. Consistently, genomic deletion of *SHE3* in vivo perturbs the nucleolar localization of Loc1p.

Results

Loc1p Binds Directly and Specifically to She2p. Loc1p was shown to copurify with She2p from yeast extracts (19). To test whether this interaction is direct, we performed pull-down experiments with recombinant maltose binding protein (MBP)-tagged Loc1p and She2p. To exclude indirect, RNA-mediated interactions, proteins were purified under stringent conditions and successful removal of nucleic acid contaminations was verified before binding experiments (*Materials and Methods*; for Loc1p, see Fig. S1*A*). She2p was retained on amylose resin in the presence of Loc1p but not with MBP alone or in the absence of an MBP-tagged protein (Fig. 1*A*). Thus, the pull-down experiments suggest a direct and quantitative interaction between Loc1p and She2p.

Loc1p is involved not only in mRNA localization but also in ribosome biogenesis. It has been proposed that the effect of Loc1p on *ASH1* mRNA localization might be only an indirect effect of impaired ribosome function (21). On the other hand, Loc1p has been found in complex with *ASH1* mRNA, She2p, and Puf6p (13, 19), suggesting a direct and functional link. We performed additional in vitro pull-down experiments in the absence of RNA and found that Loc1p interacts selectively with She2p, but not with Puf6p, His-tagged She3p (She3p-H6), or GST (Fig. 1 *A* and *B*). Thus, Loc1p binding to She2p is selective, which rather indicates a direct role of Loc1p in *ASH1* mRNA localization.

Loc1p and She2p Form a Joint Cocomplex on the E3 Zip-Code Element of the *ASH1* mRNA. Yeast three-hybrid experiments were used to identify Loc1p as an *ASH1* E1 and E3 zip-code–binding protein (13). Binding to the E3 zip-code element was further confirmed in electrophoretic mobility shift assays (EMSAs), using either yeast extracts or bacterial extracts with recombinant Loc1p. In EMSAs with purified Loc1p we show that this protein indeed binds directly to the E3 zip-code element (Fig. 1*C*). The appearance of a higher molecular weight band further indicates that a second binding event occurs at a higher protein concentration.

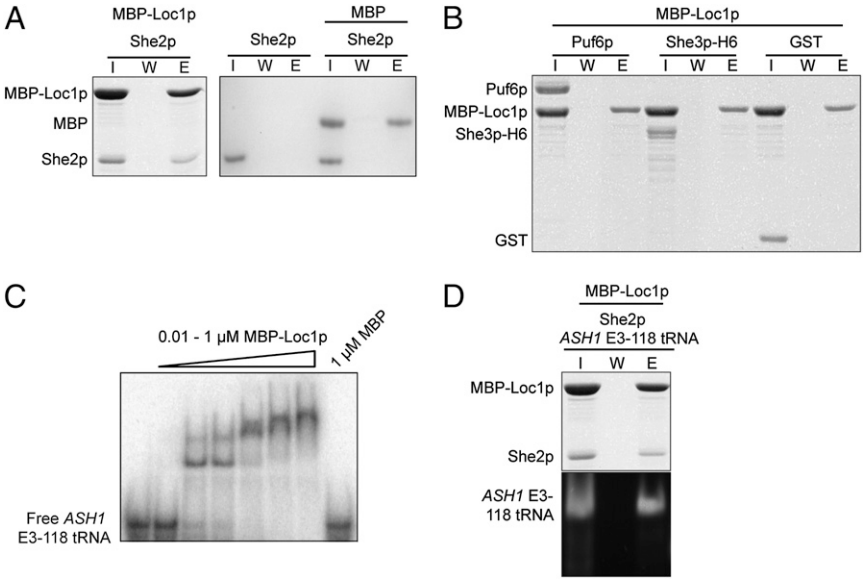

Fig. 1. Loc1p directly interacts with She2p and *ASH1* mRNA. (*A*) In pull-down experiments with amylose resin, MBP-Loc1p copurifies and thus directly interacts with recombinant She2p. Staining after SDS/PAGE was performed with Coomassie blue. (*B*) Pull-down experiments with MBP-Loc1p and Puf6p, She3p-H6, or GST. Loc1p is not able to pull down any of the tested proteins, indicating that this protein selectively interacts with She2p. Experimental setup is the same as in *A*. (*C*) EMSA with Loc1p and radioactively labeled E3 zip-code element of the *ASH1* mRNA reveals binding at nanomolar protein concentrations. (*D*) Amylose pull-down experiment with MBP-Loc1p, She2p, and the E3 element of *ASH1* mRNA suggests formation of a ternary complex. (*Upper*) Coomassie blue-stained SDS/PAGE; (*Lower*) agarose gel stained with GelRed.

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. 1*D*), 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. 2*A*, lane 3). Also Loc1p alone could be cross-linked to the RNA (Fig. 2*A*, lanes 4–6). However, this cross-linking gave a clearly detectable signal only at 1 μM protein concentration. The combination of Loc1p and She2p led to a strong increase of the Loc1p–RNA cross-link (Fig. 2*A*, 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. 2*B*). 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 μM She2p concentration (Fig. 2*B*). 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. 2*B*). Supershift experiments with increasing antibody concentrations further confirmed that She2p was indeed present in this shifted band (Fig. S2*A*, *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. S2*A*, *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.

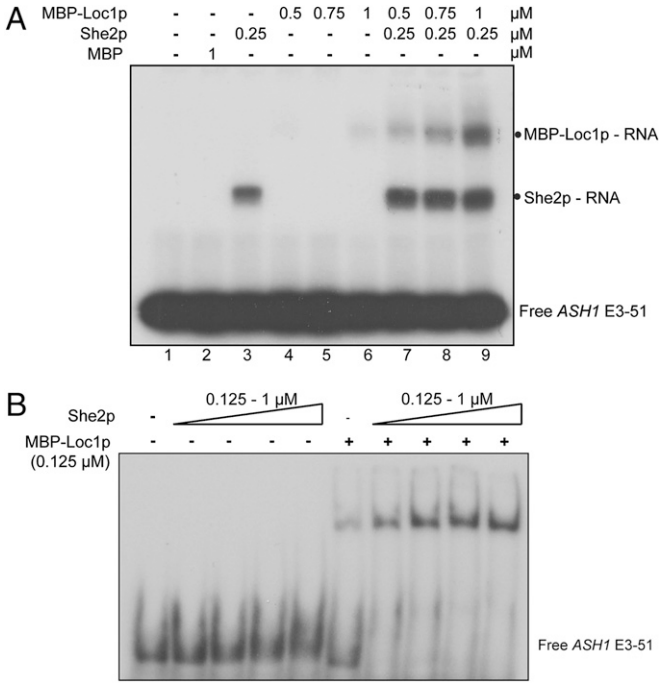

Fig. 2. She2p and Loc1p recruit each other to the *ASH1* mRNA. (*A*) UV cross-linking experiments with MBP-Loc1p, She2p, and radioactively labeled *ASH1* E3-51 RNA. In the presence of She2p and Loc1p, cross-linking of both proteins to the *ASH1* mRNA is increased (lanes 7–9). Note that in UV cross-linking 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.

Cocomplex 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. 3*A*). 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. 3*A*, asterisk). Because this band disappeared after phenol-chloroform extraction (Fig. 3*A*, *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. 3*A*, *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. 3*B*). Because She2p and Loc1p undergo a direct protein–protein interaction in the absence of RNA (Fig. 1*A*), 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. 3*B*) suggests competitive binding events, instead of the previously observed cooperative binding of She2p and Loc1p to zip-code RNA (Figs. 1*D* and 2 *A* 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. S2*B*).

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. 3*C*). Also in the cocomplex, Loc1p and She2p both efficiently cross-linked with the zip-code–containing RNA, as observed before (Fig. 2*A*). 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. 3*C*). Also in competition experiments we observed a preference for *ASH1* E3 zip-code element (Fig. S2*C*). In summary,

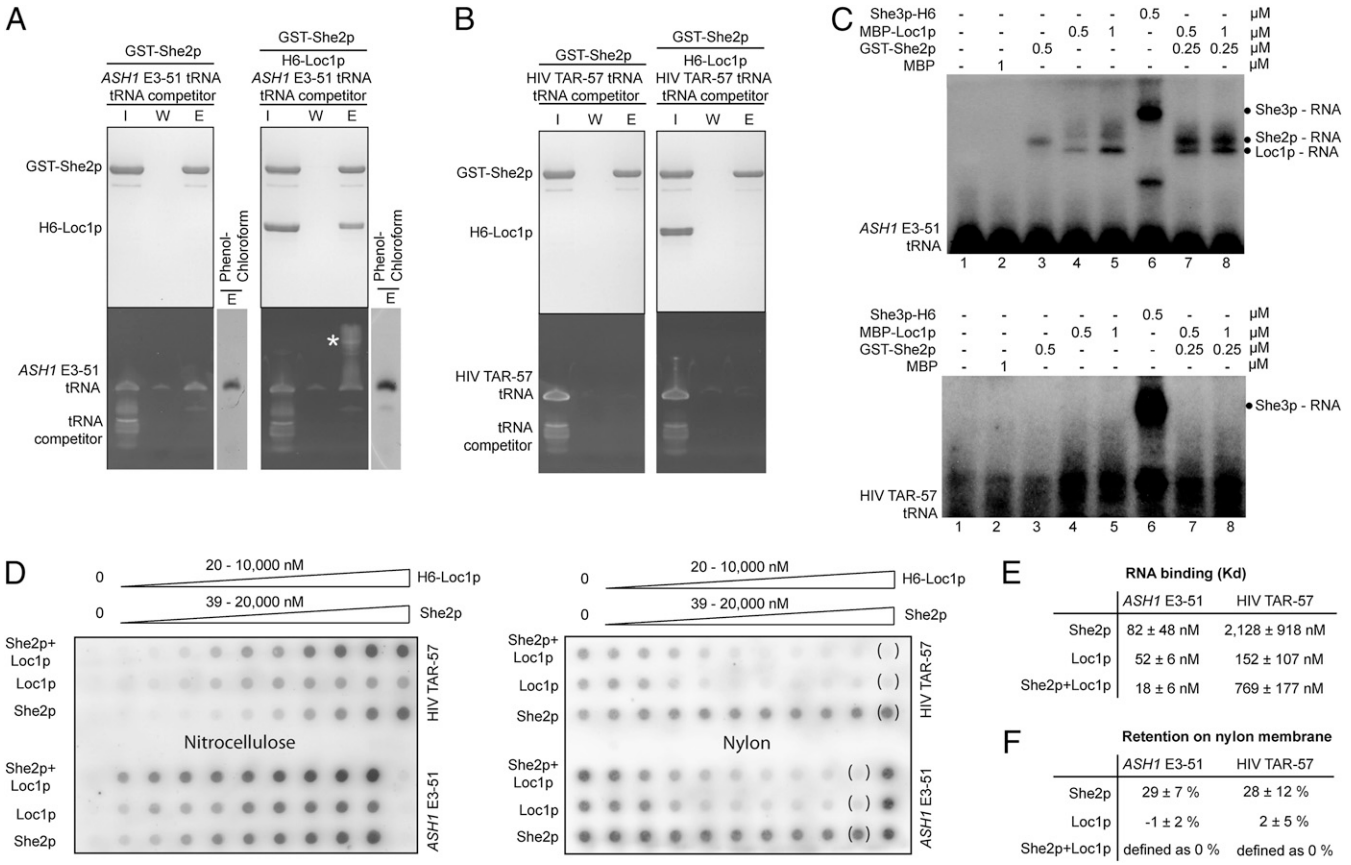

Fig. 3. The cocomplex of Loc1p and She2p mediates specific and stable binding to zip-code RNA. (*A*) In pull-down assays with glutathione Sepharose, GST-She2p copurifies *ASH1* E3-51 tRNA and H6-Loc1p. *Upper* gel shows PAGE and *Lower* gel shows an urea PAGE. The pull-down of *ASH1* E3-51 tRNA with 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. S2*B*). (*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. 1*C* and the K_ds 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 ± 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. 3*D*). In contrast, She2p alone was unable to retain a comparable amount of RNA on the first membrane (Fig. 3*D*). 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. 3*F*). 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. 1*B*). 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. 1*C* and Fig. S1 *B* and *C*). Puf6p was not retained by MBP-Loc1p on amylose beads (Fig. 4*A*), although Puf6p alone efficiently binds to E3 RNA (Fig. S1*C*) (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. S4 *A* 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. 4*B*). 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. 4*C*, lane 4; for a longer exposure of this UV cross-link, see Fig. S4*D*). Also the presence of other proteins, such as She2p (Fig. S4*C*, lanes 7 and 8), She2p and She3p (Fig. 4*C*, lanes 6 and 7, and Fig. S4*D*), or She2p and Loc1p (Fig. 4*C*, lanes 8 and 9, and Fig. S4*D*) 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 mRNP 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

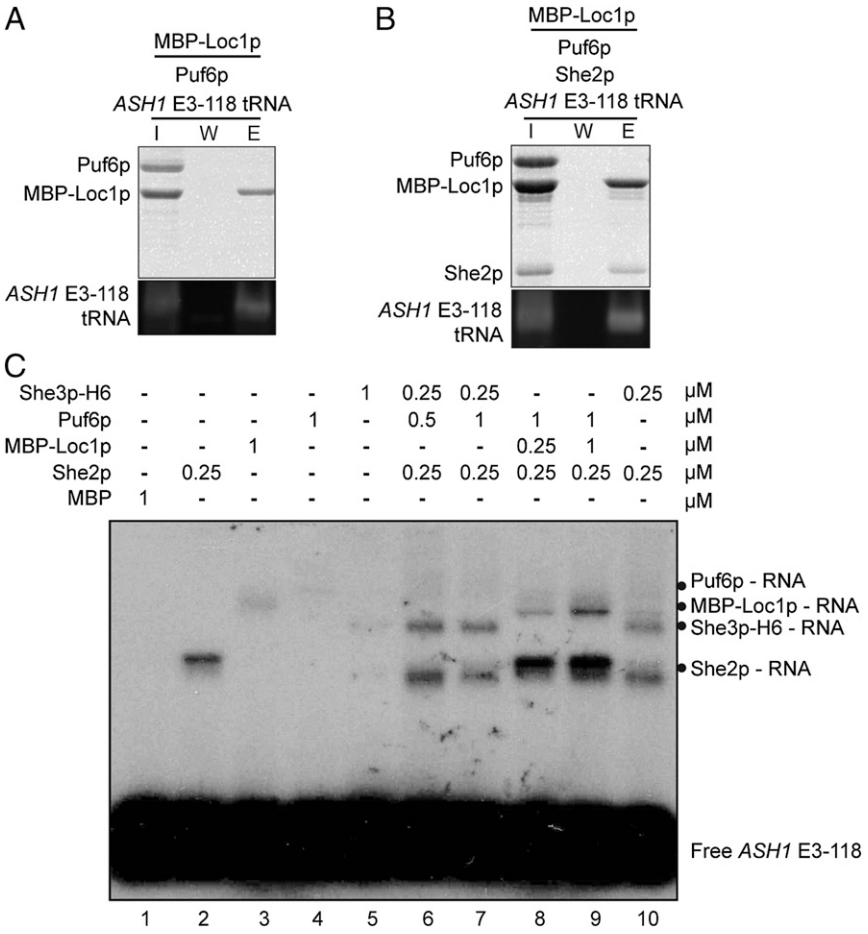

Fig. 4. Puf6p does not join the ternary complex of Loc1p, She2p, and RNA. (*A*) Pull-down experiment with amylose resin and MBP-Loc1p shows that Puf6p does not form a cocomplex with Loc1p and RNA. (*B*) Pull-down experiment with MBP-Loc1p, She2p, Puf6p, and the *ASH1* E3 zip-code RNA. All components 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. S4*D*.

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. 1*A*) and *ASH1* mRNA (Figs. 1 *C* and *D* and 2). Thus, for an active displacement, She3p would need to outcompete 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. 5*A*, 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. 5*A*, 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. 5*B*). 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. 5*C*). We conclude that She3p also outcompetes the protein interaction of Loc1p with She2p.

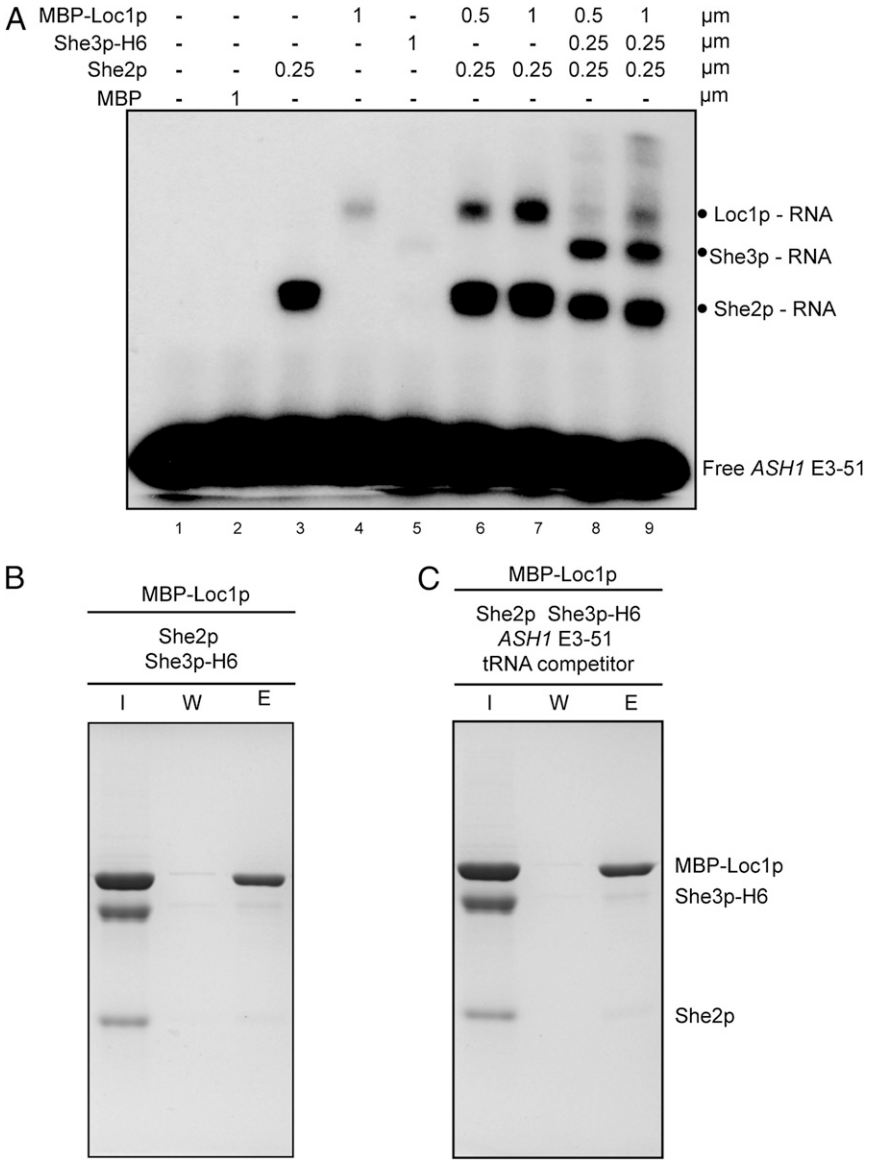

Fig. 5. She3p outcompetes Loc1p from the mRNP. (*A*) UV cross-linking experiment with She2p, She3p-H6, and MBP-Loc1p shows that in the presence of She2p and She3p Loc1p–RNA cross-linking is almost abolished. This finding suggests that She3p is able to outcompete Loc1p from its cocomplex with She2p and 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 (ΔC)] has no strong effect on in vitro binding to She3p, whereas the deletion of its protruding helix [She2p (ΔhE)] abolishes the She3p interaction (8). We first recapitulated these results (Fig. S6*A*) and then tested these mutant versions of She2p for Loc1p binding. We found that Loc1p shows reduced binding to She2p (L130Y), whereas She2p (ΔC) is bound like wild-type She2p (Fig. S6*B*). 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. 6*A*). 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 2μ plasmid and

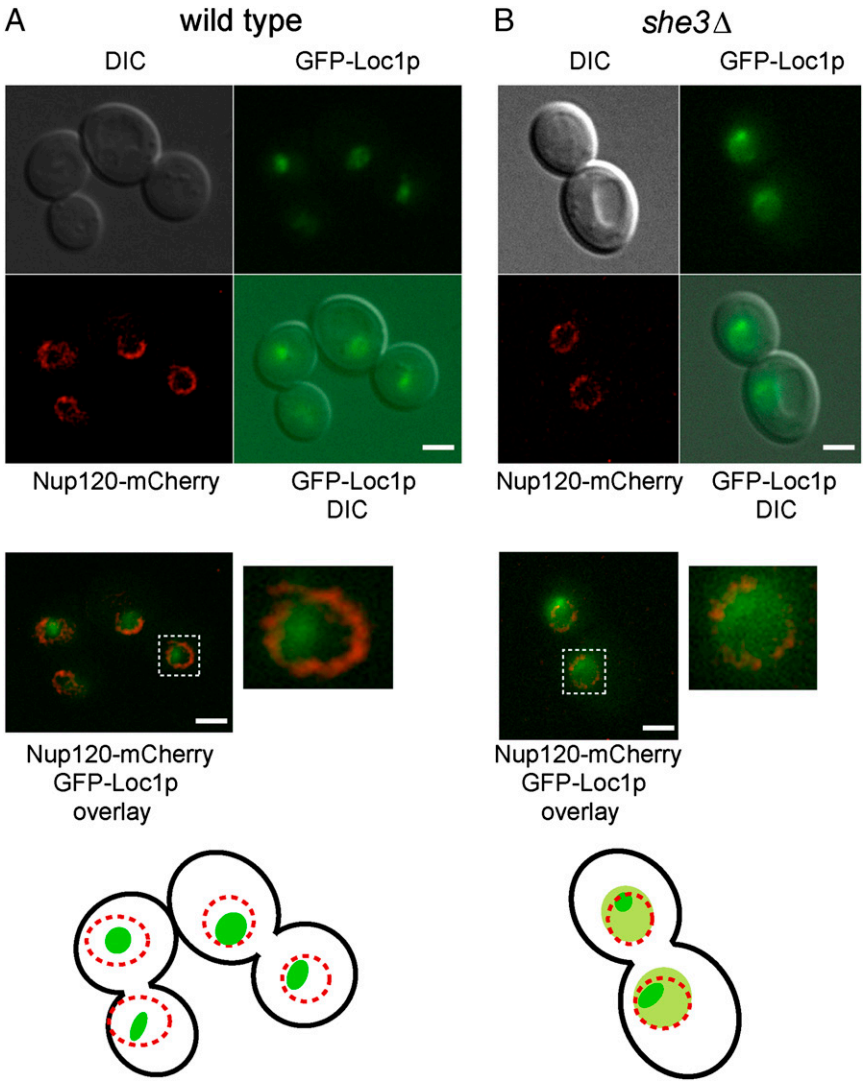

Fig. 6. (*A* and *B*) Localization of Loc1p in dividing wild-type (*A*) and *she3*Δ cells (*B*). Loc1p is visualized via GFP tag (green) and the nuclear envelope by Nup120-mCherry (red). (*A* and *B*, *Middle*) Overlays of Nup120-mCherry and GFP-Loc1p with magnifications of single cells. (Scale bars, 1 μm.) 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. 6*A*) (14). In contrast, cells that are deficient for She3p show a dispersed GFP-Loc1p staining in the nucleoplasm (Fig. 6*B*). This observation indicates that cytoplasmic She3p indeed affects Loc1p distribution in the nucle(ol)us. Occasionally, we also observed a weak cytoplasmic staining of GFP-Loc1p in *she3*Δ 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. 3*E*). 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. 3 *A* and *B* and Fig. S2*B*). 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 *puf6*Δ, 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

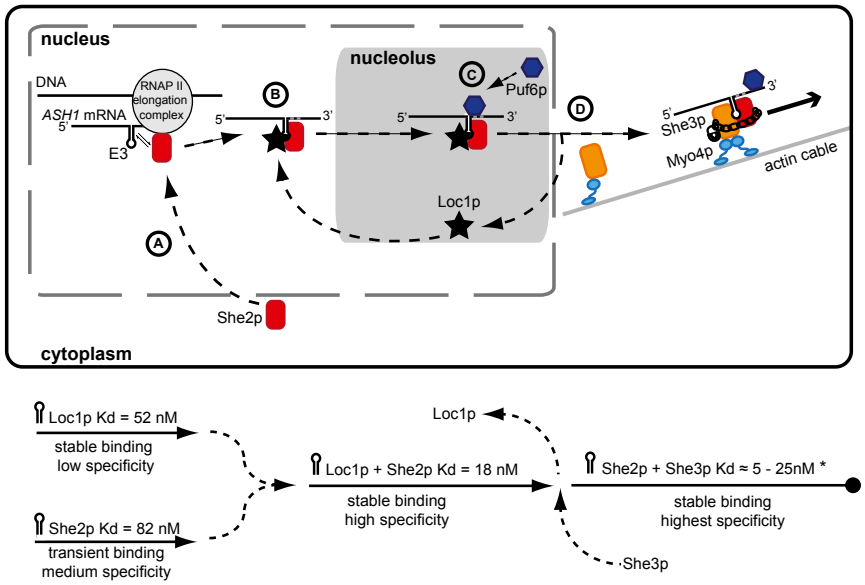

Fig. 7. Model for sequential binding and release of Loc1p to the *ASH1* mRNP. (*Upper*) She2p enters the nucleus and binds cotranscriptionally to the *ASH1* mRNA (A). Loc1p joins this subcomplex, which leads to stabilization of She2p on the *ASH1* mRNA. Loc1p contains nucleolar localization signals and 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).

His_6-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 OD_{254} to OD_{280} ratio. Proteins with an OD_{254} to OD_{280} 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 promotor sequence at the 5′ end. This fragment was used as a template for in vitro transcription (MEGAshortscript 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^{32}P]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 μL 5 nM radioactively labeled RNA, 100 μg/mL tRNA competitor, and the indicated protein concentration were mixed in HNMD buffer (20 mM Hepes, pH 7.8, 200 mM NaCl, 2 mM $MgCl_2$, 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× 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 μL 5 nM radiolabeled RNA, 100 μg/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 μL 7.5 or 10 μM protein, 4 μM 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 μL amylose resin (MBP-Loc1p), GST-Sepharose (GST-Puf6p), or Ni-NTA (She3p-H6). Binding reactions were washed four times with 200 μL and once with 50 μL 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 μL 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 μg/mL yeast tRNA competitor. Samples were applied to nitrocellulose and nylon membranes, using a Dot Blot Aparatus (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 μL 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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Supporting Information

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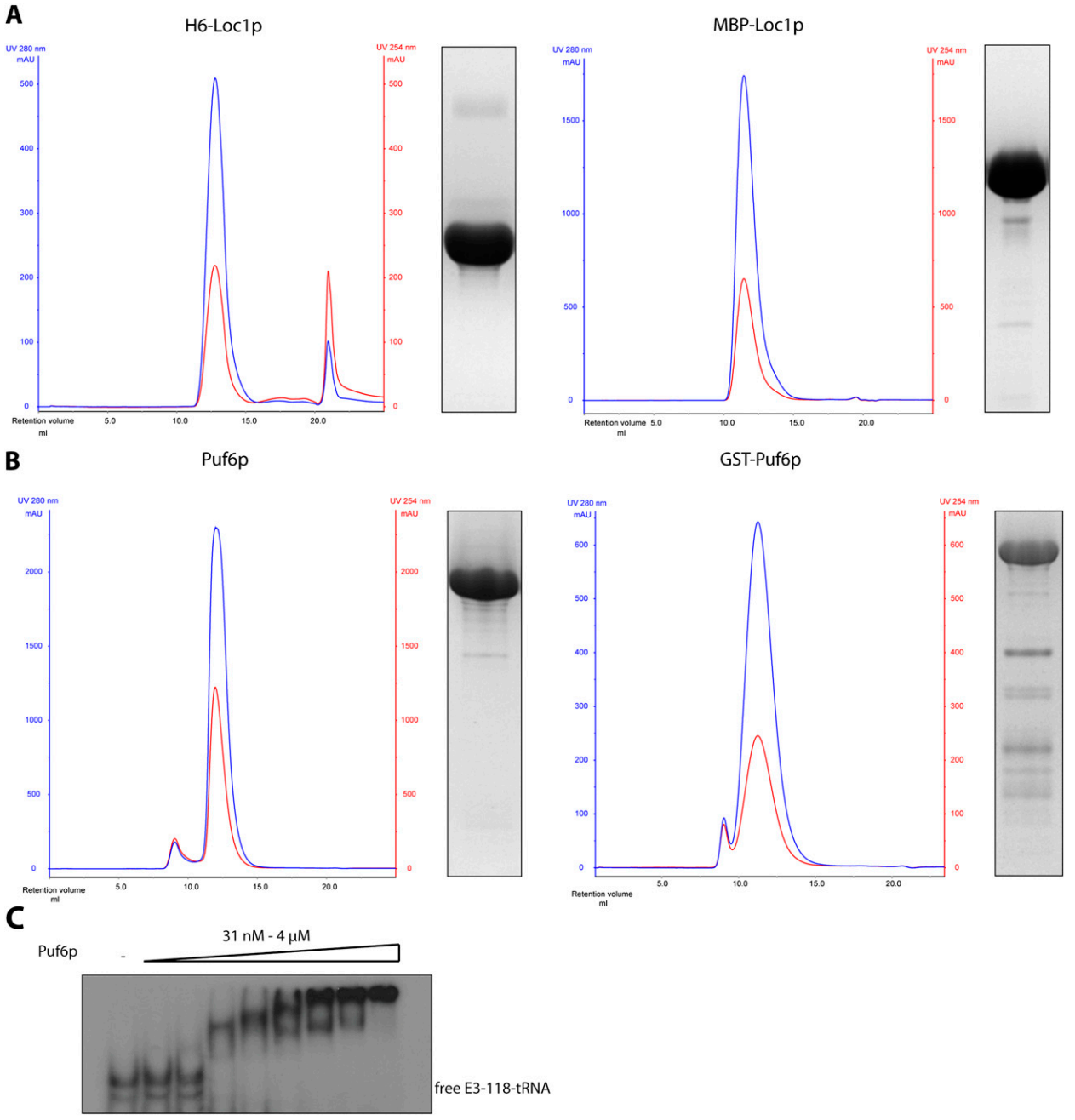

Fig. S1. Recombinantly expressed and purified proteins show high purity and are functional. (*A*) Size-exclusion chromatograms and corresponding SDS/PAGE gels stained with Coomassie blue show that His-tagged localization of asymmetric synthesis of HO 1 (*ASH1*) mRNA protein 1 (H6-Loc1p) and maltose binding protein (MBP)-Loc1p are highly pure. Observe the absorbance ratios at 254 nm (red) and 280 nm (blue) in the chromatograms, indicating that purified proteins are free of nucleic acids. Only proteins with absorbance ratios of 254/280 $\leq$ 0.6 were used for experiments. (*B*) Size-exclusion chromatograms and corresponding SDS/PAGE stained with Coomassie blue show that untagged pumilio-homology domain family protein 6 (Puf6p) is highly pure and GST-Puf6p reasonable pure. Protein preparations were free of nucleic acids. (*C*) Electrophoretic mobility-shift assay (EMSA) showing that untagged Puf6p efficiently binds to radioactively labeled *ASH1* E3-118 tRNA. This observation is consistent with previous reports (1, 2) and proves the functional activity of purified Puf6p.

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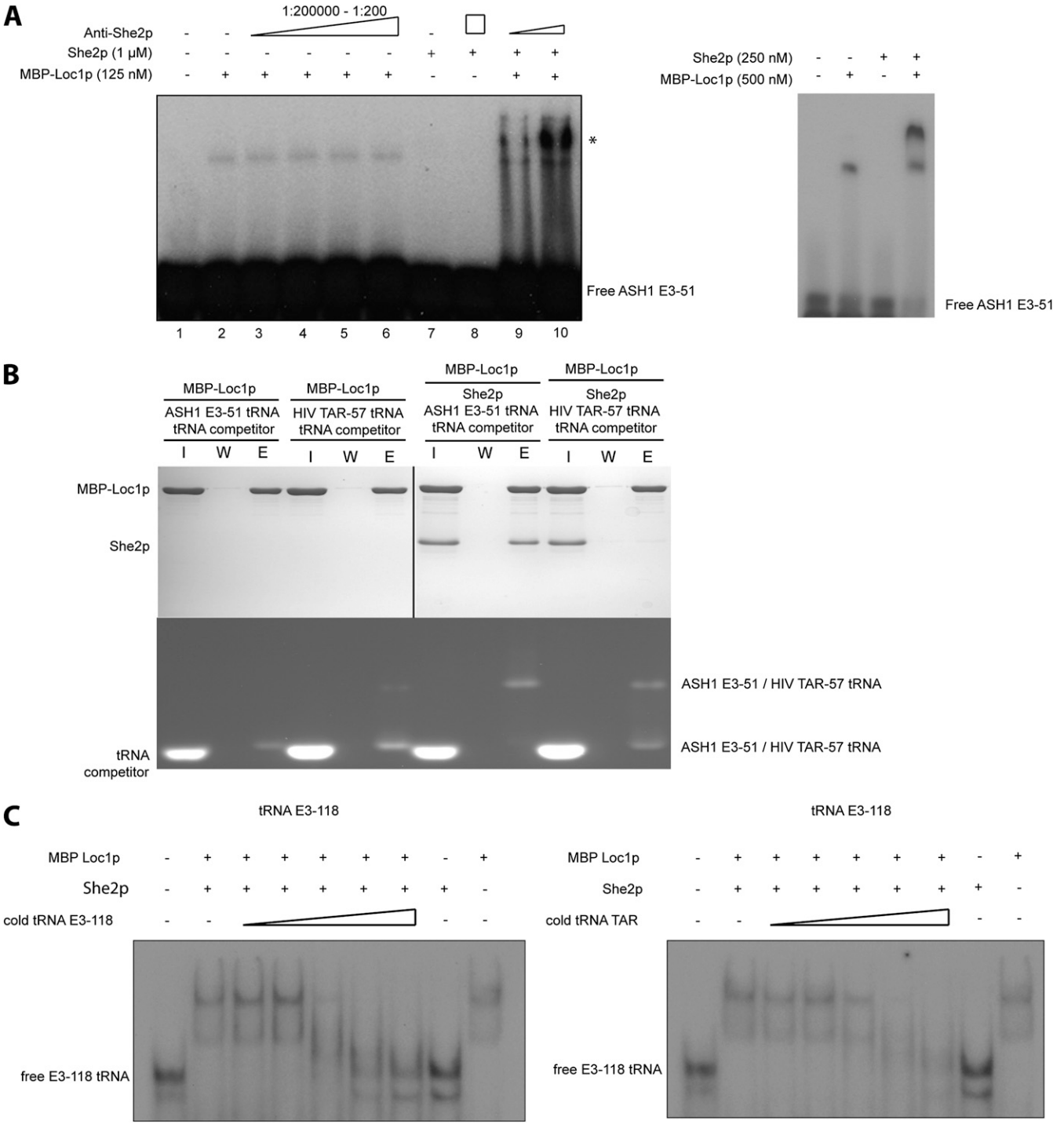

Fig. S2. EMSAs with Loc1p, Swi5p-dependent HO expression protein 2 (She2p), and *ASH1* E3 RNA. (*A*) (*Left*) Supershift EMSA with MBP-Loc1p, She2p, She2p antibody and radioactively labeled *ASH1* E3-51 RNA. Loc1p alone (lane 2) and with increasing concentrations of She2p antibody (lanes 3–6) shows one weak and constant band shift without any supershift. She2p alone at 1 μM concentration (lane 7) and in combination with She2p and 1:200 dilution of antibody (lane 8) does not yield a band shift. The combination of Loc1p, She2p, and antibody dilutions (1:200,000 and 1:20,000) leads to the formation of a supershift (lanes 9 and 10, marked with an asterisk). The supershift increases in intensity at elevated antibody concentration, indicating the presence of She2p in this complex. (*Right*) An EMSA using a 4–20% gradient gel, which resolves the different RNA–protein complexes well enough to show a higher molecular weight band for the ternary complex of MBP-Loc1p, She2p, and *ASH1* E3-51 RNA. (*B*) Pull-down experiments with MBP-Loc1p as bait and *ASH1* E3-51 tRNA, HIV TAR-57 tRNA, and She2p as prey. MBP-Loc1p alone binds *ASH1* E3-51 tRNA as well as HIV TAR-57 tRNA and thus shows no specificity in pull-down experiments. However, She2p is retained on amylose beads only in the presence of *ASH1* E3-51, indicating a preference of this ternary complex for zip-code RNA. Note that in the RNA gels stable ternary complexes are observed that migrate at a higher molecular weight, similar to the observations in Fig. 3*A* (asterisk). For further details, see legend of Fig. 3*A*. (*C*) EMSAs with excess of unlabeled cold competitor RNA show that the complex consisting of Loc1p and She2p has a preference for RNAs with zip-code elements. For competition experiments we used 1-, 2-, 5-, 25-, and 50-fold excess of cold competitor RNA over labeled E3 zip-code RNA. Because these competition experiments gave only a rough indication, more quantitative approaches were used to assess complex specificity (Fig. 3 *D–F*).

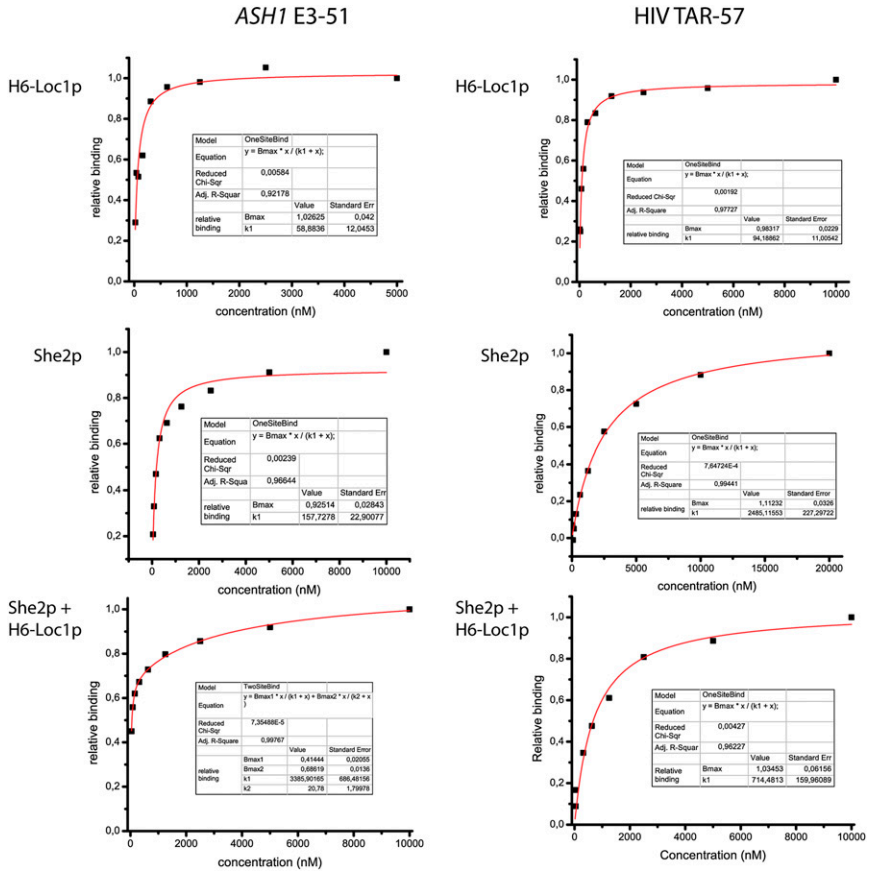

Fig. S3. Representative plots of binding curves from filter-binding assays for the determination of equilibrium-dissociation constants (K_d). Signal intensities of each black box are plotted over the respective protein concentration. K_ds were determined using either one-site binding or two-site binding fitting algorithms with the program Origin 8.6. Shown are representative fitting curves from individual experiments, with the respective fitting algorithms indicated in *Insets*. In binding experiments with individual proteins and RNA a one-site binding algorithm was used. However, the ternary complex formed by Loc1p, She2p, and E3 zip-code RNA requires a higher-order fitting function (two-site binding), indicating the presence of two RNA-interaction sites in this complex. This assumption is supported by cross-linking experiments showing that Loc1p as well as She2p contacts the *ASH1* E3 RNA upon complex formation (Fig. 2*A*). In contrast to the *ASH1* E3 zip-code RNA, data fitting of HIV-TAR binding to Loc1p and She2p demanded only a one-site binding algorithm, suggesting the absence of a ternary complex. This interpretation is consistent with those of pull-down assays (Fig. 3*B* and Fig. S2*B*) and cross-linking experiments (Fig. 3*C*).

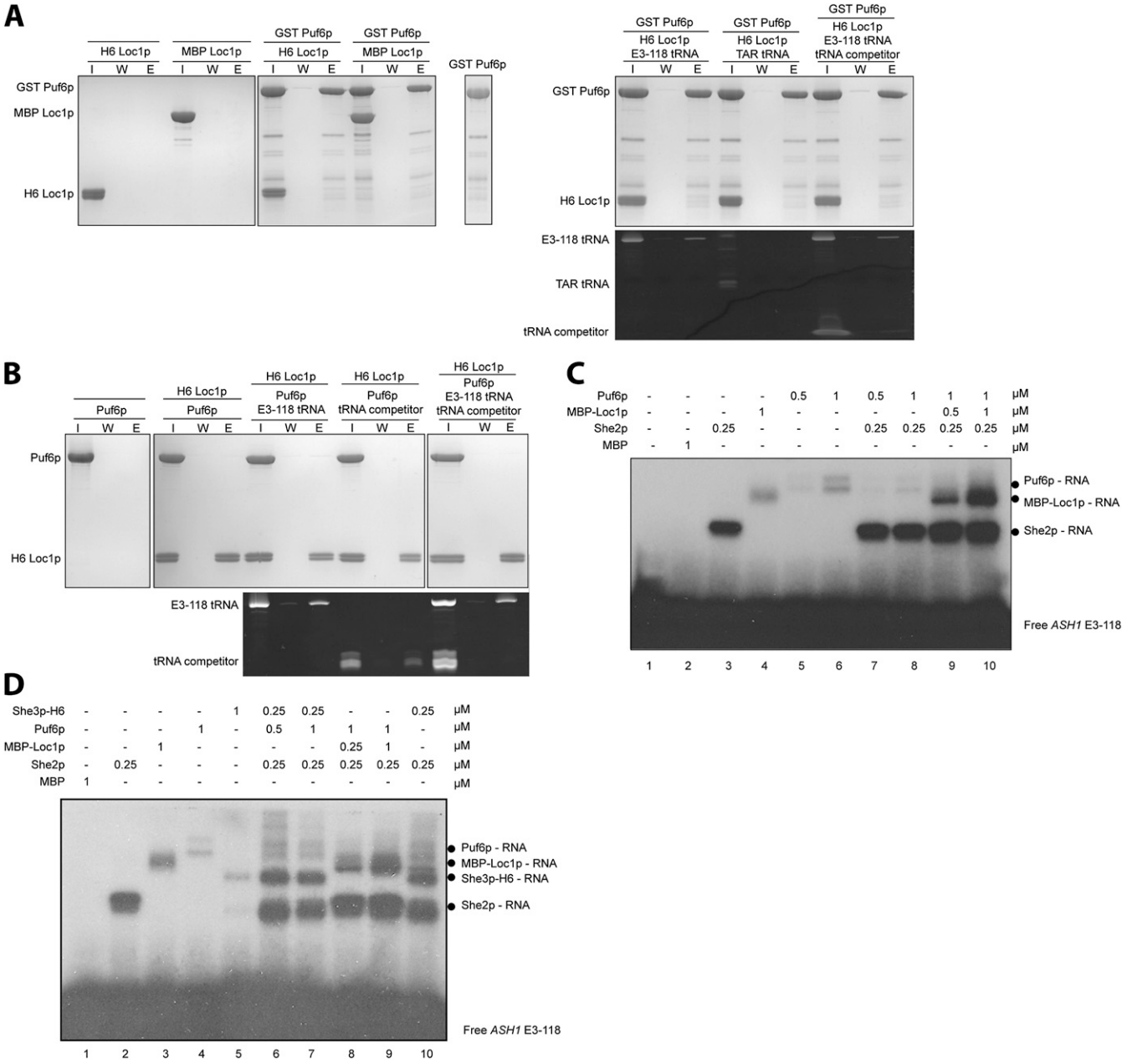

Fig. S4. Puf6p binds the *ASH1* E3 zip-code element but fails to interact with Loc1p in the absence or the presence of the *ASH1* E3 zip-code element. (*A*) Pull-down experiments with GST-Puf6p as bait and MBP-Loc1p or H6-Loc1p as prey in the presence or the absence of specific and unspecific RNA. No interaction between Loc1p and Puf6p is detected. In these experiments, more physiological pull-down conditions were applied than in Fig. 1. A total of 250 nM of each protein in 1.5 mL reaction volume was used with 50 µL of respective resin material. (*B*) Pull-down experiments with H6-Loc1p as bait and untagged Puf6p in the presence or the absence of *ASH1* E3 zip-code RNA as prey. No direct or RNA-mediated indirect interaction is observed between Loc1p and Puf6p either in the absence of RNA or in the presence of specific *ASH1* E3-118 tRNA or unspecific tRNAs. This further confirms the lack of a stable interaction between both molecules. These experiments were performed at the concentrations and volumes described in *A*. (*C*) Cross-linking experiment showing that Puf6p is not recruited to the *ASH1* E3 zip-code element by She2p alone (lanes 7 and 8) or by the She2p-Loc1p complex (lanes 9 and 10). Experimental conditions are identical to those in Figs. 2*A* and 4*C*. (*D*) Same cross-link as in Fig. 4*C* with prolonged exposure.

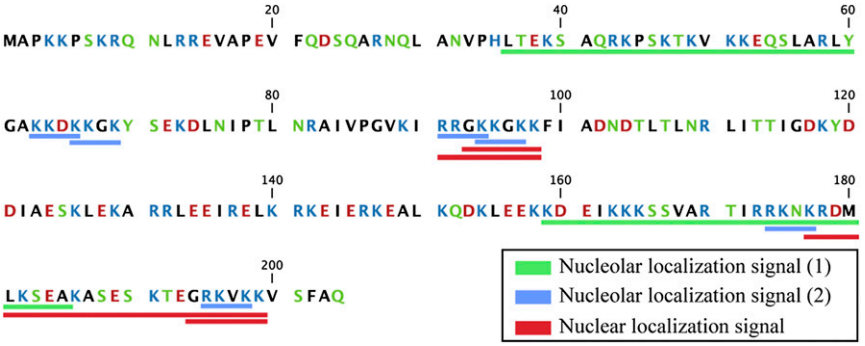

Fig. S5. Protein sequence of Loc1p and its predicted nucleolar and nuclear localization signals. Amino acids underlined in green have an extended nucleolar localization signal, as predicted by the program NoLS (1). Because NoLS has been designed to identify mammalian nucleolar localization signals, we also searched for more general nucleolar localization signals with the motif (K/R)(K/R)X(K/R) (blue) (2). Nuclear localization signals are depicted in red.

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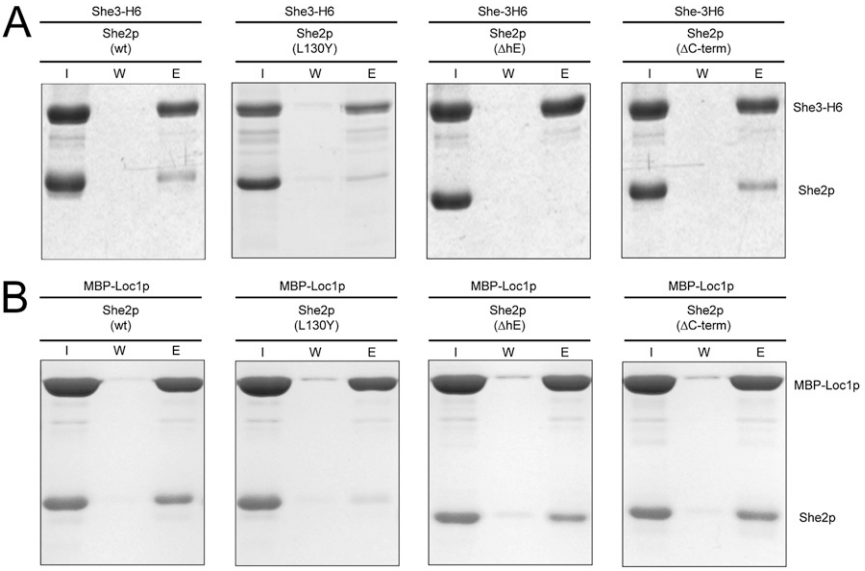

Fig. S6. Different She2p mutants that affect She3p binding also impair Loc1p binding. (*A*) In pull-down experiments with Ni-Sepharose resin and She3p-H6 the proteins She2p (WT) and She2p (ΔC-term) were efficiently copurified. In contrast, She3p does not interact with She2p (ΔhE) and has a weakened interaction with She2p (L130Y). (*B*) Amylose pull-down experiments with MBP-tagged Loc1p and She2p mutants. Whereas Loc1p showed reduced affinity for She2p (L130Y) and might have a slightly impaired interaction with She2p (ΔhE), wild-type–like binding was observed with She2p (ΔC-term). Together with competition experiments, these findings suggest that Loc1p and She3p use an overlapping but nonidentical binding surface on She2p.

Table S1. Plasmids

Plasmid name	Description	Source	PCR primers	Restriction sites
P01	pGEX-6p1 *SHE2*	(1)		
P06	pGEX-6p1 *she2* Δ C-term	(2)		
P08	pGEX-6p1 *she2* L130Y	(2)		
P14	pGEX-6p1 *she2* ΔhelixE	(2)		
P59	pET-28a *LOC1*	This study	MMO 103, MMO 104	BamHI, XhoI
P65	pET-M43 *LOC1*	This study	MMO 103, MMO 104	BamHi, XhoI
P94	pGEX-6p1 *PUF6*	(2)		
RHP 27	pFastBacDual-*SHE2*/*SHE3*-His6	(2)		
RHP 89	pBSMrna-E3-118	(2)		
RHP 90	pBSMrna-E3-51	This study	RHO 126, RHO 127	EagI, SacII
RHP 99	pBSMrna-HIV1-TAR	This study	RHO 134, RHO 135	EagI, SacII
ANP 11	pRS405-ASH1	This study	ANO 18, ANO 19	XhoI, BamHI
C3319	YEplac181-ASH1	(3)		
	pRS 314-Nup120-mCherry	(4)		

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2. Müller M, et al. (2011) A cytoplasmic complex mediates specific mRNA recognition and localization in yeast. *PLoS Biol* 9(4):e1000611.
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4. Skruzný M, et al. (2009) An endoribonuclease functionally linked to perinuclear mRNP quality control associates with the nuclear pore complexes. *PLoS Biol* 7(1):e8.

Table S2. DNA oligonucleotides

Name	Primer sequence, 5′–3′
MMO 103	AAAAGGATCCATGGCACCAAAGAAACCTTC
MMO 104	AAAACTCGAGCTATTGAGCAAATGAGAC
RHO 126	AAACGGCCGATGGATAACTGAATCTCTTTCAAC
RHO 127	AAACCGCGGCCAATTGTTTCGTGATAATGTCTC
RHO 134	AAACGGCCGGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCTGGCCGCGGAAA
RHO 135	TTTCCGCGGCCAGGTTCCCTAGTTAGCCAGAGAGCTCCCAGGCTCAGATCTGGTCTAACCAGAGAGACCCGGCCGTTT
ANO 18	AAAAAACTCGAGTCCTGTCCTATCCTTATTACGTTCA
ANO 19	AAAAAAGGATCCTGCGCAGGAGAAGTTATTAGAATGAT

Table S3. RNA sequences

RNA	Base relative to start AUG	Sequence, 5′–3′	Produced by
ASH1 E3-118	1,750–1,867	GAGACAGUAGAGAAUUGAUAC AUGGAUAACUGAAUCUCUUUCA ACUAAUAAGAGACAUUAU CACGAAACAA**UUGU**ACAU UUCUCUCC**UUGU**CUGUGCUA AAUAAACUACAAAUAAAAA	In vitro transcription
ASH1 E3-118 tRNA	1,750–1,867	GGCUACGUAGCUCAGUU GGUUAGAGCAGCGGCCGGAG ACAGUAGAGAAUUGA UACAUGGAUAACUGAAUCUC UUUCAACUAAUAAGAGACAUUAUCA CGAAACAAUUGUACAUUUC UCUCCUUGUCUGUGCUAA AUAAACUACAAAUAAAAAUG GCCGCGGGUCACAGGUUCGA AUCCCGUCGUAGCCACCA	Expression in *Escherichia coli*
ASH1 E3-51 tRNA	1,771–1,821	GGCUACGUAGCUCAGUUG GUUAGAGCAGCGGCCG AUGGAUAACUGAAUCUC UUUCAACUAAUAAGAGAC AUUAUCACGAAACAAUU GGCCGCGGGUCACAGGU UCGAAUCCCGUCGUAGCCACCA	Expression in *E. coli*
ASH1 E3-51	1,771–1,821	AUGGAUAACUGAAUCUC UUUCAACUAAUAAGAGACAU UAUCACGAAACAAU	Chemical synthesis (Dharmacon)
HIV TAR-57 tRNA		GGCUACGUAGCUCAGUUGG UUAGAGCAGCGGCCGGG UCUCUCUGGUUAGACCAGAUC UGAGCCUGGGAGCUCUCUGG CUAACUAGGGAACCUGGCC GCGGGUCACAGGUUCGAA UCCCGUCGUAGCCACCA	Expression in *E. coli*

Underlined sequences correspond to the E3-118 or TAR sequences in tRNA fusion constructs. Sequences in boldface type indicate the Puf6p binding sites within the E3-118 zip-code element.

Table S4. Yeast strains used in this study

Yeast strain	Genotype
RJY4358	LOC1-GFP::HIS3MX6 YEplac181-ASH1 pRS314-Nup120-mCherry
RJY4359	LOC1-GFP::HIS3MX6 she3Δ::URA3 YEplac181-ASH1 pRS314-Nup120-mCherry

3.2 Original Publication 2

"*ASH1* mRNP-core factors form stable complexes in absence of cargo RNA at physiological conditions."

In budding yeast, a synergistic complex formed by She2p, She3p, Myo4p, and the *ASH1* mRNA is required for the localization of this transcript to the daughter cell tip. The molecular motor protein Myo4p drives the processive movement along actin cables. However, Myo4p is monomer in solution and thus unprocessive on its own. The formation of the synergistic complex induces dimerization of the motor, thereby enabling processive movement. Recently, two studies were published that used *in vitro* motility assays of reconstituted *ASH1* complexes to answer the question, which components are necessary for complex motility. The outcomes of the studies, however, were contradictory on the requirement of cargo RNA for complex assembly and processive movement. This publication addressed the dispute by measuring complex stability in absence of RNA. By showing that Myo4p dimerization *per se* does not require *ASH1* mRNA the study confirmed that complex assembly and processive movement takes place in absence of RNA.

For the reconstitution of complexes, I expressed and purified recombinant She2p, She3p and Myo4p variants. I performed pull-down experiments and proved that those three factors interact stably. Furthermore, I performed pull-down experiments at elevated temperatures (RT and 30 °C) and salt concentration of 140 mM sodium chloride that closely resemble physiological conditions within the cell. In order to proof whether the full complex remains intact after much longer time, I performed size-exclusion chromatography followed by static light scattering at a reduced flow rate. Complementing experiments were performed by Franziska Edelmann from the Niessing group.

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ASH1 mRNP-core factors form stable complexes in absence of cargo RNA at physiological conditions

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Keywords: *ASH1* mRNA, budding yeast, *in vitro* reconstitution, myosin, Myo4p, mRNP, macromolecular assembly, RNA localization, She3p, She2p

Asymmetric *ASH1* mRNA transport during mitosis of budding yeast constitutes one of the best-studied examples of mRNA localization. Recently, 2 studies used *in vitro* motility assays to prove that motile *ASH1* mRNA-transport complexes can be reconstituted entirely from recombinant factors. Both studies, however, differed in their conclusions on whether cargo RNA itself is required for particle assembly and thus activation of directional transport. Here we provide direct evidence that stable complexes do assemble in absence of RNA at physiologic conditions and even at ionic strengths above cellular levels. These results directly confirm the previous notion that the *ASH1* transport machinery is not activated by the cargo RNA itself, but rather through protein-protein interactions.

Introduction

Directional transport of mRNAs by motor-containing particles and subsequent local translation is a common control mechanism of gene expression (reviewed in ref. [1,2]). Due to the fact that in most organisms such transport complexes consist of several dozens of factors, the molecular principles of complex assembly and regulation are not well understood. However, mRNA-localization complexes in the bakers yeast *Saccharomyces cerevisiae* consist of only about half a dozen components. For this very reason, yeast has emerged as a particularly well-suited model system to study mechanistic principles of mRNA transport (reviewed in ref. [3,4]).

During mitosis, budding yeast undergoes unequal cell division, resulting in a larger mother and a smaller daughter cell. While in the mother cell the so-called HO endonuclease mediates genomic recombination in the MAT gene locus, this event does not occur in the daughter cell (reviewed in ref. [5]). The result is a mother cell specific conversion of the mating type from a to α or *vice versa*. Because the daughter cell does not undergo mating-type switching, both progenies adopt different cell fates.

Mating-type switching is inactive in the daughter cell due to the specific expression of Ash1p, the inhibitor of HO endonuclease.[6,7] This local inhibition is achieved by the selective, motor-dependent transport of the *ASH1* mRNA into the daughter cell and its subsequent translation of Ash1p at the bud tip.[6,8,9] In addition to *ASH1* mRNA, about 30 other transcripts are transported by this complex into the daughter cell.[10-14]

The motile SHE-transport complex consists of the RNA-binding proteins She2p and She3p, as well as the type-V myosin Myo4p.[8,15,16] She2p binds to the *ASH1* mRNA already co-transcriptionally[17,18] and forms a nuclear complex.[19-22] After nuclear export, it is joined by the cytoplasmic complex of She3p and Myo4p to form the motile transport complex.[15,16,20,23] In this mature complex, She2p and She3p interact to mediate the specific recognition of localizing RNAs.[17]

Recent studies dissected the exact stoichiometric ratios of the core factors in the *ASH1* mRNP. Type-V myosins need to form dimers in order to move processively along actin filaments. Therefore it was surprising that Myo4p alone was found to be a monomer and thus is non-processive.[24-27] She3p is a dimer that constitutively interacts with a single Myo4p motor.[28] She2p forms a tetramer that contains 2 RNA binding sites.[28-30] This She2p-tetramer formation is required for *in vitro* complex assembly,[30] *in vitro* motility,[31,32] and *ASH1* mRNA localization *in vivo*.[30] More recently, determination of the exact stoichiometric ratio of the core factors within the mature *ASH1* mRNP revealed that one She2p tetramer binds 2 Myo4p-She3p complexes. Thus the mature transport complex contains 2 Myo4p molecules and indeed shows processive movement.[28] While a study from Heym and colleagues came to the conclusion that mRNA itself is dispensable for the activation of the myosin motor,[28]

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a second study by Sladewski and colleagues provided evidence that mRNA may be essential for motility.[33]

A number of experimental differences in the single-particle motility assays have been discussed to be potentially responsible for these seemingly contradicting results.[28,34,35] These technical differences include the choice of actin from different organisms, the use of actin bundles or single filaments, and reconstitution with wild-type She2p or a quadruple cysteine-mutant version of the protein. Another major difference is that Heym assembled *ASH1* mRNPs at the correct stoichiometric ratios (2 × RNA, 4 × She2p, 4 × She3p, 2 × Myo4p),[28] whereas Sladewski assembled complexes with significant deviation of stoichiometric ratios from the correct assembly (ratio of She3p to Myo4p of 1:1 instead of 2:1 and a ratio of RNA to Myo4p with a 20- to 280-fold excess of the motor).[33]

In a recent commentary, Sladewski and colleagues now offer a new explanation.[35] The authors suggest that particle movement in absence of RNA, as reported by Heym and colleagues,[28] must have been artificially induced by protein-protein interactions at low ionic-strength (50 mM KCl) conditions. The authors further suggest that ". . . a stable Myo4p-She3p-She2p complex does not form at 140 mM KCl *in vitro* and thus Myo4p would be non-motile in the cell without cargo."[35] Unfortunately, no reference is given to substantiate this statement and to our knowledge no experiment has been published that directly assesses the SHE-complex assembly at different salt concentrations. The aim of this study is to test whether zip-code RNA is indeed required to assemble the Myo4p-containing transport complex at physiologic salt concentrations and temperature.

Results and Discussion

Previous studies already showed that a stable complex of She2p, She3p, and Myo4p is formed in presence of *ASH1* mRNA *in vitro* at high ionic strength (200 mM sodium chloride) (**Fig. 2C** in ref. [28]). To answer the question whether such a complex would also assemble in absence of RNA, we first performed pull-down experiments with She2p, She3p, and the C-terminal part of Myo4p (Myo4p-C) (**Fig. 1**). At 4 °C, we observed a robust co-precipitation of She2p and She3p with GST-Myo4p-C at the physiological salt concentration of 140 mM sodium chloride (**Fig. 1A**). Stable interactions were also observed in pull-down experiments when the ionic strength was increased to 200 mM sodium chloride (**Fig. 1A**). Even an experimental setup of 140 mM sodium chloride and the optimal yeast-growth temperature of 30 °C leads to a stable co-precipitation of the proteins (**Fig. 1E**). No differences could be detected when performing the pull-down experiments at room temperature (data not shown). In order to test whether this complex depends on the previously described specific interactions, we included in our pull-down experiments a She2p mutant (Δhelix E) that is unable to interact with She3p.[30] Independent of ionic strength and incubation temperature, this mutant failed to interact with GST-Myo4p-C and She3p (**Fig. 1B and 1E**), indicating that the observed complex formation (**Fig. 1A and 1E**) is indeed specific. Together these results clearly demonstrate that Myo4p, She3p, and She2p form a stable and specific complex in absence of RNA at experimental setups closely resembling physiologic conditions.

These observations are also in line with size-exclusion chromatography experiments. At 200 mM sodium chloride the RNA-free complex of She2p, She3p and Myo4p-C is stable enough to co-elute after about 24 minutes (i.e. 12 ml) of chromatography (**Fig. 2**). This ternary complex does not form with the above-described She2pΔhelix E mutant, again confirming that this interaction is specific.

In order to evaluate whether full complexes would still remain intact even after much longer time, we performed static light-scattering measurements after size-exclusion chromatography at reduced flow rates. In this experiment a main complex peak eluted at 135–140 minutes of chromatography (**Fig. 3**). The measured molecular mass shows great differences between both sides of this peak (red line in **Fig. 3**) and has a median measured molecular mass of 248 kDa (**Fig. 3**). The slope with the lower elution volume (to the left) shows a molecular mass of 420 kDa indicating the presence of the fully assembled complex with a calculated molecular weight of 434 kDa. Thus, these data indicate that the majority of particles have disassembled, but that a subfraction of the mature complex still remains assembled after more than 2 hours of size-exclusion chromatography.

Together our data demonstrate that the interactions suggested by Sladewski and colleagues to be an artifact of low ionic-strength conditions are robust binding events, even at salt concentrations above the physiological ionic strength and at 30 °C. These findings are also in line with previously published work, which demonstrated that the key interaction for complex assembly, She2p-She3p, is quite insensitive to high salt concentrations. For instance, at 200 mM sodium chloride, defined RNA-free complexes of She2p and She3p co-elute as a single peak in size-exclusion chromatography (**Figs. 2C, S3B** in ref. [17]; see also pull-down in **Figure 2A** of the same reference). Again, this complex does not form in the She2p mutant (Δhelix E) that is deficient for She3p interaction (**Table 1** and **Figure 6A-C** in ref. [17]) and no processive movement is observed with these components at low ionic strength (**Fig. 4F** in ref. [28]).

The same insensitivity to high salt concentrations is true for the interaction of GST-Myo4p-C and full-length She3p. Here a robust co-precipitation of both proteins is observed in pull-down experiments at 4 °C using 140 and 200 mM sodium chloride (**Fig. 1C**), or even at 30 °C using 140 mM sodium chloride (data not shown). The stability of this protein-protein interaction was already reported by Heuck and colleagues showing that Myo4p-C together with the N-terminal part of She3p are pulled down with each other at salt concentrations up to 1 M sodium chloride (**Fig. 1D** in ref. [36]).

It should also be pointed out that motilities observed in the study by Heym and colleagues show the same velocities and run lengths in the presence or absence of RNA, or even with complexes that contain a She2p mutant deficient for RNA bunding.[28] In summary, we consider the robustness of the She2p-She3p-Myo4p interaction as well as the same motile properties of both, RNA-containing versus RNA-lacking particles, as a valid indication for a well-defined complex activation via interactions

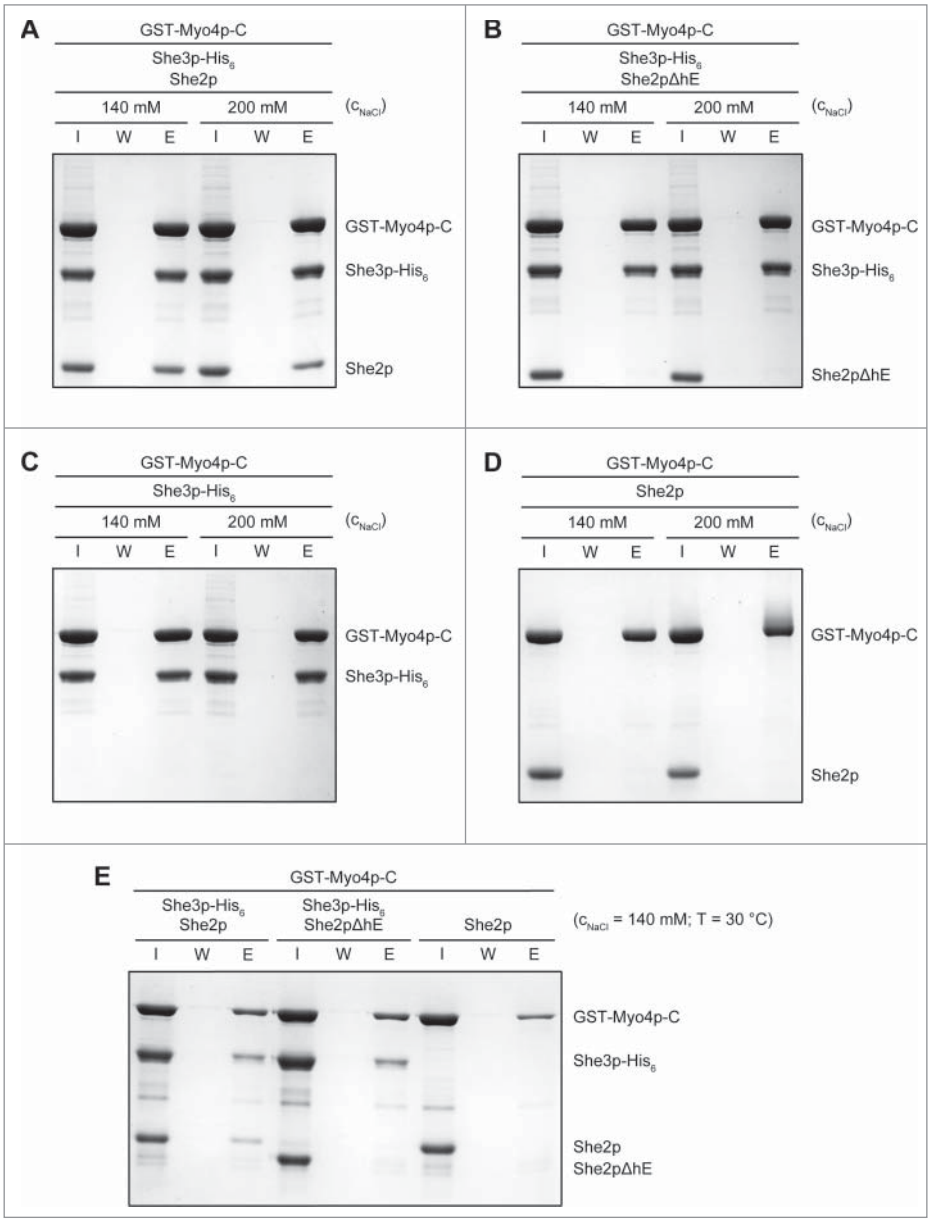

Figure 1. GST pull-down experiments were performed with Glutathione FF-sepharose and GST-Myo4p-C as bait. (**A**) In absence of RNA and at an ionic strength resembling the physiological level (140 mM sodium chloride), She2p and She3p efficiently co-elute as one complex. The same is true for even higher salt concentrations (200 mM sodium chloride). (**B**) In contrast, the She2p (Δhelix E) mutant failed to co-elute with She3p and Myo4p-C at either salt concentration. This mutant was previously shown to be unable to interact with She3p[17] and incapable of forming processive complexes with the other core factors in single-particle motility assays.[28] (**C**) A stable complex of GST-Myo4p-C and full-length She3p is also formed in the absence of She2p or the She2p mutant at both salt concentrations. Together these results clearly demonstrate the robustness of this ternary protein complex in absence of RNA. (**D**) In a control experiment, She2p failed to interact with GST-Myo4p-C in absence of She3p. (**E**) At 30 °C and 140 mM sodium chloride, She2p, but not She2p (Δhelix E), co-elutes with She3p and GST-Myo4p-C. Thus, these 3 factors form a stable complex at near-physiologic conditions, suggesting that their interaction is stable enough to mediate the activation of motility in absence of RNA.

between She2p and She3p, for which RNA-binding is dispensable.

An RNA-free assembly of the SHE-transport machinery is unlikely to occur *in vivo* and very difficult to generate in mutant yeast strains. For this very reason, *in vitro* reconstitution experiments with recombinant factors proved to be essential to understand basic mechanisms of complex assembly and motor activation.

We would like to add that when comparing size-exclusion chromatography profiles in presence[17] or absence of RNA (this study), in our hands the RNA-containing particles appear to show higher stability. It indicates that RNA binding further stabilizes the complex. For understanding the physiological meaning of this observation, it has to be considered that *ASH1* particles transport their cargo RNA in less than 2 minutes into the daughter cell.[37–39] Thus, the additional RNA-mediated stabilization of particles that are already stable over several minutes in absence of RNA (**Figs. 1–3**) seems rather non-essential for transport *per se*. It is of course possible that binding of localizing RNA has a positive impact on motility *in vivo*. However, to us it seems rather likely that RNA-mediated stabilization might play a more prominent role in processes with a longer time scale, for instance during particle anchoring at the bud tip. In addition it is possible that RNA binding helps fulfilling alternative functions, such as supporting translational repression by the formation of large, inaccessible particles.[34]

Materials and Methods

Protein expression and purification: The C-terminal cargo-binding region of Myo4p (amino acids 923–1471) was expressed as N-terminal His_6-fusion and GST-fusion protein in *E.coli* BL21 (DE3)Star. Cells were sonicated at 4 °C in lysis buffer (10 mM Hepes/NaOH pH 7.5; 500 mM NaCl; 1 mM EDTA; 15 mM Imidazol; 1 mM DTT). After centrifugation the cleared supernatant was applied to a His FF column (GE Healthcare). The protein was further purified using ion exchange and size-exclusion chromatography as previously described.[25] She2p, She2p (Δhelix E), and She3p were expressed and purified as previously described.[17,29,30] The absence of RNA contaminations was confirmed by measuring the ratio of OD 260/280.[40]

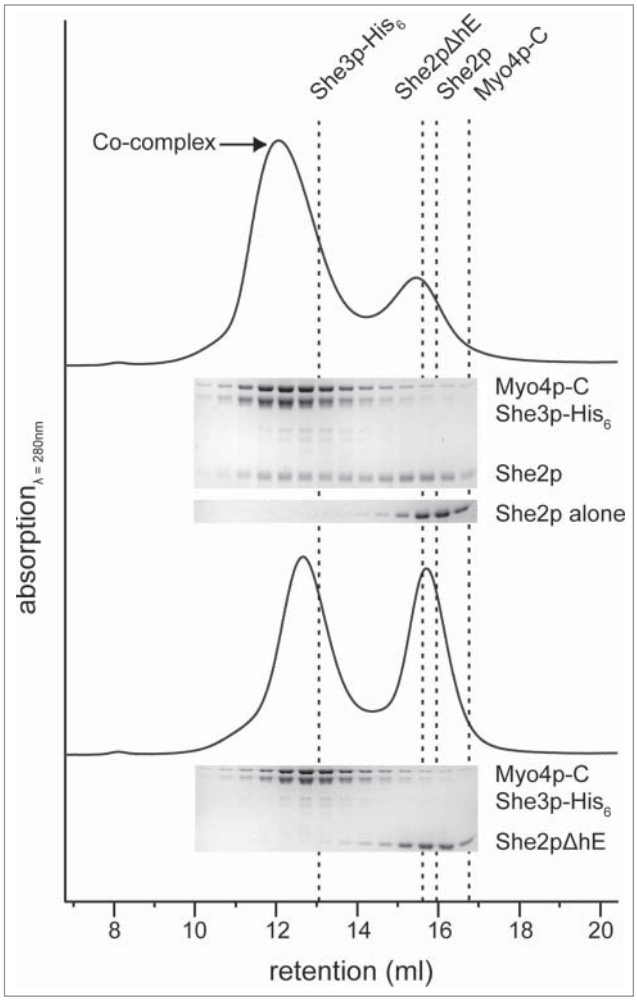

Figure 2. Size-exclusion chromatography of co-complexes with She2p, She3p, and Myo4p-C. Whereas wild-type She2p forms a stable complex with She3p and Myo4p that co-elutes from a Superose 6 column (upper part), the She3p interaction-deficient She2p (Δhelix E) mutant fails to do so (lower part). These findings are in line with previous results (**Fig. 1**) and further confirm that this protein complex efficiently forms in absence of RNA. Co-elution of the RNA-free complex after about 24 minutes (12 ml) of chromatography suggests that complexes are stable enough to mediate bud-tip localization within 2 minutes. Shown are elution profiles from size-exclusion chromatography and denaturing PAGE from corresponding elution fractions. As control also She2p alone was applied to the chromatography (upper part; gel below the PAGE of complex elution). Dashed lines indicate the peak maxima of individual proteins.

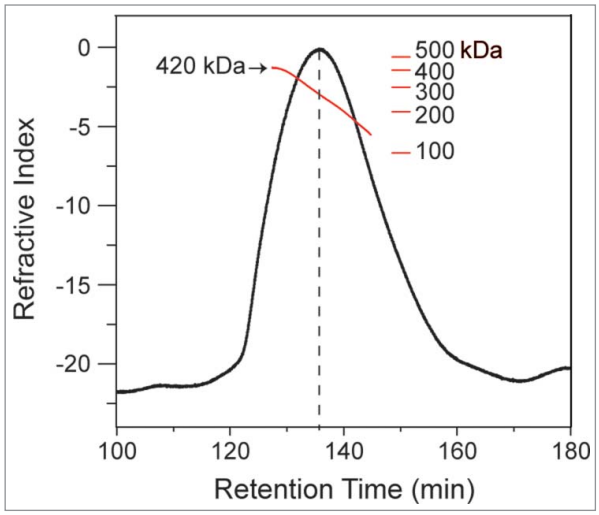

Figure 3. Static-light scattering experiments to determine the molecular mass of complexes. In absence of RNA, a portion of She2p, She3p, and Myo4p-C remain bound to each other as stoichiometric complexes even after 2 hours of chromatography. The determined median molecular weight of the complex in the peak is 248 kDa. However, the molecular mass distribution (red) on the left side shows a molecular weight of 420 kDa, indicating that a portion of She2p, She3p, and Myo4p-C remain bound to each other as stoichiomentric complexes. The expected molecular weight of the assembled complex is 434 kDa. As control, the molecular mass of the complex formed by She3p and Myo4p was analyzed. The measured molecular weight of 154 kDa (not shown), is in agreement with the calculated molecular weight of 160 kDa (2 × She3p and 1 × Myo4p).

In vitro pull-down experiments: Protein samples were mixed in their correct stoichiometric ratios, using 10 μM She2p wt/Δhelix E, 10 μM She3p-His_6 and 5 μM GST-Myo4-C in a final volume of 100 μl pull-down buffer (20 mM Hepes pH 7.8, 140 mM or 200 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT). After centrifugation for 10 min, 16100 × g, 4 °C, 95 μl of the supernatant were incubated with 45 μl Glutathione Sepharose beads (GE Healthcare) for 30 min at 4 °C on an overhead shaker. Binding reactions were washed 4 times with 200 μl pull-down buffer and each time spun down at 400 × g, 4 °C for 1 min. The last washing step was performed with 41 μl pull-down buffer. Bound proteins were eluted with 41 μl pull-down buffer, supplemented with 10 mM glutathione (reduced). In pull-down experiments at room temperature and at 30 °C all experimental steps were performed at the indicated elevated temperature. On SDS-PAGE, 10 % of the input, 20 % of the last wash step, and 20 % of the elution were analyzed by Coomassie blue staining.

Size-Exclusion Chromatography was essentially performed as described before,[17] using a Superose 6 10/300 GL column (GE Healthcare) in HNMD buffer (20 mM Hepes (pH 7.8), 200 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT) and a flow-rate of 0.5 ml/min at 4 °C.

Static Light-Scattering experiments were performed after size-exclusion chromatography with a Superose 6 10/300 GL (GE Healthcare) and a flow rate of 0.1 ml/min at 4 °C, using a 270 Dual Detector and a VE3580 RI Detector from Malvern. System calibration was performed with 100 μl BSA at a concentration of 4 mg/ml. Sample concentrations were in the range of 1.9 to 3.1 mg/ml in a total volume of 100 μl. Complexes were assembled at stoichiometric ratios and applied to size-exclusion chromatography. For data analysis the Malvern OmniSEC 5.02 software was used. For molecular weight determination the average value of 2 independent experiments was used.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

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3.3 Unpublished Manuscript

"A conserved, intrinsically disordered RNA-binding motif operates as RNA-folding catalyst."

In budding yeast, Loc1p is a nucle(ol)ar RNA-binding protein that is, on the one hand, required for *ASH1*-mRNA localization and, on the other hand, involved in the biogenesis of the large ribosomal subunit. This manuscript proves that Loc1p is an intrinsically unstructured protein with powerful RNA-annealing activity. Furthermore, Loc1p contains eleven motifs that are able to bind, anneal, and stabilize dsRNA. However, the combination of several motifs dramatically increases annealing activity. NMR analysis of a single motif revealed its structural flexibility and that it gains rigidity in complex with double-stranded RNA. A proteome-wide search of proteins with multiple motifs shows an enrichment of factors involved in ribosome biogenesis and other RNA-metabolic processes. Furthermore, also *Schizosaccharomyces pombe*, and *Escherichia coli* show a similar enrichment of factors with multiple motifs indicating a conserved function. Both, the annealing activity and the stabilization of dsRNA make it tempting to speculate that such a motif might have been involved in the co-evolution of ribozymes.

For the biochemical characterization I purified Loc1p, Hfq, Imp4p and variants thereof. I performed fluorescence-based EMSA experiments showing that Loc1p binds indiscriminately to single-stranded as well as double-stranded RNA or DNA and hybrids thereof. I implemented both fluorescence-based and radioactive chaperone assays and discovered the ATP-independent annealing activity of Loc1p that exceeds the activity of well-studied chaperones (Hfq and Imp4p). I performed a peptide tiling array that led to the identification of the motif mediating RNA binding. Furthermore, I detected the cooperative chaperone activity of motifs by analyzing the activity of peptides and proteins with different motif counts. I designed a suitable RNA and purified labeled peptide for NMR experiments that were performed in collaboration with Dr. Janosch Hennig (group of Prof. Sattler; TUM Munich). In addition, I performed co-crystallization of a single motif and RNA, which resulted in crystals diffracting up to 3.1 Å. Furthermore, I performed proteome-wide database searches for factors yielding multiple motifs in different organisms and performed gene ontology analysis of these factors.

4 Discussion

4.1 Loc1p in the nuclear *ASH1* mRNP assembly and reassembly

Loc1p, She2p, and the* ASH1 *mRNA form a stable and specific complex

According to the current experimental evidence, *ASH1*-mRNA localization already starts in the nucleus with the cotranscriptional recruitment of She2p to nascent mRNA (Müller et al., 2011; Shen et al., 2010). However, *in vitro* binding studies detected only a transient interaction of She2p and *ASH1* LEs with a medium affinity (Müller et al., 2011; Müller et al., 2009). Therefore, it seems likely that a nuclear complex is required to stably tether She2p on the *ASH1* mRNA. The integrity of the nuclear *ASH1* mRNP was previously analyzed with co-immunoprecipitations of TAP-tagged She2p (Gu et al., 2004; Shen et al., 2009). Besides *ASH1* mRNA, Loc1p and Puf6p were identified as components of the mRNP.

Here, I report that Loc1p binds specifically to She2p in presence and absence of RNA. Loc1p furthermore stabilizes the RNA interaction of She2p. Whereas Loc1p alone has only limited specificity towards LE containing RNAs (approximately three-fold better K_d), the She2p-Loc1p complex has a high specificity towards LE containing RNAs (approximately 40-fold better K_d). In pull-down experiments this ternary complex is only formed in presence of *ASH1* LE which further proofs the specificity of the complex. Thus, I conclude that Loc1p stably and specifically associates She2p with nuclear *ASH1* mRNA and that in this ternary complex both She2p and Loc1p cooperatively interact with the RNA, leading to a gain of mRNP stability and specificity (Figure 4-1; Publication 1; Niedner et al., 2013). Consistent with this model, a nuclear-localization deficient She2p strain (She2pM5) has been reported to show a strong reduction of *ASH1* mRNA and She2p-M5A co-immunoprecipitated with TAP-tagged Loc1p (Shen et al., 2009).

But where exactly within the nucleus does Loc1p join the She2p-*ASH1* mRNP? Loc1p could join the complex during transcription which would lead instantly to stable and specific complex formation. Chromatin immunoprecipitations by Shahbabian and colleagues (Shahbabian et al., 2014) support this mechanism, since

the authors show that Loc1p, like She2p, interacts cotransciptionally with the *ASH1* mRNA. Furthermore, this cotransciptional recruitment depends on She2p (Shahbabian et al., 2014), proving that only a Loc1p-She2p complex stably and specifically interacts with *ASH1* mRNA. Together with the fact that Loc1p, like She2p, was co-purified in co-immunoprecipitation of the transcription elongation factor Spt5 in yeast (Lindstrom et al., 2003) a cotranscriptional joining of Loc1p is likely.

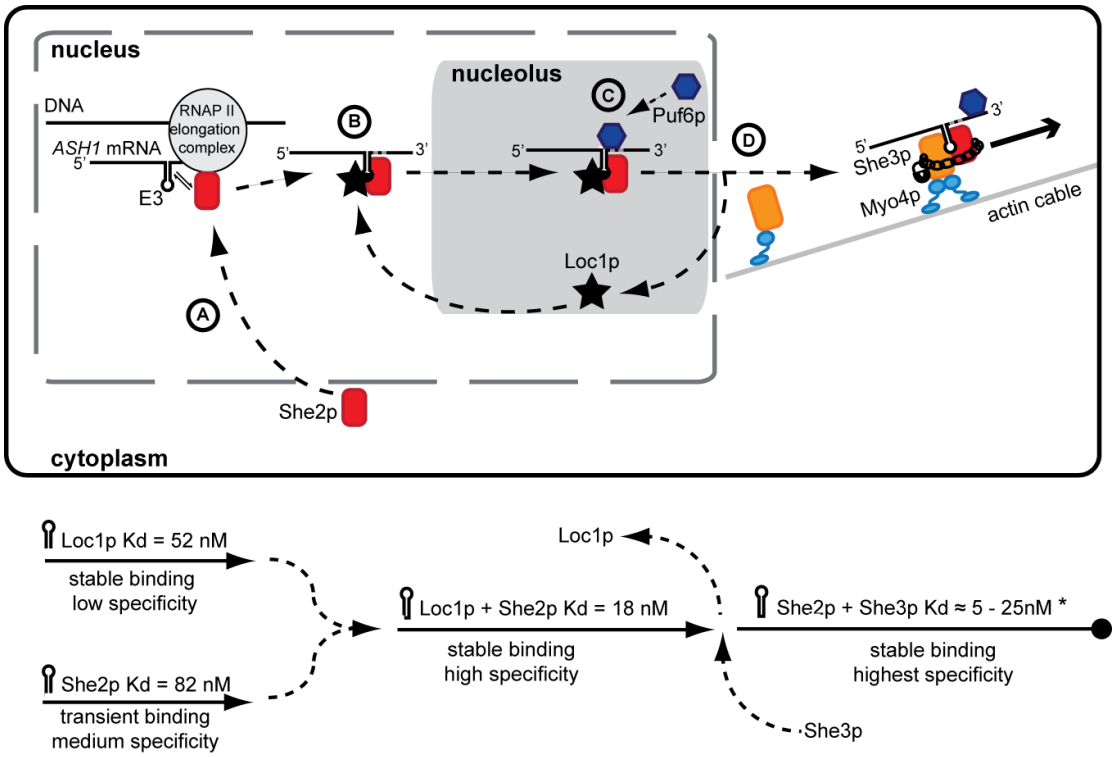

Figure 4-1 Model of sequential *ASH1*-mRNP maturation: The picture was taken from Niedner and colleagues (Niedner et al., 2013) **Upper part:** She2p enters the nucleus and interacts cotranscriptionally with the *ASH1* mRNA (A). Loc1p joins the complex leading to a stabilization of She2p on *ASH1* mRNA. Loc1p contains several nucleolar localization signals and might recruit She2p into the nucleus (B). Within the nucleus Puf6p could join the complex in a recruitment independent manner (C). At or close to the nuclear pore the complex is reorganized. The formation of the synergistic complex between She2p and She3p outcompetes Loc1p from the mRNP and ensures that Loc1p is omitted from the cytoplasmic *ASH1* mRNP. **Lower part:** Schematic drawing of the RNA-binding properties during the stepwise assembly of the mRNP. Affinity, specificity and complex stability improve from the initial nuclear binding events to the assembly of the mature transport complex. The gradient in RNA binding provides an explanation for directionality of complex formation. The asterisk indicates that this K_d has been estimated from figure 3C of the study by Müller and colleagues (Müller et al., 2011).

Nuclear complex formation is not limited to *S. cerevisiae*. Also in *D. melanogaster* nuclear priming is necessary for correct localization of an mRNA. Splicing of *osk* mRNA creates a LE, called spliced *oskar* localization element (SOLE), that ,together with the deposition of the exon junction complex, is required for the localization of *osk* mRNA to the posterior pole of the oocyte (Ghosh et al., 2012). Another example indicating the importance of nuclear mRNPs is the interaction of ZBP1 and ZBP2

with β-*actin* mRNA. ZBP1 localizes β-*actin* mRNA to the growth cones of fibroblast (Ross et al., 1997) and binds the RNA already cotranscriptionally. This mRNA association of ZBP1, however, depends on the recruitment by ZBP2 which is a predominantly nuclear factor (Gu et al., 2002; Pan et al., 2007). ZBP2, like Loc1p, is only a constituent of the nuclear mRNP and is omitted from the cytoplasmic mRNP (Gu et al., 2002).

Nucleolar trespassing of the ASH1 *mRNP*

Loc1p, as well as Puf6p, is a nuclear protein enriched in the nucleolus of the cell (Du et al., 2008; Huh et al., 2003; Urbinati et al., 2006). She2p and *ASH1* mRNA accumulate in the nucleolus when RNA export is blocked (Du et al., 2008), indicating that the *ASH1* mRNP trespasses the nucleolus. Loc1p contains several nucleolar localization signals. Therefore, a hub protein independent localization to the nucleolus is probable. In contrast, She2p does not contain a nucleolar localization signal and, thus, probably needs a hub protein to enter the nucleolus. Therefore, it is tempting to speculate that Loc1p, besides stabilizing the She2p *ASH1*-mRNA interaction, piggybacks She2p and the *ASH1* mRNA into the nucleolus of the cell (Figure 4-1). Such an indirect nucleolar recruitment of proteins lacking a nucleolar localization signal has previously been reported for Nucleolin and Nucleophosmin (Emmott and Hiscox, 2009).

Puf6p is not recruited to the nuclear ASH1 *mRNP*

Previous studies identified Puf6p as a component of the nuclear mRNP (Gu et al., 2004; Shen et al., 2009). In a study using a nuclear-localization deficient She2p strain (She2pM5), much less *ASH1* mRNA and She2pM5 was co-immunoprecipitated with TAP-tagged Puf6p (Shen et al., 2009), further supporting that Puf6p joins the nuclear *ASH1* mRNP. However, *in vitro* reconstitution failed to detect an interaction of Puf6p with She2p (Müller et al., 2011), with Loc1p, or with the Loc1p-She2p complex (Publication 1; Niedner et al., 2013). Also in presence of *ASH1* mRNA no association or recruitment of Puf6p was detectable (Publication 1; Niedner et al., 2013). Hence, there must be another explanation for the She2p dependent association of Puf6p with the *ASH1* mRNP. Since Puf6p is a nucleolar protein, an obvious possibility is that it can join the *ASH1* mRNA only in the

nucleolus and that Loc1p and She2p are required to locate *ASH1* mRNA in the nucleolus (Figure 4-1).

However, a study by Shahbabian and colleagues (Shahbabian et al., 2014) offers a different mechanism. Based on chromatin immunoprecipitations, the authors suggest that Puf6p is also cotranscriptionally recruited to the *ASH1* mRNA. But the enrichment over background of Puf6p to the E3 element (6-fold) seems to be rather low, if one compares it with the enrichment over background of Loc1p (50-fold) (Shahbabian et al., 2014), raising the question how significant this enrichment really is. A recent study by Qui and colleagues (Qiu et al., 2014) suggests that Puf6p binds RNA and DNA indiscriminately in a sequence-independent manner which might explain the low enrichment of Puf6p on *ASH1* E3. Although PUF proteins usually bind ssRNA sequence specifically with a crescent-shaped RNA-binding domain comprising eight PUM repeats (Wang et al., 2002; Wang et al., 2013), Puf-A, the human ortholog of Puf6p, adopts an L-shaped structure comprising eleven PUM repeats that contact mainly the phosphate backbone of the dsDNA with conserved basic residues (Qiu et al., 2014). Nucleic acid-binding studies as well as mutational analysis proved that this binding mode also applies for Puf6p (Qiu et al., 2014). This study, together with previous work from the Niessing lab (Müller et al., 2011; Müller et al., 2009), render it unlikely that Puf6p undergoes specific interactions with *ASH1* mRNA.

Shahbabian and colleagues also report an interaction between Loc1p and Puf6p and an association of Puf6p with the complex formed by She2p, Loc1p, and the *ASH1* mRNA, based on pull-down experiments (Shahbabian et al., 2014). These results contradict my own experimental evidence and might be due to experimental conditions and differently tagged proteins. Experimental differences for example are the usage of heparin (Shahbabian et al., 2014) versus tRNA (Publication 1; Niedner et al., 2013) or the elution of pull-downs with a gentle eluent like imidazole (Publication 1; Niedner et al., 2013) versus incubation at 95 °C with Lämmli-buffer (Shahbabian et al., 2014).

Complex reorganization upon nuclear export

After nucleolar trespassing (Du et al., 2008) the *ASH1* mRNP is exported into the cytoplasm were She2p, She3p, and the *ASH1* mRNA form a stable, specific, and

synergistic complex (Müller et al., 2011). She3p is a strictly cytoplasmic protein stably tethered to Myo4p (Bohl et al., 2000; Huh et al., 2003; Takizawa and Vale, 2000). Since Loc1p is a nucleolar protein (Huh et al., 2003; Urbinati et al., 2006), it has to be removed upon export of the *ASH1* mRNP. In *in vitro* reconstitution experiments I could show that addition of She3p to the nuclear *ASH1* mRNP displaces Loc1p from the complex and leads to the formation of the synergistic She2p-She3p mRNP (Publication 1; Niedner et al., 2013). This offers a mechanism where a specific and stable nuclear complex is replaced by the formation of a cytoplasmic complex with an even higher specificity and stability. This stepwise increase in specificity and stability provides the required directionality of complex formation.

But where exactly could such a outcompetition take place, if Loc1p is localized in the nucle(ol)us and She3p in the cytoplasm? Although heterokaryon assays proved the nuclear localization of Loc1p (Long et al., 2001), they cannot exclude that a minor protein subfraction shuttles between the nucleus and the cytoplasm. Therefore, complex reorganization might take place at or close to the nuclear pore (Figure 4-1). In agreement with this hypothesis, the localization of GFP-tagged Loc1p is altered upon genomic deletion of *SHE3*. In detail, Loc1p becomes dispersed within the nucleus and occasionally a cytoplasmic signal close to the nuclear membrane is observed when *SHE3* is deleted (Publication 1; Niedner et al., 2013). This is consistent with a defective complex reorganization at or close to the nuclear pore which would result in a delay in Loc1p disassembly and subsequent reimport into the nucleus.

mRNP reorganization upon nuclear export has also been reported for the *Vg1/VegT* mRNP in *X. laevis*. The nuclear complex is composed of the proteins hnRNP1, Vg1RBP/Vera and 40LoVe, as well as the mRNAs (Kress et al., 2004; Lewis et al., 2008). Thereby, 40LoVe assembly depends on recruitment by hnRNP1 (Czaplinski et al., 2005; Czaplinski and Mattaj, 2006; Kroll et al., 2009). Besides the incorporation of cytoplasmic factors, the reorganization of the complexes effects especially the interaction between hnRNP1 and Vg1RBP/Vera. Whereas the two factors interact directly within the nucleus, only an RNA-dependent interaction between both proteins is detectable in the cytoplasmic mRNP (Kress et al., 2004; Lewis et al., 2008).

4.2 Stable complex formation of *ASH1* mRNP core factors does not require RNA

Motile *ASH1* mRNPs consist of the RNA-binding proteins She2p and She3p as well as the type-V myosin-motor protein Myo4p (Bohl et al., 2000; Jansen et al., 1996; Kruse et al., 2002; Long et al., 2000). Both She2p and She3p are required for the specific recognition of target mRNA (Müller et al., 2011). Myo4p, as a type-V myosin, needs dimerization to processively move along actin cables (Vale, 2003). Since Myo4p alone is a monomer and thus unprocessive (Dunn et al., 2007; Heuck et al., 2007; Reck-Peterson et al., 2001), complex formation has to induce motor dimerization, allowing for mRNP transport. Recently, the exact stoichiometry of the motile *ASH1* mRNP has been determined. She3p is a dimer that constitutively interacts with one Myo4p molecule (Heym et al., 2013; Shi et al., 2014). She2p is a tetramer (Müller et al., 2009) that binds two *ASH1* LEs and two She3p dimers, thus incorporates two Myo4p molecules into the complex allowing for processive movement of *ASH1* mRNPs (Heym et al., 2013). However, single molecule motility studies differ in their conclusion on the necessity (Sladewski et al., 2013) or dispensability (Heym et al., 2013; Krementsova et al., 2011) of cargo RNA for motor activation.

My experiments confirmed that She2p, She3p, and Myo4p form a stable complex also in absence of *ASH1* mRNA (Publication 2; Edelmann et al., 2015). This complex is even present in pull-down experiments under conditions resembling the physiological salt concentration and the yeast growth temperature (140 mM NaCl; 30 °C). In analytical size-exclusion chromatography all components elute in a single peak indicating stable interaction of the components. Also after more than 2 hours of size-exclusion chromatography, a sub-fraction of the mature complex is still intact. Its molecular mass was determined by static light scattering to be 420 kDa, which is in agreement with the calculated molecular weight of 434 kDa .

Within the cell, the occurrence of RNA-free complexes is unlikely. However, it is mechanistically interesting to understand whether RNA has an influence on motor dimerization. Previous studies on subcomplexes already indicated a stable interaction in absence of RNA. Even by using 1 M NaCl the She3p-Myo4p subcomplex still elutes in a pull-down experiment (Heuck et al., 2010) and the She2p-She3p complex is stable in pull-down experiments and analytical size-

exclusion chromatography at salt concentration of 200 mM NaCl (Müller et al., 2011). Therefore, the detected stability of the reconstituted complex consisting of She2p, She3p, and Myo4p is consistent with previous work and suggests that motor dimerization and activation is mediated primarily by protein-protein interactions.

4.3 Loc1p in ribosome biogenesis

Based on the phenotype of the genomic deletion of *LOC1*, Loc1p has been implicated in ribosome biogenesis. Deletion of *LOC1* results in half-mer polyribosomes (Harnpicharnchai et al., 2001) and a delay in the processing at sites A_0, A_1, and A_2 (Urbinati et al., 2006). These processing sites are the first steps of 35S pre-rRNA processing and occur already cotranscriptionally (Figure 1-3; Kos and Tollervey, 2010; Woolford and Baserga, 2013). Co-purifications of Loc1p with pre-60S particles (De Marchis et al., 2005; Horsey et al., 2004; Saveanu et al., 2003) further suggest that Loc1p is involved in the biogenesis of the large ribosomal subunit.

rRNA and tRNA modifications are affected in the* loc1Δ *strain

The *LOC1* genomic deletion strain exhibits a slow-growth phenotype and the amount of 80S ribosomes purified from this strain is severely reduced (Manuscript) which underlines the importance of Loc1p as a constituent in ribosome biogenesis. To determine whether ribosomes derived from the *loc1Δ* strain show abnormalities, I analyzed in collaboration with Caterina Brandmayr (group of Prof. Carell; LMU Munich) the rRNA modification pattern in the *loc1Δ* strain. Indeed, rRNA modifications were significantly reduced for a variety of modifications (Manuscript). This indicates that Loc1p is not involved in the modification of a specific nucleotide and that at least some of these changes are likely to be an indirect effect. This is also resembled by the changes in tRNA modifications in the *loc1Δ* strain where most modification rates are increased (Manuscript). Such an effect has been recently reported to be caused by stress (Chan et al., 2010; Chan et al., 2012), resulting in codon-biased protein translation (Endres et al., 2015; Gu et al., 2014).

Loc1p interacts with ribosomes and rRNA with low specificity

Micro arrays of TAP-tagged Loc1p identified co-purified rRNAs (Manuscript; collaboration with Prof. André Gerber; University of Surrey). Furthermore, Loc1p interacts with mature prokaryotic and eukaryotic ribosomes (Manuscript; previous work of Roland Heym), indicating a rather unspecific interaction with mature ribosomes. However, since Loc1p is a nucleolar protein (Huh et al., 2003; Urbinati et al., 2006), an interaction with ribosome precursors is more likely *in vivo*. Consistent with the binding to ribosomes from different species, EMSA experiments showed that Loc1p indiscriminately binds to RNA and DNA and does not prefer double-stranded or single-stranded nucleic acids (Manuscript). This implies an interaction most likely via their phosphoribose backbone. But why does Loc1p affect specifically ribosome biogenesis and not other RNA-based processes? One possibility is that defective ribosome biogenesis is only the most dramatic and thus most visible defect amongst others. However, the alterations in ribosome biogenesis might also be simply explained by the nucleolar localization of Loc1p (Huh et al., 2003; Urbinati et al., 2006), which restricts Loc1p functionality to this process.

Loc1p is a folding catalyst bearing eleven RNA-binding motifs

Assembly factors of ribosome biogenesis possess various functions (Paragraph 1.3). In the unpublished manuscript, I used different *in vitro* chaperone assays to show that Loc1p stabilizes energetically favored RNA structures and anneals single stranded nucleic acids (Manuscript). The annealing activity of Loc1p even exceeds the activity of the well-studied U3 snoRNA chaperone Imp4p (Gerczei and Correll, 2004; Gerczei et al., 2009). On the other hand, Loc1p fails to separate double stranded RNAs. Using peptide-tiling arrays I identified eleven iterations of a positively charged RNA-binding motif within the Loc1p sequence. Surprisingly, a single motif was able to anneal single-stranded DNA, however, multimerization of motifs resulted in a large increase in annealing activity (Manuscript). This indicates that motifs act cooperatively in annealing. Since an artificial protein comprising ten RNA-binding motifs has an annealing activity comparable to wild-type Loc1p (Manuscript), such iterations of motifs appear sufficient to recapitulate the annealing activity of the wild-type protein *in vitro*.

Loc1p is an intrinsically unstructured protein

The overall fold of Loc1p was analyzed by SAXS and NMR spectroscopy (Manuscript; previous work of Marisa Müller in collaboration with Irina Anasova; group of Prof. Sattler; TUM Munich). SAXS experiments already indicated that Loc1p is unstructured. Subsequent ^{1}H-^{15}N HSQC spectra of Loc1p showed an accumulation of signals between 8.4 and 7.6 ppm, confirming that this protein is indeed unstructured in large parts. Also the addition of RNA does not increase the spectral dispersion of Loc1p, indicating that this protein is also unstructured in large parts while interacting with RNA.

Motif-RNA interaction

In collaboration with Janosch Hennig (group of Prof. Sattler; TUM Munich) I used NMR experiments to confirm the interaction of a single, Loc1p-derived RNA-binding motif with dsRNA. However, peptides without a motif failed to interact with dsRNA. Binding of the motif did not alter the secondary structure of the dsRNA, which indicates a likely interaction with the phosphoribose backbone. The motif, however, gains rigidity in complex with RNA, but does not adopt a secondary-structure fold. Furthermore, binding of the motif leads to a thermal stabilization of the dsRNA structure, whereas peptides without a motif destabilized the stem region (Manuscript). This motif-dependent thermal stabilization also explains the folding catalyst activity of a single motif at the molecular level. Based on its structural and chemical features, I named the repeating sequence elements of Loc1p positively-charged unfolded nucleic acid-binding motif (PUN).

Evolutionary aspects of the PUN motif

PUN motifs likely interact with the phosphoribose backbone of the RNA which implicates a rather general function in RNA metabolism. When also considering their stabilizing activity of RNA secondary structures, PUN motifs are likely to facilitate efficient RNA folding in the nucleolus of budding yeast. By using proteome-wide bioinformatics approaches, I searched for proteins in *S. cerevisiae* and *S. pombe* with at least five PUN motifs, as well as for *E. coli* proteins containing at least three PUN motifs (Manuscript). Proteins matching these criteria were analyzed for their gene ontology terms. PUN-motif containing *S. cerevisiae* and *S. pombe* proteins show a significant enrichment in the nucleus and nucleolus of the

cell. Nucleic acid metabolic processes, rRNA metabolic processes, and ribosome biogenesis are gene ontology terms that showed a high enrichment and low P-values in the analysis. This high similarity between both species is surprising, since *S. pombe* and *S. cerevisiae* have been evolutionary separated since more than 330 million years (Sipiczki, 2000) and are thus very distinct. Also *E. coli* proteins containing three PUN motifs are involved in similar processes. Thus PUN-motif containing proteins from different organisms and even different kingdoms of life are involved in closely related processes that require ribozyme activity, like ribosome biogenesis.

Already several years ago it has been proposed that in an ancient RNA-dominated world short peptides could have protected RNAs against degradation or stabilized a certain RNA conformation, thus fostering co-evolution of both molecules (Poole et al., 1998). The formation of large RNPs, like ribosomes, was perhaps also possible because incorporated peptides extended the structural and functional capabilities of RNA (Cech, 2009; Noller, 2004). Herschlag and colleagues (Herschlag et al., 1994) tried to reconstitute such a scenario. They used a mutated and thus inactivated hammerhead ribozyme and attempted to restore the catalytic activity by adding of short peptides. A positively charged peptide showing unspecific RNA binding was not only able to restore the catalytic activity but also annealed RNA molecules. PUN motifs or related sequences might have played a similar role during evolution. It might therefore well be possible that this is the reason why PUN motifs are present in large RNPs with ribozyme activities in very different species.

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6 Abbreviations

°C	Degree Celsius
g	standard gravitiy
µ	Micro
Å	Angstrom
A_{260}	Absorption at 260 nm
A_{280}	Absorption at 280 nm
ATP	Adenosine triphosphate
BicD	*Bicaudal-D*
cycB	*CyclinB*
D. melanogaster	*Drosophila melanogaster*
DNA	Deoxyribonucleic acid
ds	double-stranded
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylendiaminetetraacetic-acid
EJC	Exon junction complex
EMSA	Electrophoretic mobility shift assay
FISH	Fluorescence in situ Hybridization
g	gram
G. gallus	*Gallus gallus*
GTP	Guanosin triphosphate
HEPES	Hydroxyethyl piperazine-enthansulfonic acid
HO endo-nuclease	Homothallic switching endonuclease
K	Kelvin
Kd	Equilibrium dissociation Constant
l	liter
LC	Liquid chromatography
LE	Localization element
m	Milli
M	Molar
MBP	Maltose-binding protein
mRNA	messenger RNA
mRNP	messenger ribonucleo-protein
MS	Mass spectrometry
M. smegmatis	*Mycobacterium smegmatis*
min	Minutes
n	nano
NMR	Nuclear magnetic resonance
nos	*nanos*
OES	Oocyte entry signal
osk	*oskar*
PMSF	Phenylmethylsulfonyl-fluoride
RNA	Ribonucleic acid
rRNA	ribosomal RNA
PAGE	Polyacrylamide gel Electrophoresis
PUN	Positively-charged unfolded nucleic acid-binding motif
S	Svedberg
SAXS	Small angle X-ray scattering
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodiumdodecylsulfate
SHE	Swi5p-dependent HO expression
SLS	Static light scattering
snoRNA	small nucleolar RNA
SOLE	Spliced *oskar* localization element
ss	single-stranded
S.pombe	*Schizosaccharomyces pombe*
TAP-Tag	Tandem affinity purification Tag
TBE	TRIS-Borat-EDTA buffer
TRIS	Tris- (hydroxymethyl)-aminomethane
tRNA	transfer RNA
UTR	Untranslated region
UV	Ultraviolet

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