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

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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 physiological 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-transcriptionally17,18 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.30,31 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.29 While a study from Heym and colleagues came to the conclusion that mRNA itself is dispensable for the activation of the myosin motor,28
a second study by Sladewski and colleagues provided evidence that mRNA may be essential for motility.\textsuperscript{33}

A number of experimental differences in the single-particle motility assays have been discussed to be potentially responsible for these seemingly contradicting results.\textsuperscript{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 \textit{ASH1} mRNA particles at the correct stoichiometric ratios (2 × RNA, 4 × She2p, 4 × She3p, 2 × Myo4p),\textsuperscript{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).\textsuperscript{33}

In a recent commentary, Sladewski and colleagues now offer a new explanation.\textsuperscript{35} The authors suggest that particle movement in absence of RNA, as reported by Heym and colleagues,\textsuperscript{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 \textit{in vitro} and thus Myo4p would be non-motile in the cell without cargo.”\textsuperscript{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 \textit{ASH1} mRNA \textit{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.\textsuperscript{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 binding.\textsuperscript{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.
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 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. 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.

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 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. She2p, She2p (Δhelix E), and She3p were expressed and purified as previously described. The absence of RNA contaminations was confirmed by measuring the ratio of OD 260/280.
**In vitro pull-down experiments**: Protein samples were mixed in their correct stoichiometric ratios, using 10 μM She2p wt/Δhelix E, 10 μM She3p-His6 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 MgCl2, 2 mM DTT). After centrifugation for 10 min, 16,100 × 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 Coo- massie blue staining.

**Size-Exclusion Chromatography** was essentially performed as described before,17 using a Superox 6 10/300 GL column (GE Healthcare) in HNMD buffer (20 mM Hepes (pH 7.8), 200 mM NaCl, 2 mM MgCl2, 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 Superox 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.

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