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Of social molecules

The interactive assembly of *ASH1* mRNA-transport complexes in yeast

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Asymmetric, motor-protein dependent transport of mRNAs and subsequent localized translation is an important mechanism of gene regulation. Due to the high complexity of such motile particles, our mechanistic understanding of mRNA localization is limited. Over the last two decades, *ASH1* mRNA localization in budding yeast has served as comparably simple and accessible model system. Recent advances have helped to draw an increasingly clear picture on the molecular mechanisms governing *ASH1* mRNA localization from its co-transcriptional birth to its delivery at the site of destination. These new insights help to better understand the requirement of initial nuclear mRNPs, the molecular basis of specific mRNA-cargo recognition via cis-acting RNA elements, the different stages of RNP biogenesis and reorganization, as well as activation of the

motile activity upon cargo binding. We discuss these aspects in context of published findings from other model organisms.

Introduction

mRNA localization is a universal feature throughout eukaryotes with important roles in cell polarity and differentiation, as well as embryonic development and body axis determination (reviewed in refs. 1–3). In somatic tissues mRNA localization is required for the formation and function of highly differentiated cells like neurons, neuroglia and epidermal cells. In higher eukaryotes, several dozens of factors usually co-immunoprecipitate in localizing messenger ribonucleoprotein particles (mRNPs).[4-6] This complexity is much reduced in protozoans like fungi.[7] However, for most protozoan model organisms, including the filamentous plant pathogen *Ustilago maydis*, the number of core factors remains unknown (reviewed in refs. 3 and 8). Several localizing mRNPs in higher and lower eukaryotes associate with membranes and vesicles (reviewed in refs. 9 and 10). Also the reported numbers of specifically recognized RNA species vary dramatically from thousands[11-13] to only several dozens.[14] Most likely these numbers extensively depend on the threshold for the definition of specific binding and usually drastically decrease when cross-validation is performed.[14] Obviously, a model system with a much lower complexity is needed to understand the mechanisms underlying mRNA localization.

In the past 20 years, budding yeast *Saccharomyces cerevisiae* has emerged as a particularly well-suited model system to understand basic principles of the assembly and function of mRNA-transport complexes (reviewed in refs. 9 and 15). In haploid yeast cells, asymmetric cell division results in progenies with opposing mating types of either *MATa* or *MAT*α (reviewed in ref. 16). The mating type is altered in the mother cell by transcription of the site-specific endonuclease HO (Homothallic

switching)[17] and an HO-dependent genomic recombination in the *MAT* gene locus. Since *HO* is only expressed in the mother cell, the mating type of the daughter cell remains unchanged.

In 1996 two concurrent studies showed that the protein Ash1p (asymmetric synthesis of *HO*) localizes preferentially to the daughter cell, where it inhibits *HO* transcription and thus mating-type switching.[18,19] A third concurrent study reported the identification of five so-called *SHE* genes (*SWI5*-dependent *HO* expression), whose protein products are required for the asymmetric distribution of Ash1p.[20] Only one year later two laboratories published the surprising finding that the *ASH1* transcript and not its protein product is actively transported into the daughter cell.[21,22] The locally restricted translation of the *ASH1* mRNA causes the Ash1p-dependent repression of *HO* transcription in the daughter cell, thereby leading to the selective inhibition of mating-type switching (reviewed in ref. 16).

The *SHE1* gene identified in a genetic screen by Jansen and colleagues encodes the type V myosin (MyoV) motor Myo4p.[20] MyoV motors constitute the main group of myosins for cargo transport (reviewed in ref. 23). The N-terminus consists of an ATP-hydrolyzing motor domain, followed by a calmodulin-bound lever arm, a coiled-coil dimerization domain, and a globular cargo-binding domain at its C-terminus. Dimeric MyoV binds to and moves processively along actin filaments (reviewed in ref. 23). She2p is an *ASH1* mRNA-binding protein[24-26] with a domain fold that lacks similarity to previously described RNA-binding domains.[27] She3p was initially shown to be an adaptor protein in the transport complex.[24,25,28] She4p/Dim1p belongs to the UNC-45/CRO/SHE4 (UCS)-domain containing protein family and binds to Myo4p and other myosins in yeast.[29,30] It has been suggested to be required for the structural integrity of Myo4p[29,30] and as determinant of Myo4p step-size on

actin filaments.[31] However, its exact role is still not fully understood. She5p/Bni1p is a member of the formin family that is involved in the nucleation of linear actin filaments and thus required for cytoskeletal integrity during cell division (reviewed in ref. 32). Initial experiments already suggested that She2p is the core *ASH1* mRNA-binding protein, which is bound by Myo4p-associated She3p to form an active transport complex (**Fig. 1A**).[24,25,28]

Besides these essential *SHE* factors, auxiliary proteins were discovered that also associate with the *ASH1* mRNA. Their deletion resulted in the reduction, but not abolishment of *ASH1* mRNA localization. These factors include the nucleolar ribosome biogenesis factor Loc1p, as well as the translational repressors Puf6p and Khd1p.[33-39] Aside from *ASH1* mRNA, about 30 other transcripts are transported into the daughter cell.[7,40-43]

The assembly of the *ASH1* mRNA-transport complex already occurs co-transcriptionally with the binding of the RNA-binding protein She2p to so-called localization or zip-code elements of the nascent *ASH1* mRNA (**Figs. 1A and 2A**).[44] This core complex is joined by Loc1p, Puf6p, and Khd1p.[34-38,45,46] While Loc1p remains in the nucleus, the rest of the *ASH1* mRNP exports into the cytoplasm. There, She2p interacts with She3p (**Fig. 1A**).[24,25,47] Since She3p itself is constitutively bound to the Myo4p motor,[24,25,48-51] this interaction results in the formation of the mature transport complex. Together with its translational repressors, the *ASH1* mRNP moves along actin filaments until it reaches the tip of the bud cell. At the bud tip, translation is activated. The result is Ash1p-dependent inhibition of the HO endonuclease and the prevention of mating-type switching in the daughter cell (reviewed in ref. 16).

Although the initial genetic screen did not offer any link to membrane transport, a subsequent screen revealed that two components of the mRNA transport

complex, Myo4p and She3p are also required for the early inheritance of cortical endoplasmic reticulum (ER) to the bud cell (**Fig. 1A**).[52] Vice versa, a subset of proteins required for cortical ER inheritance were also shown to affect mRNA localization,[43,53] suggesting a direct link between both processes.

While a concomitant review by Singer-Krüger and Jansen in this issue gives an overview on all the different mRNA transport processes present in yeast, this review focuses on recent advances of *ASH1* mRNP function. In the remaining part of this review, we summarize the major progress made in the last five years to understand (1) why localizing mRNAs and transport-core factors are already assembled in mRNPs within the nucleus, (2) how specificity for localizing mRNAs is achieved, (3) what importance has mRNP reorganization at specific cellular sites, (4) how processive motility of the *ASH1* mRNP is achieved, (5) how ER inheritance is connected to mRNA localization, and (6) what effect multimerization of sub-complexes has on the function of localizing mRNPs.

Recent Advancements in Understanding *ASH1* mRNP Function

Why do we need initial nuclear mRNPs?

Although the assembly of nuclear sub-complexes with localizing RNAs has been described in different organisms, to date it is not well understood why the nuclear history of mRNPs is important for cytoplasmic mRNA localization. Also in the budding yeast such initial nuclear complexes have been described. The core RNA-binding protein She2p shuttles between nucleus and cytoplasm.[48] An RNA-binding deficient version of She2p accumulates in the nucleolus,[45] indicating that She2p is exported with its target mRNA into the cytoplasm. Consistently, blocking nuclear mRNA export with a temperature-sensitive mutant of the export factor *MEX67* traps She2p in the nucleus.[48] In addition, mutant versions of She2p, which are unable to

enter the nucleus, show impaired cytoplasmic mRNA localization and upregulation of *ASH1* mRNA translation.[45,46] Together these findings indicate that nuclear mRNA binding of She2p is required to mediate cytoplasmic inhibition of translation during transport.

More recently, it was shown that this nuclear association of She2p with *ASH1* mRNA occurs already co-transcriptionally via binding to an RNA-polymerase II-associated heterodimeric protein complex, consisting of Suppressor of Ty's 4 and 5 (Spt4/5).[44] Based on these data Chartrand and colleagues proposed a model, in which She2p first associates with RNA polymerase II at the *ASH1* locus and then selectively translocates onto the nascent *ASH1* transcript to form a first, specific nuclear mRNP (**Fig. 1A**).

For this intriguing model an open question concerns the specificity of co-transcriptional She2p recruitment to the *ASH1* locus. In a second study a very similar approach was used to assess the association of She2p with chromatin and nascent RNA. However, it failed to confirm the previously reported selective enrichment of She2p at the *ASH1* locus, but rather showed a broad, unspecific enrichment of She2p at chromatin.[47] Although it would make sense to selectively tether She2p co-transcriptionally to localizing mRNAs, it seems to require additional experimental proof to adequately understand these events.

A related unresolved issue is the association of She2p with the transcription machinery and its implications on specificity. The Spt4/5 complex acts as general RNA polymerase II-dependent elongation factor.[54,55] In contrast, She2p is selectively required for *ASH1* mRNA recognition and localization. Considering that RNA-polymerase II is much more abundant in cells than She2p[56] and that RNA-polymerase II as well as the Spt4/5 complexes associate with a large number of loci, it remains

unclear how She2p would efficiently find the few transcription sites of localizing mRNAs. Thus, more work seems to be required to fully understand how the She2p-dependent co-transcriptional recruitment results in the formation of specific *ASH1* mRNPs.

In the nucleus this complex is joined by the translational repressors Puf6p and Khd1p (**Fig. 1A**).[34-37] In experiments using a She2p mutant that lacks a nuclear localization signal less *ASH1* mRNA is associated with Puf6p. Thus, She2p seems to be important for the formation of nuclear Puf6p-containing mRNPs.[46] Consistent with this finding, in cells with a cytoplasmically tethered mutant of She2p, translational repression is impaired during transport.[45] The third factor interacting with the *ASH1* mRNA in the nucleus is Loc1p (**Fig. 1A**).[38,45] This protein contains a number of potential nucleolar localization signals and directly interacts with She2p in a specific manner.[57] Furthermore, the incorporation of Loc1p into the *ASH1* mRNA-She2p complex enhances the specificity and stability in vitro.[57] Loc1p is therefore likely to stabilize and direct the *ASH1*-She2p mRNP into the nucleolus, where it is loaded with the translational repressor Puf6p (**Fig. 1B**).[36,37]

Also examples from higher eukaryotes have been described, where initial nuclear mRNPs are important for cytoplasmic mRNA localization. For instance, neuronal Staufen2 is implicated in cytoplasmic RNA localization to dendrites, thereby likely modulating synaptic plasticity.[58] In similarity to studies with She2p, a mutant of Staufen2 with deficiency in RNA binding accumulates in the nucleolus.[59] This finding suggests the formation of initial nuclear mRNPs and co-export of Staufen2 with its target mRNA into the cytoplasm.

A mechanistically very interesting and well understood example is the requirement of nuclear splicing of *oskar* mRNA in the oocyte of the fruit fly

Drosophila melanogaster for its cytoplasmic localization to the posterior pole.[60] For correct RNA localization two structural elements within the RNA are prerequisite: the spliced oskar localization element (SOLE)[61] and the *oskar* 3′UTR containing a dimerization element (**Fig. 2B**).[62] In the unspliced *oskar* pre-mRNA two halves of a bipartite zip-code element are separated by an intron. The splicing reaction at the first exon-exon junction joins both halves, and thus creates this so-called SOLE. Upon splicing the exon junction complex (EJC) is deposited on the mRNA and contributes to the correct localization of the RNA to the posterior pole.[61]

Another example for nuclear priming of mRNPs can be found in *β-actin* mRNA localization, which facilitates neurite outgrowth and cell migration. *β-actin* mRNA is already bound co-transcriptionally by its dedicated RNA-binding protein and localization factor zipcode-binding protein 1 (ZBP1). This nuclear recruitment of ZBP1 to *β-actin* mRNA depends on ZBP2.[63,64] Since a knock-down of ZBP2 inhibits neurite outgrowth, formation of these nuclear mRNPs seems to be a prerequisite for functional cytoplasmic *β-actin* mRNA localization.

Also the localization of *Vgl1* and *VegT* mRNA in the African clawed frog *Xenopus laevis* requires nuclear mRNP assembly. Already in 2004, Kress and colleagues proposed the formation of a nuclear core complex consisting of heterogeneous nuclear ribonucleoprotein 1 (hnRNP), Vg1 RBP/vera, and zip-code containing mRNA that is necessary for mRNP localization to the vegetal pole of the oocyte.[65]

Molecular Mechanisms of Specificity in mRNA Recognition

Cis-acting regions in localizing mRNAs: The zip-code elements

Localizing mRNAs contain cis-acting localization elements, also termed zip-code elements, that are recognized by the transport machinery for their localization

(reviewed in ref. 66). Zip-code elements are sufficient to mediate localization, even when placed into heterologous mRNAs.[67] In budding yeast, the *ASH1* mRNA has four zip-code elements (**Fig. 2A**). Three of them, termed E1, E2A, and E2B, reside in the coding region, whereas the most 3′-located E3 zip-code element is found directly after the stop-codon. Although all four elements are specifically bound by the SHE complex,[47] they show no obvious sequence conservation and great differences in their predicted secondary structures (**Fig. 2A**). Khd1p binds to a region that includes the E1 element and Puf6p interacts with sequences in the E3 element.[34,36] This E3 zip-code element appears to play a particularly important role. While showing the highest affinity for She2p, it is the only element in the *ASH1* mRNA that resides outside of the open reading frame (**Fig. 2A**), and has a binding site for Puf6p.[26,36,67] Secondary structure predictions of all four zip-code elements indicate stem loops with mismatches like bulges and internal loops (**Fig. 2A**). The only reported consensus between these elements is the presence of a CGA-base triplet and a single cytosine separated by six bases either 3′ or 5′ from the triplet.[68] While this small motif is found in all four elements of the *ASH1* mRNA and also in the localizing RNAs *IST2* and *YMR171c*, it is lacking in some other localizing RNAs, such as the *EAR1* zip-code (**Fig. 2A**). Furthermore, within the *ASH1* zip-code elements, this motif is located in different secondary structures, can have an inverse 3′ to 5′ orientation, the sequence of its base triplet can be permutated, and the spacing of this bipartite motif seems variable (**Fig. 2A**). For several of the other localizing mRNAs, no defined zip-code element has been identified to date. Thus, further work will be required to understand what structural and sequence features define a zip-code element for SHE-dependent localization.

Since the zip-code binding mode in budding yeast is not well understood, a comparison with zip-code elements in higher eukaryotes might offer common themes. For instance, it seems to be a general rule that zip-code elements consist of double-stranded regions with hairpins and well-defined mismatches (reviewed in ref. 69). Such mismatches appear to be important to promote specificity for protein interactions. An exception to this rule is the zip-code element *β-actin* mRNA,[70] which consists of a single-stranded bipartite recognition motif.[71,72]

In particular in *Drosophila* a number of such zip-code elements has been identified for mRNAs expressed either during oogenesis or embryogenesis. They include the *Gurken Localization Signal* (GLS),[73] *Bicoid Localization Element* (BLE),[74,75] the above-described SOLE element[61] and the *Oocyte Entry Signal* (OES)[76] of the *oskar* mRNA (**Fig. 2B**), and a 44nt *Transport/Localization Sequence* (TLS) in the *fs(1)K10* mRNA (**Fig. 2C**).[77] Although these localizing mRNAs have distinct functions in development, at least some of them appear to have related zip-code elements. For instance, the *fs(1)K10* mRNA normally localizes to the apical regions of the embryo. When its zip-code element TLS is expressed earlier during oogenesis as part of a heterologous RNA, this element also mediates its localization in its new environment.[77,78] A second example is the OES, which mediates the transport of *oskar* mRNA from the nurse cells into the oocyte.[76] When ectopically expressed, OES-containing RNAs are also localized in blastoderm-stage embryos, in follicular epithelial cells, and in larval salivary glands.[76] This observation indicates that the localization machinery might be reused during different developmental stages of the fruit fly. Furthermore, *fs(1)K10* TLS and the *oskar* OES are interchangeable.[76] This is surprising because both zip-code elements share no obvious homology on the level of

sequence and secondary-structure predictions (**Fig. 2B and C**), thereby being very reminiscent of the *ASH1* zip-codes (**Fig. 2A**).

In comparison to double-stranded DNA binding motifs it is much more difficult to define consensus protein-binding motifs in RNAs. A main reason is that RNAs fold in a much more diverse manner, more similar to proteins than to DNA. As a consequence it is difficult to predict secondary or even tertiary structures with high reliability (reviewed in ref. 79). Thus, in most cases, secondary structure predictions and sequence similarities are insufficient to identify zip-code elements and to understand their function.

The example of the *fs(1)K10* TLS from *Drosophila* shows that three-dimensional structural information are extremely useful to overcome this limitation. The 44nt long TLS zip-code element is a stem-loop structure with an A'-form helix.[77] This unusual helix has a widened major grove that is required for dynein-dependent localization to apical regions of the *Drosophila* embryo (**Fig. 2C**). A second example where structural information are necessary to explain zip-code binding is ZBP1 from chicken and its RNA target *β-actin* mRNA. Here a 54nt long single-stranded zip-code element with a bipartite binding motif is necessary and sufficient for the localization of reporter mRNAs.[70-72] Important for specificity are not only the two binding motifs but also their exact spacing. Other interesting examples, where cis-acting targeting elements lead to transport of distinct mRNAs can be found in neurons. For instance the small non-coding brain cytoplasmic RNA 1 (*BC1*) is localized to dendrites of rodents,[80] as well as Mauthner axons and dendrites of goldfish,[81] where it regulates translation of target genes.[82,83] Dendritic localization elements can also be found in the α-calcium/calmodulin-dependent protein kinase II (*α-CaMKII*) mRNA. Here several cis-acting elements mediate site-directed transport.[84-88]

RNA-Binding Domains for mRNA Recognition

It is interesting to note that only two of the RNA-binding proteins in the *ASH1* mRNP bear known RNA-binding domains. The first one is the auxiliary factor Puf6p, which belongs to the highly conserved PUF (Pumilio and FBP) family of RNA-binding proteins. PUF proteins are characterized by a Pumilio-homology domain (PUM-HD).[89,90] The second is Khd1p, which contains three K-homology (KH) RNA-binding domains.[91,92] In contrast, no predictable RNA-binding domain can be found in the auxiliary factor Loc1p. Also the two zip-code recognizing RNA-binding proteins She2p and She3p lack any recognizable canonical RNA-binding domain.

A number of abundant RNA-binding domains have been identified in the past. Beside the above-described PUM-HD and the KH domains, other examples for wide-spread RNA-binding domains include the double-stranded RNA-binding domain (dsRBD),[93-95] the RNA-recognition motif (RRM),[96,97] zinc fingers (ZF),[98,99] PAZ domain,[100,101] PIWI domain,[102-104] and the Sterile alpha motif/pointed (SAM) domain.[105] Thus, at first glance this apparent lack of known domains in the *ASH1* mRNP appears unusual.

However, recent studies suggest that also other RNA-binding proteins for mRNA localization lack identifiable RNA-binding domains. For instance, in *Drosophila* the RNA-binding protein Egalitarian shows no sequence similarity to known RNA-binding domains or motifs.[106] Furthermore, two recent systematic screens for mRNA-interacting proteins demonstrated that at least one-third of the identified factors lack identifiable RNA-binding domains and were previously not predicted to bind RNA.[107,108] These findings suggest that the RNA-interactome is larger than previously anticipated and that the high number of non-canonical RNA-binding proteins in the *ASH1* mRNP might not be unusual after all.

Specific She2p-Loc1p mRNP in the Nucleus

In vitro binding experiments showed that the initial nuclear mRNP consisting of *ASH1* mRNA and She2p has a low specificity and complex stability.[26,47] Both of these features seem incompatible with the idea that She2p stably and selectively escorts localizing mRNAs from their site of transcription to the final destination at the tip of the daughter cell. This discrepancy was resolved by the recent finding that the joining of Loc1p is able to overcome this limitation. Loc1p binds simultaneously and directly to the *ASH1* mRNA as well as to She2p, resulting in a ternary complex with much improved stability and specificity (**Fig. 1A and B**).[57] Although it is unclear where exactly in the nucleus Loc1p joins the complex, it is reasonable to assume that this event should occur as early as possible, perhaps during or shortly after transcription. The requirement of the joint RNA-binding of two proteins is important also in subsequent stages of *ASH1*-mRNP function.

Such a cooperative RNP binding can also be found in the claw frog *Xenopus laevis*. Vg1 RBP/Vera, hnRNP1 and 40LoVe associate with *Vg1* mRNA already in the nucleus and all factors affect vegetal mRNP localization.[109-111] 40LoVe binds the localization element of the *Vg1* mRNA (VLE) with high affinity but low specificity. Mutation or deletion of binding motifs of hnRNP1 and Vg1 RBP/Vera within the VLE abolishes also the binding of 40LoVe.[109,111] Therefore hnRNP1 and/or Vg1 RBP/Vera might be the recruitment factors that provide specificity for the localizing mRNAs.

Specific She2p-She3p mRNP in the Cytoplasm

Since no Loc1p is present in the cytoplasm, the exported She2p-*ASH1* mRNA complex would be unstable in absence of another stabilizing co-factor. This problem

is overcome by the joining of She3p. Besides being a myosin adaptor, the strictly cytoplasmic She3p is a rather unspecific RNA-binding protein that also undergoes protein-protein interactions with She2p.[47] The joining of She2p and She3p with *ASH1* mRNA in the cytoplasm results in the formation of a highly specific, high-affinity co-complex with each of the four zip-code elements of the *ASH1* mRNA (**Fig. 1A**).[47] This synergistic complex together with its associated motor Myo4p and the translational repressors Puf6p and Khd1p constitutes the mature *ASH1* transport complex. Interestingly, there is a gradual increase of binding affinity for the RNA from the co-transcriptional recruitment to the assembly of the mature cytoplasmic transport complex, ensuring directionality of assembly (**Fig. 1B**).

From these recent insights on cargo-RNA binding in the nucleus and in the cytoplasm, it became clear that only the joint RNA binding of two proteins achieves specific zip-code recognition. In the nucleus She2p requires Loc1p and in the cytoplasm She3p takes over from Loc1p to form a highly specific ternary complex with She2p and zip-code RNA. These observations in yeast might serve as an emerging concept for zip-code recognition also in other organisms.

In the *Drosophila* embryo, the apical localization of *fs(1)K10* and *hairy* mRNA depend on dynein-containing particles. This mRNP contains the two interacting proteins Bicaudal D and Egalitarian, which in co-complex show higher affinity and specificity than the Egalitarian mRNP alone.[106] Although similar to the joint binding of She2p and She3p, this effect is less pronounced.

Recent studies on ZBP1 demonstrated how two of its KH domains mediate specificity for *β-actin* mRNA localization.[71,72] Chao and colleagues combined NMR data with a crystal structure to show that this zip-code element is single stranded with a bipartite recognition motif, which is recognized by two KH domains forming a

defined di-domain.[71,72] The zip-code element wraps around this di-domain and binds at two opposing sites of the protein.[72] Whereas each binding motif interacts with one KH domain only with medium specificity, the spacer of defined length ensures the precise coordination of both binding events, thus mediating specificity in RNA recognition.

Interestingly, the overwhelming majority of mRNA-binding proteins in higher eukaryotes have more than one RNA-binding domain (reviewed in ref. 112). For instance the protein Vigilin consists of 14 KH domains and the RNA-binding transcription factor III A (TFIIIA) contains nine zinc-finger motifs. Such proteins can also contain combinations of different types of RNA-binding domains, like the above-described ZBP1, which has four KH domains and two additional RRMs. It has been previously speculated that the combined action of two or more domains within one protein might be required to achieve high specificity.[112]

Complex Reorganization at Nuclear Pore and at Bud Tip

The presence of a specific and stable nuclear mRNP indicates the need for reorganization processes to ensure the formation of the subsequent complex (e.g., mature cytoplasmic complex). Since Loc1p is a strictly nucle(ol)ar protein, it has to be removed from the *ASH1* mRNP prior to its cytoplasmic transport. Indeed, in vitro pull-down experiments and UV-crosslinking studies demonstrated that the cytoplasmic co-complex of She2p and She3p out-completes and displaces Loc1p from the mRNP (**Fig. 1B and C**).[57] As Loc1p binds to both, the zip-code RNA and She2p, the out-competition by She3p must occur in a specific manner at the protein and the mRNA. She3p itself is strictly cytoplasmic and constitutively bound to the type V myosin Myo4p,[24,25,48-51] whereas Loc1p has only been reported in the nucleus.[38,39,45] Thus Loc1p is likely removed from the complex close to the nuclear

pore complex (**Fig. 1C**) during or directly after nuclear export. In cells with a genomic deletion of *SHE3*, the nuclear distribution of Loc1p is altered and a subfraction of Loc1p is observed in the cytoplasm, proving that complex remodeling at the nuclear pore depends on She3p.[57]

Recently it was shown that phenylalanine-glycin repeat domains of yeast nucleoporins (Nup) are necessary for complex remodeling shortly after mRNP export. Nup159 and Nup42 position mRNPs for remodeling by the RNA helicase Dbp5, which triggers the release of mRNP from the transport receptor Mex67-Mtr2 and their export into the cytoplasm.[113] The dependence of nuclear export of She2p on Mex67 indicates that remodeling of the *ASH1* mRNP at the nuclear pore might involve the same molecular pathway.

Once the cytoplasmic mRNPs are assembled, they move toward the plus ends of actin filaments at the bud tip, where translation is activated. About ten years ago it was shown that efficient *ASH1* localization and localized translation involves complex reorganization at the bud tip.[114] This conclusion is consistent with the finding that proper localization at the bud tip requires the mRNA to bear a start codon,[34,115] suggesting that active translation might potentially be involved in anchoring.

More recently two studies demonstrated that both translational repressors, Khd1p and Puf6p, are phosphorylated at the bud tip, thereby reducing their affinity for the *ASH1* mRNA (**Fig. 1C**).[35,37] The posttranslational modifications result in reduced RNA binding, the release of *ASH1* mRNA, and translation of the Ash1p. Phosphorylation-dependent release of localizing mRNAs at their site of destination has also been observed in higher eukaryotes.[116] Hence, it might serve as general principle for translational activation at the end of mRNA transport.

Understanding Motile Activity of Localizing ASH1 mRNPs

In vivo the *ASH1* mRNP moves along actin filaments to the bud tip within 1–2 min,[117,118] where it is anchored.[119] Recently, in vitro reconstitution experiments with single-particle motility assays proved that the core factors Myo4p with its bound calmodulins and light chains, as well as She3p and She2p are sufficient to assemble a functional mRNA-transport complex.[120,121] Directional particle motility was also achieved in vitro when either cargo RNA was absent or when an RNA-binding deficient mutant of She2p was used for complex reconstitution.[120] Thus, the cargo RNA itself is dispensable for processive movement of the transport complex in vitro. Instead, the interaction of She2p with Myo4p-bound She3p activates the motile activity.[120]

Unraveling the exact molecular composition of the *ASH1* mRNP started already in 2007. At this time, it was reported that the Myo4p motor alone is monomeric and non-processive.[49,122,123] She3p binds to the rod of Myo4p and forms a single-headed myosin-She3p complex.[49,123] Other prerequisites for motile activity turned out to be the tetrameric state of She2p[26] and the need for at least two Myo4p molecules in one complex.[124] Subsequently, results from a cryo electron-microscopy study indeed showed that two Myo4p motors form a dimer-like structure in the particle.[125] Today the stoichiometric ratios and absolute numbers of molecules in a fully reconstituted and motile *ASH1* mRNP are known.[120] One She2p tetramer binds two zip-code RNAs, thereby forming an initial nuclear complex. This pre-formed mRNP interacts with two complexes of a She3p dimer coupled to one Myo4p monomer.[120] Recently Shi and colleagues confirmed this She3p-Myo4p heterotrimer with the crystal structure of the C-terminal domain of Myo4p together with the N-terminal part of She3p.[51] The mature SHE core complex has a defined stoichiometric

ratio, consisting of two *ASH1* zip-codes, a She2p tetramer, two She3p dimers and two Myo4p monomers. This complex moves processively along actin filaments (**Fig. 1A and D**).[120] From these studies, one of the most surprising findings was the observation that actin transport complexes can also be assembled in absence of RNA.

In case of dynein-dependent transport in the *Drosophila* embryo, the RNA-cargo seems to play a more active role. Beside the protein levels of Egalitarian and Bicaudal D also the dosage of zip-code elements is responsible for a preferential minus-end directed movement.[126-128]

The ER Inheritance Connection

Interestingly, part of the localizing mRNA pool moves together with ER tubules to the bud.[53,129] Furthermore, some of the proteins required for cortical ER inheritance also affect the bud localization of a subset of mRNAs.[53] Because the majority of these co-migrating RNAs encode proteins involved in membrane biogenesis and function, their co-transport with ER implies a functional connection between ER and mRNA. Although She2p itself is dispensable for ER inheritance, its genomic deletion results in loss of localization of the above-described subset of mRNAs.[43,53] The recent observation that She2p directly associates with membranes independently from She3p or Myo4p[129,130] indicates that membrane-tethered She2p is responsible for binding of mRNAs to the cortical ER (**Fig. 1A and E**).

In membrane-free *ASH1* mRNPs, She3p is required for specific RNA recognition of She2p.[47] In the case of membrane-bound She2p it is still an unresolved issue whether She3p is also required for specific RNA-recognition. A number of recent studies suggest that such tethering of mRNAs to ER in a signal-recognition particle (SRP)-independent fashion is a rather common mechanism (reviewed in ref. 9 and 10). Since cortical ER is inherited into the daughter cell also in a *SHE2*-deficient

strain,[52] a second open question remains how She3p and Myo4p are tethered to the ER (**Fig. 1E**). Complex reconstitution experiments showed that She2p is required for Myo4p dimerization and is thus needed for processive movement (reviewed in ref. 15). It is therefore also unclear how Myo4p is activated in absence of She2p to mediate efficient ER inheritance (**Fig. 1E**).

The Concept of Multimerization for mRNP Localization

In yeast, multiple zip-code elements exist in the *ASH1* mRNA and in few other localizing transcripts.[24,41,42,115,117,131] Considering the observation that each motor-containing SHE complex contains two binding sites for zip-code elements,[120] every mRNA with multiple zip-codes should potentially be able to concatenate SHE complexes into larger particles. This hypothesis is supported by the in vivo observation that each transport particle contains several *ASH1*-mRNA molecules,[132,133] suggesting complex oligomerization also in the cell (**Fig. 1F**). In vitro reconstitution experiments with RNAs bearing two zip-code elements confirmed that zip-code-mediated oligomerization of SHE complexes can occur.[120]

In single particle motility assays Sladewski and colleagues found that mRNAs with multiple zip-code elements show increased processivity of movement.[121] While these particles were produced at non-stoichiometric ratios, a second study with mRNPs assembled with their correct stoichiometries did not find such zip code-dependent differences in motility.[120] Although the latter study suggests that there is no positive influence on processive movement caused by RNA-mediated oligomerization, the possibility exists that due to diverging subcellular localizations such strong sub-stoichiometric ratios could occur in the cell. However, due to the fact that stoichiometrically assembled *ASH1* particles achieved run-lengths and

velocities[120] comparable to in vivo observations,[67,118,119] it is unclear if a positive effect of multiple zip-codes on processivity would be required in vivo.

There could also be other reasons why multimerization by multi-zip code mRNAs is important for a yeast cell. For instance it could be advantageous to co-transport several mRNAs with a similar function. So-called post-transcriptional operons might be better co-regulated if they are transported in the same particles.[134,135] This interpretation would be beneficial for instance for mRNAs being associated with cortical ER. The majority of these mRNAs encode for proteins in membrane biogenesis and function.[53] Double-fluorescence staining in vivo indeed showed that two different RNAs can be co-transported in the same particle,[118] suggesting that co-regulation could indeed occur.

ASH1 mRNA is repressed during its transport by Puf6p and Khd1p.[34-37] For the other localizing mRNAs it is unclear, whether translational repression occurs by Puf6p and Khd1p. It is therefore tempting to speculate on translational repression of a subset of localizing mRNAs exclusively via the previously described multimerization of complexes into larger SHE particles.

The impact of mRNP mulitmerization has already been reported for *oskar* mRNA transport in *Drosophila* oocytes. The binding of the polypyrimidine tract-binding protein/hnRNP (PTB) to multiple sites of the *oskar* 3′UTR mediates the formation of higher-order RNPs and translational repression.[136] The 3′UTR of *oskar* mRNA also contains a dimerization element that interacts via kissing-loop between two RNA molecules and contributes to the enrichment of *oskar* mRNP at the posterior pole of the oocyte.[62] Such an RNA-mediated dimerization might also participate in the above described PTB-dependent translational repression of *oskar* mRNA.

RNA- and DNA-binding proteins show a significant enrichment of low-complexity (LC) regions when compared with the entire proteome.[137] For a long time, the functional implications of such low-complexity regions in RNA- and DNA-binding proteins were not well understood. A recent study found that (G/S-Y-G/S) motif-enriched LC regions in RNA-binding proteins like Fused in Sarcoma/Translocated in Sarcoma (FUS), Heterogeneous nuclear ribonucleoproteins A1 and 2 (hnRNPA1/2), Cytotoxic Granule-Associated RNA Binding Protein 1 (TIA1), Cytoplasmic polyadenylation element-binding protein 2 (CPEB2), or the Fragile X-Mental Retardation Protein (FMRP) can form reversible amyloid-like fibers in vitro.[138] In cells, the LC region of FUS was shown to be required for its localization to stress-granules.[138] Several of the RNA-binding proteins identified in this study are found in sub-cellularly localized mRNPs. These include FMRP, IGF-II mRNA-binding proteins (IMP), HRP40, Barentz, Pumilio, and the hnRNP F/H homolog Glorund. Two subsequent studies demonstrated that the LC region of FUS mediates its RNA-dependent association with RNA polymerase II, indicating that RNA-dependent oligomerization might regulate transcriptional events.[139,140] It appears reasonable to assume that also in localizing mRNPs the LC regions of RNA-binding proteins play a functional role. They could offer a new organizing principle for oligomerization and assembly, and add an additional layer of regulation to this process.

Outlook

It is interesting to see that mechanistic key findings from yeast are conserved in other species. Synergistic RNA binding of zip-codes, the requirement of a nuclear history for proper cytoplasmic mRNA localization, complex reorganization at the nuclear pore and at the site of destination, as well as complex oligomerization of

mRNPs are common principles found not only in yeast. Thus, this model organism will continue to provide essential information for understanding such general principles of mRNA localization.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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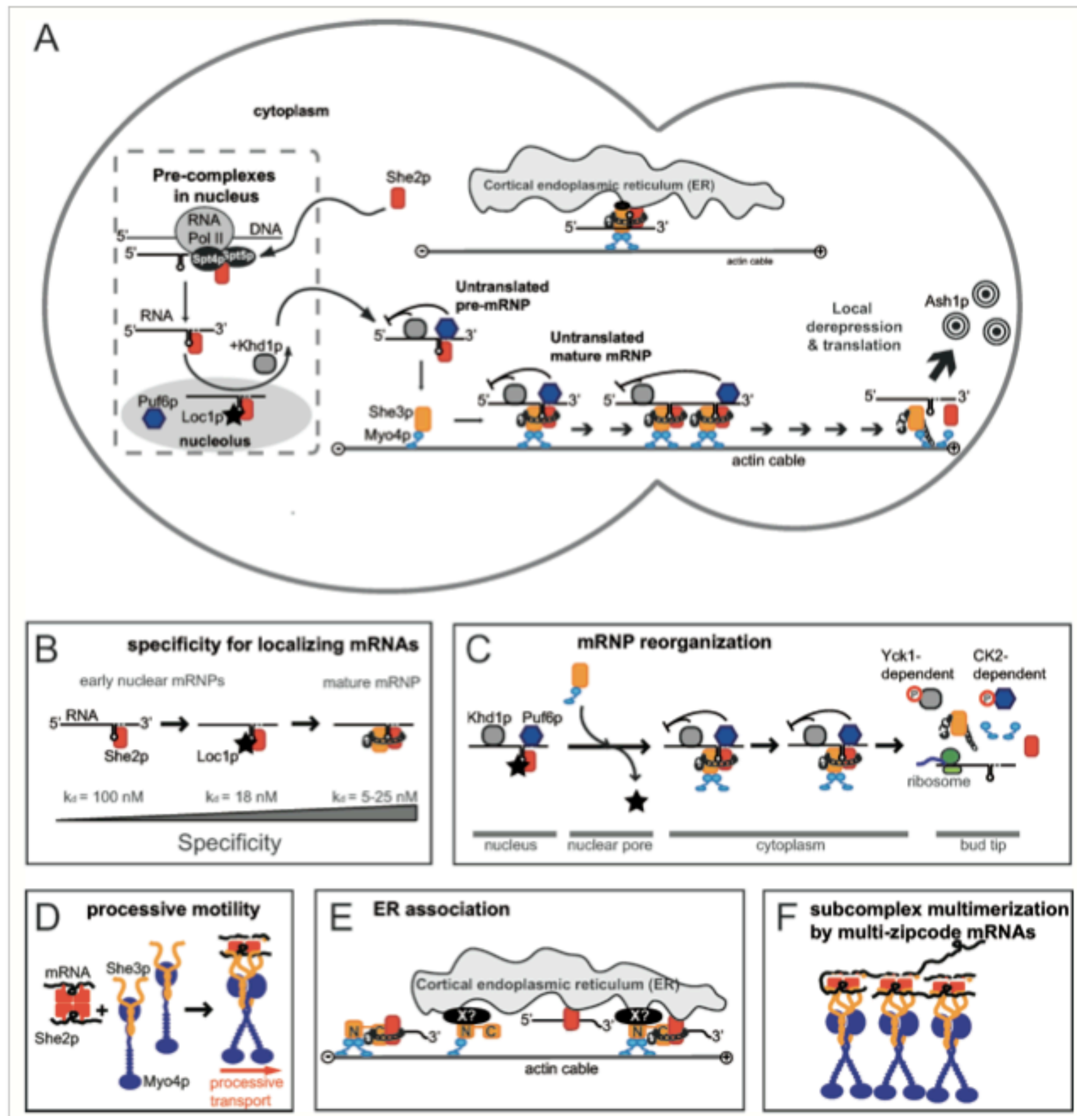

Figure 1. Schematic drawings of *ASH1*-mRNA localization in budding yeast. (**A**) Overview cartoon showing the major components of the transport complex and their sites of action. Left side indicates the mother cell, right side the daughter cell. (**B**) Directionality of *ASH1*-mRNP assembly is warranted by a gradient of affinity and specificity from the initial assembly of the co-transcriptional mRNP to the mature cytoplasmic transport complex. (**C**) Schematic representation of complex reorganization at the nuclear pore and at the bud tip. (**D**) Cartoon showing the stoichiometries and number of molecules assembling into a motile transport complex.[120] (**E**) Scheme of the ER association of the *ASH1* mRNP and its sub-complexes. For a more detailed assessment of ER inheritance, please see the concomitant review by Singer-Krüger and Jansen.

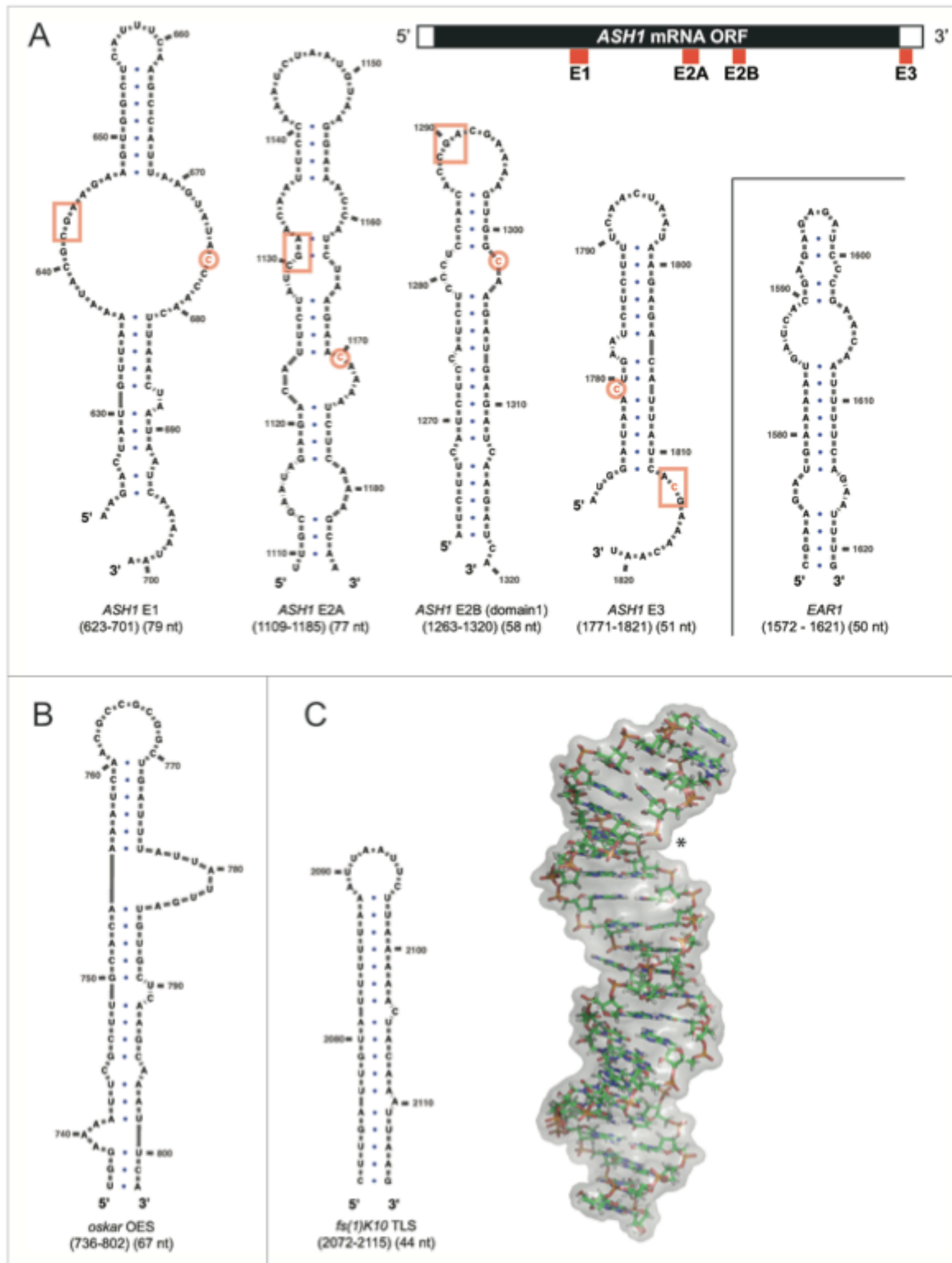

Figure 2. Secondary structures of zip-code elements (**A**) Selection of known zip-code elements bound by the She2p-She3p complex in yeast, as predicted by the mfold web server.[141] Cartoon on top shows that in the *ASH1* mRNA, the zip-code elements E1, E2A, and E2B are part of the open reading frame, whereas E3 is located directly 3′ to the stop codon. Also the *EAR1* mRNA contains an experimentally confirmed zip-code element. In the secondary structure predictions, boxed base triplets and a single encircled cytosine mark a

previously identified consensus recognition motif.[68] Note that the *EAR1* zip-code lacks this motif, that the motif can occur in inversed 3′ to 5′orientation, and that the base triplet is also found in permutations of its sequence. Also the location of the bipartite motif with regard to the secondary structure varies, indicating that the basic features of specific zip-code recognition are still not well understood. (**B**) *OES* secondary structure from chemical probing data.[62,76] (**C**) Secondary structure (left) and three-dimensional NMR structure (right) of *fs(1)K10 TLS* (PDB-identifier: 2KE6).[77] Asterisk shows widened major grove that is required for dynein-dependent localization.