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mRNA localization in metazoans: A structural perspective

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ABSTRACT

Asymmetric localization of mRNAs is a widespread gene regulatory mechanism that is crucial for many cellular processes. The localization of a transcript involves multiple steps and requires several protein factors to mediate transport, anchoring and translational repression of the mRNA. Specific recognition of the localizing transcript is a key step that depends on linear or structured localization signals, which are bound by RNA-binding proteins. Genetic studies have identified many components involved in mRNA localization. However, mechanistic aspects of the pathway are still poorly understood. Here we provide an overview of structural studies that contributed to our understanding of the mechanisms underlying mRNA localization, highlighting open questions and future challenges.

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Introduction

The localization of mRNAs is a gene regulatory mechanism, widespread in eukaryotes, that is crucial for many processes, including patterning of embryonic axes, asymmetric cell division, cell migration and synaptic plasticity (reviewed in Holt and Bullock, 2009; Martin and Ephrussi, 2009).[1,2] Among metazoans, the fruit fly *Drosophila melanogaster* has been most extensively studied, given the genetic accessibility of this system. During *Drosophila* oogenesis and early development, hundreds of mRNAs have been shown to localize in very defined subcellular patterns.[3-5] Among vertebrates, a model system that has been classically used is *Xenopus laevis*.[1,6] Cell cultures of mammalian neuronal cells and fibroblasts are also widely used to explore how mRNA localization contributes to neuronal function and cell motility, respectively. Cultured cells-based systems have allowed transcriptome-wide characterization of mRNA localization. For example, several mRNAs have been shown to be localized in dendrites, in axons or in cell protrusions in these systems.[1,6] Many mRNA-localizing factors initially identified in *Drosophila* have homologs in vertebrates, including Staufen,[7-9] Bruno,[10,11] Bicaudal D (BicD),[12] ZBP1[13-15] and Pumilio (Pum).[16] In vertebrates some of the proteins that are involved in mRNA localization in *Drosophila* are also involved in mRNA localization, for example, mammalian Staufen and Fragile X Mental Retardation Protein (FMRP) are required for mRNA localization in neurons,[17-20] and the Exon Junction Complex (EJC) component eIF4AIII is involved in dendritic mRNA localization.[21] Genetic studies in *Drosophila* have provided detailed part-lists of mRNA localization components. However, our mechanistic understanding lags behind the genetic characterization of this pathway.

Here we review the contribution of structural studies to the mechanistic understanding of mRNA localization. We will discuss how target mRNAs are recognized, repressed and transported to their target site within the cell. We will only briefly touch on RNA recognition mediated by canonical RNA-binding domains (such as KH, RRM, dsRBD), for which we refer to more focused reviews.[22-24] We will also not address the mechanisms of motor function, mRNA localization studies in fungi, nor provide an in-depth description of pathways intersecting with mRNA localization (e.g. mRNA decay), which have been reviewed elsewhere.[25-28]

Steps in mRNA localization

The key step in mRNA localization is the specific recognition of the transcript, which depends on *cis*-acting elements, generally found in 3′ untranslated regions (3′UTR) of mRNAs (reviewed in Besse and Ephrussi, 2008; Meignin and Davis, 2010; Medioni et al., 2011)[6,29,30] (Fig. 1). These elements are recognized by *trans*-acting factors, or RNA-binding proteins (RBPs). Many RBPs contain several domains, that can contribute to specific target recognition or mediate the recruitment of additional protein factors.[31] In the case of active localization, mRNAs are transported along the cytoskeleton either by dynein or kinesin motor proteins (mediating microtubule minus end- or plus end-directed transport, respectively) or myosins (which move along actin filaments). Alternatively, localized expression can be achieved by selective translational repression or degradation of the target mRNA in parts of the cell. Often, the localized messenger ribonucleoprotein particle (mRNP) is maintained at the destination site by anchoring to the cytoskeleton, generally actin filaments. Translational repression of the mRNP before localization is fundamental for both active and passive

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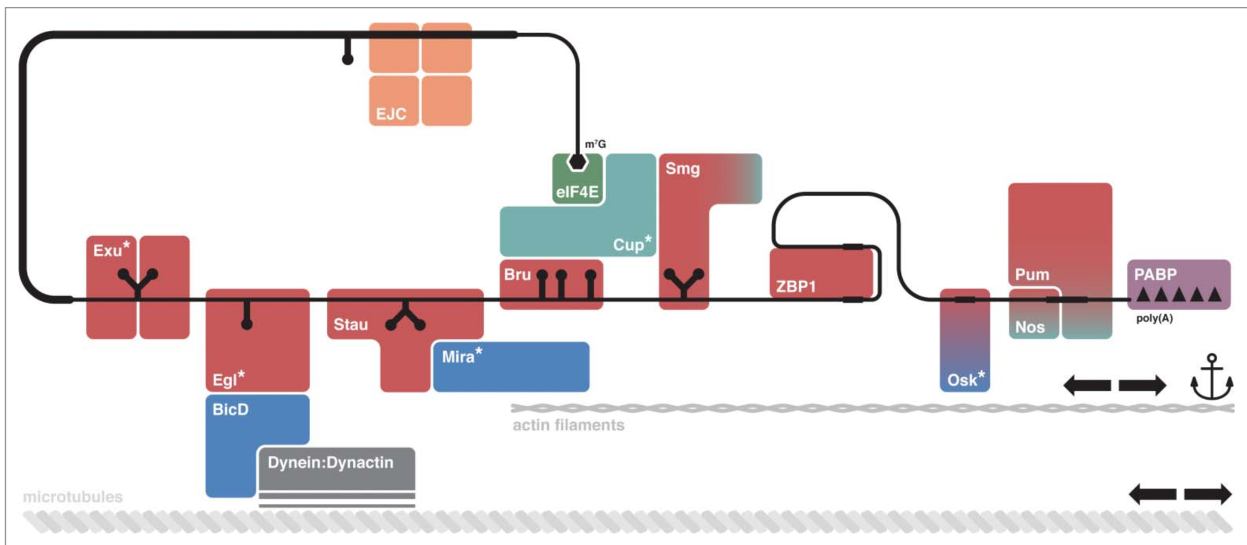

Figure 1. mRNA localization requires multiple factors. The mRNA to be localized is recognized by RNA-binding proteins (RBPs, in red) that interact with linear or structured localization signals, or *cis*-acting elements, on the mRNA. These RBPs can recruit adaptor proteins (in blue), which mediate anchoring or transport of the localizing messenger ribonucleoprotein complex (mRNP) along the cytoskeleton through the action of molecular motors (in gray). Other proteins (in teal) are required to maintain the mRNP in a translationally repressed state, for example by competing directly with components of the translation machinery (e.g.: Cup) or by recruiting the CCR4:NOT complex to promote shortening of the poly(A) tail on the target mRNA (e.g.: Smg, Nos, Pum). Special RBPs are represented by the Exon Junction Complex (EJC, in orange), the cap-binding protein eIF4E (in green) and the Poly(A)-Binding Protein (PABP, in purple). Asterisks (*) indicate proteins with no known ortholog in vertebrates. Not all of the proteins depicted here will associate at the same time point, or to the same mRNA.

localization mechanisms, to prevent protein expression at ectopic sites in the cell. Such repression may persist at the destination site (e.g., during *Drosophila* oogenesis), until it is relieved by an extrinsic cue. This translational activation is currently one of the least understood steps of the mRNA localization pathway.

RNA localization signals

A variety of mRNA localization signals has been identified to date, ranging from linear sequences to defined secondary structures. These signals are critical for providing recognition sites for proteins of the localization machinery.[32-34]

Recognition of linear signals

One of the best characterized examples of a linear localization signal is the zipcode element on *β-actin* mRNA, recognized by the Zipcode-Binding Protein 1 (ZBP1).[13,35] ZBP1 regulates local translation of *β*-actin mRNA at the base of activated dendritic spines in hippocampal neurons.[36] The crystal structure of human ZBP1 RNA-binding domains (hnRNP K homology, or KH domains) revealed an intramolecular pseudo-dimer arrangement that positions the RNA-binding surfaces on opposing faces of the protein. This induces the looping of the bipartite zipcode element around ZBP1 (Fig. 1, 2a). Binding of both zipcode elements enhances the affinity of ZBP1 for β-*actin* mRNA. Since the 2 zipcode elements can only bind if they are separated by a spacer sequence of defined length, this ensures specificity in mRNA recognition.[37] The occurrence of multiple domains to obtain high RNA-binding affinity and specificity, often combined with the remodelling of the target mRNA (as in the β-*actin* mRNA and ZBP1), is a recurrent feature of RNA-protein complexes (reviewed in Lunde et al., 2007).[31]

Another example is provided by the CUG-binding protein 1 (CUGBP1). The human CUGBP1 protein is a member of the CUGBP1 and ETR-like factors (CELF) protein family, which includes the *Drosphila* translational repressor Bruno (Bru). CELF family members share a conserved domain architecture, characterized by two adjacent RNA recognition motifs (RRMs) in the N-terminal region, and a third C-terminal RRM, which is separated from the others by a long, non-conserved, linker.[10] The two N-terminal RRM domains of CUGBP1 bind with similar affinities to their respective target sequences (UGUU/G). NMR studies indicate that the two domains tumble independently in solution in the absence of the RNA substrate.[38] However, in the presence of an RNA molecule comprising two recognition signals the two RRM domains are found in a compact and rigid arrangement (Fig. 2b), which explains the observed binding cooperativity.[38] While the short linker between RRM1 and RRM2 of CUGBP1 favors cooperative binding of the two domains on adjacent sequences on the target mRNA, the longer, and probably disordered, linker that connects RRM3 could allow for recognition of a more distant sequence element, perhaps belonging to a different mRNA molecule. This mechanism could help in creating a large messenger ribonucleoprotein particle (mRNP) for transport, and would also contribute to maintaining the translational repression of the target mRNA, as has been proposed for *Drosophila* Bru in *oskar* (*osk*) mRNA regulation.[39] In addition, such unstructured regions can cooperate in RNA binding: the linker region N-terminal to the third RRM in both human CUGBP1 and *Drosophila* Bru extends the binding surface of the RNA-recognition motif, which increases RRM3 affinity for its target mRNA.[40,41]

Another way to achieve recognition of an RNA sequence element with high affinity and specificity is by combining multiple copies of a simple repeated structural motif within a protein domain. This approach is used by members of the Pumilio

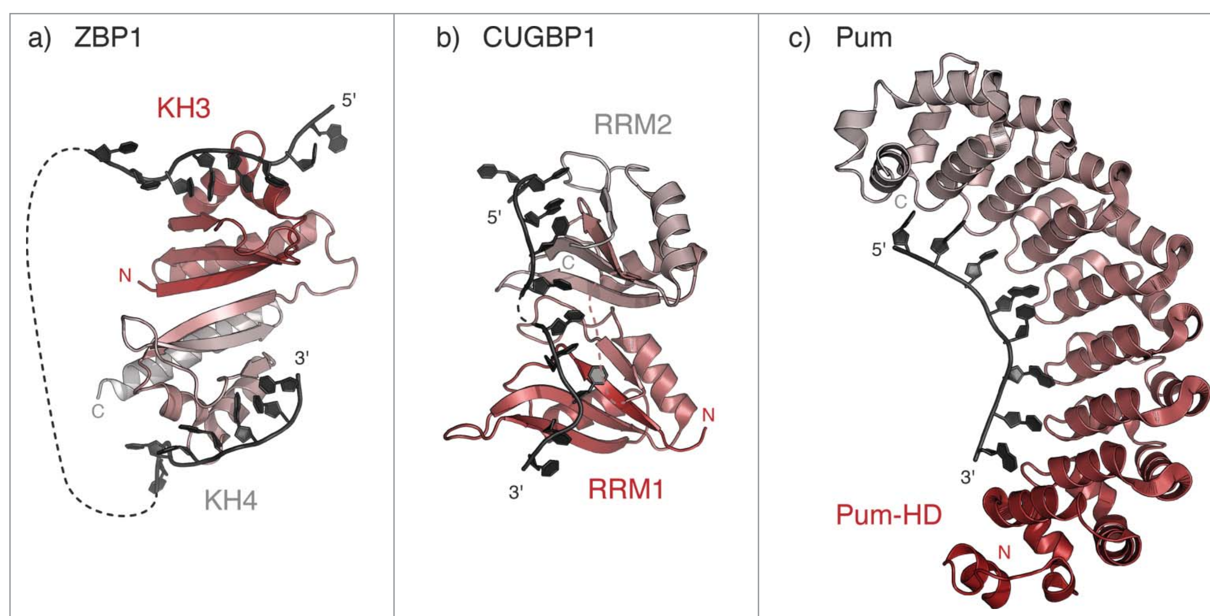

Figure 2. Recognition of linear localization signals. (a) Model of RNA binding by KH3 and KH4 domains of the human ZBP1 homolog IMP1 (PDB 3KRM[37]), showing the pseudo-dimer arrangement that positions the RNA-binding surfaces on opposing directions of the structure. The RNA molecules bound to KH3 and KH4 are derived from the structure of *Gallus* ZBP1 (PDB 2N8L and 2N8M, respectively[128]), superposed using the cealign command in Pymol v1.7 (www.pymol.org). (b) Model of RNA binding by RRM1 and RRM2 domains of human CUGBP1 (PDB 3NNH and 3NMR, respectively). The relative orientation of the two RRM domains is modeled as described in Teplova et al., 2009[38]. (c) Crystal structure of *Drosophila* Pum in complex with the Pum Recognition Element (PRE) from *hunckback* (*hb*) mRNA (PDB 5KLA[76]).

and *fem-3* mRNA binding factor (Puf) protein family, of which *Drosophila* Pumilio (Pum) is the founder (reviewed in Wickens et al., 2002).[16] Puf proteins are characterized by the presence of a sequence-specific RNA-binding domain known as the Pum homology domain (Pum-HD), comprising 8 sequence repeats of three helices each, plus N- and C-terminal flanking regions.[42,43] This helical domain is reminiscent of the Armadillo repeat domain, a protein-protein interaction module found in a wide range of proteins with diverse functions, including nucleo-cytoplasmic transport, intracellular signaling, and cytoskeletal organization (reviewed in Hatzfeld et al., 1999; Coates, 2003).[44,45] The Pum-HD domain contacts RNA through its concave surface, where each nucleotide is recognized by a triumvirate of amino acid side chains at conserved positions within the helical repeats[46] (Fig. 2c). In addition, the Pum-HD domain can interact with the CCR4:NOT deadenylation complex and promote shortening of the poly(A) tail on the target mRNA.[47,48] In this way, Puf proteins control stability and translation of a variety of different mRNAs, recognized through a Pum response element (PRE) in their 3′UTRs.

Recognition of structured signals

Given the difficulty in predicting tertiary or even secondary structures of RNAs with high reliability, the characterization of structured localization signals has proven more challenging and relies on experimental verification.[49] This is well exemplified by the *Drosophila fs(1)K10* Transport and Localization Signal (*K10* TLS), which mediates minus-end-directed, dynein-dependent transport along the microtubules during early *Drosophila* development. The structure of this localization element, derived by NMR spectroscopy, has revealed a stem-loop conformation in which purine-purine stacking within the double stranded (ds) region forces the stem to adopt an unusual A′-form conformation. This conformation has 2 widened major grooves which are oriented at 90° to one another in contrast to the standard A-form dsRNA where major and minor grooves have similar widths (Fig. 3). This specific arrangement (reminiscent of B-form dsDNA) is required for the localization of K10 TLS-containing mRNAs *in vivo*[33] (Fig. 3a). Other mRNAs that are transported at various stages during *Drosophila* oogenesis and early development toward the minus end of microtubules also contain localization signals that could form hairpins of similar structure but of different primary sequence as the *K10* TLS.[33] This suggests that widened major grooves in dsRNA, rather than being used for primary sequence recognition, represent an unusual structural feature that can be sensed by a common localization machinery. Indeed, several of these elements, including the *K10* TLS, are recognized by the protein Egalitarian (Egl).[50] Egl does not contain a canonical RNA-binding domain, but rather contacts RNA through a large region including a domain that displays homology to 3′-5′ DEDD exonucleases (Exo-domain), which usually catalyze the exonucleolytic cleavage of nucleic acids.[50,51] Mutation of the putative catalytic residues within Egl Exo-domain does not affect mRNA localization *in vivo* or RNA-binding activity *in vitro* indicating that, if the protein is a functional exonuclease, this activity is dispensable for protein function.[50,52] Similarly, the protein Exuperantia (Exu) uses a catalytically inactive Exo-domain to bind and localize *bicoid* (*bcd*) mRNA during early oogenesis in *Drosophila*.[53] Both Egl and Exu recognize mRNA signals with defined secondary structures, though the molecular details of the interaction are not known.

To date, there are very few examples of structured localization signals in complex with RNA-binding proteins. One is provided by the RNA-binding domain of yeast Vts1 in complex

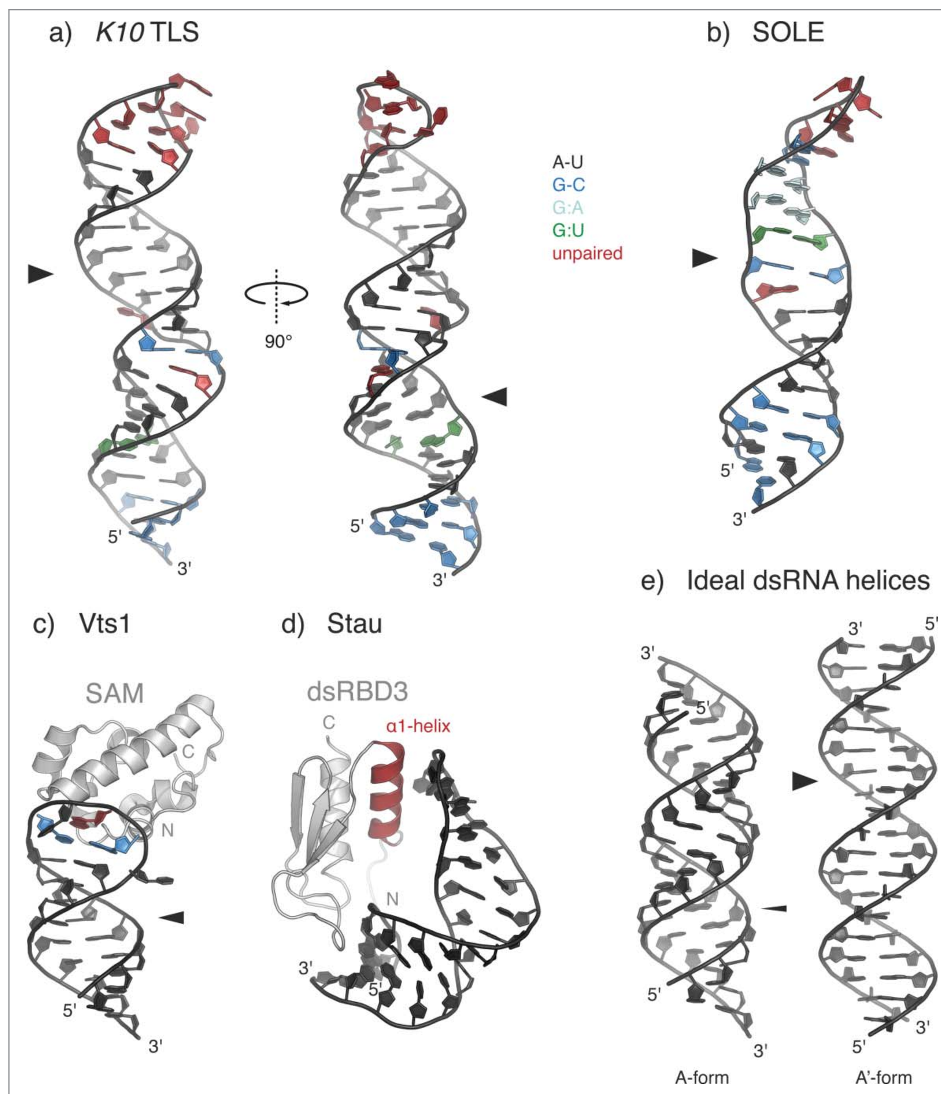

Figure 3. Recognition of structured localization signals. (a-b) Solution structures of the Transport and Localization Signal from *K10* mRNA (*K10* TLS; a) (PDB 2KE6[33]) and of the SOLE hairpin from *osk* mRNA (b) (PDB 5A17[70]). A-U base pairs are shown in black; G-C base pairs in blue; G:A base pairs in cyan; G:U base pairs in green; unpaired residues (loops and bulges) are highlighted in red. Both stem-loops present widened major grooves (indicated by broad arrowheads) as compared with the common A-form of dsRNA helices (e). (c) Solution structure of yeast Vts1 Sterile α motif (SAM) bound to the Smg Recognition Element (SRE) (PDB 2ESE[58]). The loop residues forming a base pair are colored in blue; the specifically recognized G residue at position 3 of the loop is highlighted in red. The position of the major groove is indicated by an arrowhead. In the case of SRE RNA, the major groove has an intermediate width between that of the K10 TLS and that of an ideal A-form dsRNA and it is not involved in Smg/Vts1 recognition.[54] (d) Solution structure of the third dsRNA-binding domain (dsRBD3) of *Drosophila* Stau bound to an artificial stem-loop sequence (PDB 1EKZ[68]). The N-terminal α-helix (α1-helix), which is potentially involved in sequence-specific contacts with the RNA loop, is highlighted in red. (e) Cartoon representation of ideal dsRNA structures (generated in Coot[129]), assuming the canonical A-form (left) or the unusual A'-form conformation (right), which is reminiscent of the B-form of dsDNA. Major grooves are indicated by arrowheads (thin for A- and broad for A'-form, respectively).

with the Smaug (Smg) Recognition Element (SRE) RNA (Fig. 3c). Vts1 binds RNA through its Sterile α motif (SAM), a domain consisting of 5 α-helices arranged in a globular bundle, that was previously thought to be solely a protein-protein interaction motif.[54,55] The SRE consists of a stem-loop structure, but only the 5 residues of the loop (CUGGC) are directly contacted by a shallow, positively-charged surface patch on the SAM domain. The only residue that is specifically recognized is the G at position 3 within the pentaloop, while the other protein-RNA interactions involve non-sequence specific contacts with the RNA phosphate oxygens.[56-58] However, the formation or stabilization of a base pair within the pentaloop upon protein binding also seems to be important, suggesting that the G3 nucleotide base within the loop is recognized within a specific structural context.[56,57] The sequence of the stem, as long as base-pairing is preserved, does not influence Vts1 binding affinity.[54] Yeast Vts1 is a homolog of *Drosophila* Smg, a protein that contributes to anterior-posterior axis determination in the *Drosophila* embryo. Smg represses *nanos* (*nos*) mRNA translation everywhere in the embryo but at the posterior pole plasm. This generates a gradient of Nos protein emanating from the posterior of the embryo. Such gradient is required to trigger a series of downstream events that result in proper abdominal segmentation.[59] Smg also binds mRNA by its SAM domain, suggesting evolutionary conservation, but the details of this interaction are not known. A SAM-like domain is also present in Exu and mutational analysis suggests that it is important for RNA binding. However, in Exu there is an additional surface not present in Smg or Vts1 that contributes to mRNA binding.[53]

Another conserved mRNA-localization protein, Staufen (Stau), presents a distinct RNA-binding mechanism. Members of the Stau protein family are important for mRNA localization in many organisms and contain several copies of dsRNA-binding domain (dsRBD), a domain that was first identified in the *Drosophila* Stau protein.[7] In *Drosophila*, Stau is required for *oskar* (*osk*) mRNA localization at the posterior pole during oogenesis, for *bcd* mRNA anchoring at the anterior pole in late oogenesis and early embryogenesis, and for *prospero* (*pros*) mRNA transport during neuroblast asymmetric cell division.[60-64,65,66] Structural characterization of the third dsRBD of *Drosophila* Staufen in complex with a non-physiologic stem-loop RNA sequence showed that the dsRBD3 interacts with the RNA sugar-phosphate backbone, without making direct contacts with the RNA bases. Some sequence specificity could be provided by the interaction of the first α-helix with the nucleotide bases in the hairpin loop[67,68] (Fig. 3d), although structural studies on a physiologic substrate would be required to confirm this.

For other localized mRNAs, the protein factors required for the recognition of their structured localization signals are still unidentified. This is the case of the Spliced *osk* Localization Element (SOLE)[69,70] (Fig. 3b). The SOLE is generated upon splicing of the first intron of *osk* and it is required for plus end-directed, posterior localization of *osk* mRNA during oogenesis in *Drosophila*.[69] *Osk* localization also requires the deposition of the EJC upstream of the first exon-exon junction. The isolated SOLE assumes a stem-loop structure capped by a 5 nucleotide loop, and, like the *K10* TLS, displays a widened major groove.[70] A protein binding partner of the SOLE has yet to be identified.

Combinatorial regulation mechanisms (The importance of a bigger picture)

In many of the examples described above, the specificity of the RNA-binding proteins (such as Vts1[56], Egl,[50] Stau,[23] Exu[53]) appears to be less stringent *in vitro* than *in vivo*.[31,71] This could be due to the use of isolated RBDs in *in vitro* assays. The inclusion of multiple domains, where present, would be important in future studies to better approximate *in vivo* conditions. In other cases, the binding affinity and specificity of an RNA-binding protein (RBP) for its target mRNA is modulated by the interaction with a protein partner. Such cases highlight the importance of structural studies focusing on multiprotein complexes together with their bound mRNA targets. The effects of partner proteins on mRNA binding could be mediated by partner-dependent stabilization of RBPs, as has been proposed for *Drosophila* Egl and Bicaudal D (BicD).[50,72,73] Alternatively, binding partners could alter RBP binding specificity either by promoting conformational changes or by contributing additional surfaces for binding. A striking example is provided by the complex of Pum and Nanos (Nos). These proteins co-regulate several mRNAs in *Drosophila*, including repression of maternal *hunchback* (*hb*) mRNA at the posterior pole of the embryo, and of *Cyclin B* (*CycB*) mRNA in primordial germ cells and germline stem cells.[74,75] The crystal structure of the Pum:Nos complex bound to *hb* and *CycB* recognition elements revealed that Nos not only induces conformational changes in Pum that increase its affinity for RNA, but also directly contacts the RNA upstream of the Pum canonical recognition sequence[76] (Fig. 4a–b). In this way, Nos adds upstream recognition specificity, thereby relaxing the requirement for a perfect Pum-binding consensus sequence. The Pum:Nos complex can thus regulate a broader range of mRNA targets than Pum alone. Cooperativity is also exhibited by the proteins Sex-lethal (Sxl) and Upstream of N-Ras (Unr). In *Drosophila* females, the two proteins bind adjacent sequences in the 3′UTR of *male-specific lethal 2* (*msl2*) mRNA, which encodes for the limiting component of the dosage compensation complex and repress its translation.[77] The crystal structure of the Sxl:Unr:*msl2* mRNA ternary complex showed how the 5′ end of the RNA sequence is recognized by Sxl, while the 3′ end is sandwiched by both proteins. The formation of such a "triple zipper" allows additional protein:RNA contacts that would not be possible with either protein alone[78] (Fig. 4b–c).

Transport and anchoring

Long-range active transport usually depends on microtubules, with kinesin and dynein motors mediating plus end- and minus-end directed movement, respectively. There are very few instances where all biochemical links between the localizing mRNA and the molecular motor have been identified. These include the complex linking *ASH1* mRNA to myosin 4 motor during localization to the bud of *Saccaromyces cerevisiae* (reviewed in Niedner et al., 2014)[26] and *Drosophila* BicD, a protein linking localizing mRNAs to the dynein motor during minus end-directed mRNA localization (reviewed in Hoogenraad and Akhmanova, 2016)[79] (Fig. 5a). BicD is the founder of a conserved family of motor adaptor proteins, which, in addition to coupling the dynein motor to various cargoes, stimulates processive dynein motility by stabilizing dynein interaction with its constitutive cofactor dynactin.[80-82] While the N-terminal 2 coiled-coil domains of BicD associate simultaneously with dynein and dynactin, stabilizing their interaction,[82] the C-terminal part of BicD can recognize different protein partners, that in turn mediate the transport of specific cargoes.[83] In *Drosophila*, one of the most important partners of BicD is Egl, which is required for dynein-dependent localization of a variety of transcripts.[50] Another BicD-associated factor is FMRP, which has an important role in mRNA localization in neurons and is mutated in the most common inherited form of cognitive deficiency in humans.[84] All existing data point to BicD binding to one partner at a time, and in some cases through overlapping interaction surfaces.[83] Though we can draw a link between localizing mRNPs and dynein-mediated transport, little is known about kinesin-mediated transport.

Another key adaptor protein in mRNA localization is Miranda (Mira). Mira is required for the segregation of the cell fate determinants Brain tumor (Brat), Prospero (Pros) and Numb to the basal cortex during the asymmetric cell division of neuroblasts in *Drosophila*.[66,85-88,89,90] In addition, Mira directly interacts with the dsRNA-binding protein Stau to localize *pros* mRNA in an actin-dependent manner both in neuroblasts and epithelial cells[66,88,91] (Fig. 5b). In this case, the connection with the cytoskeletal motor is still unknown.

The mechanism by which *osk* mRNA is anchored at the posterior cortex during *Drosophila* oogenesis and early

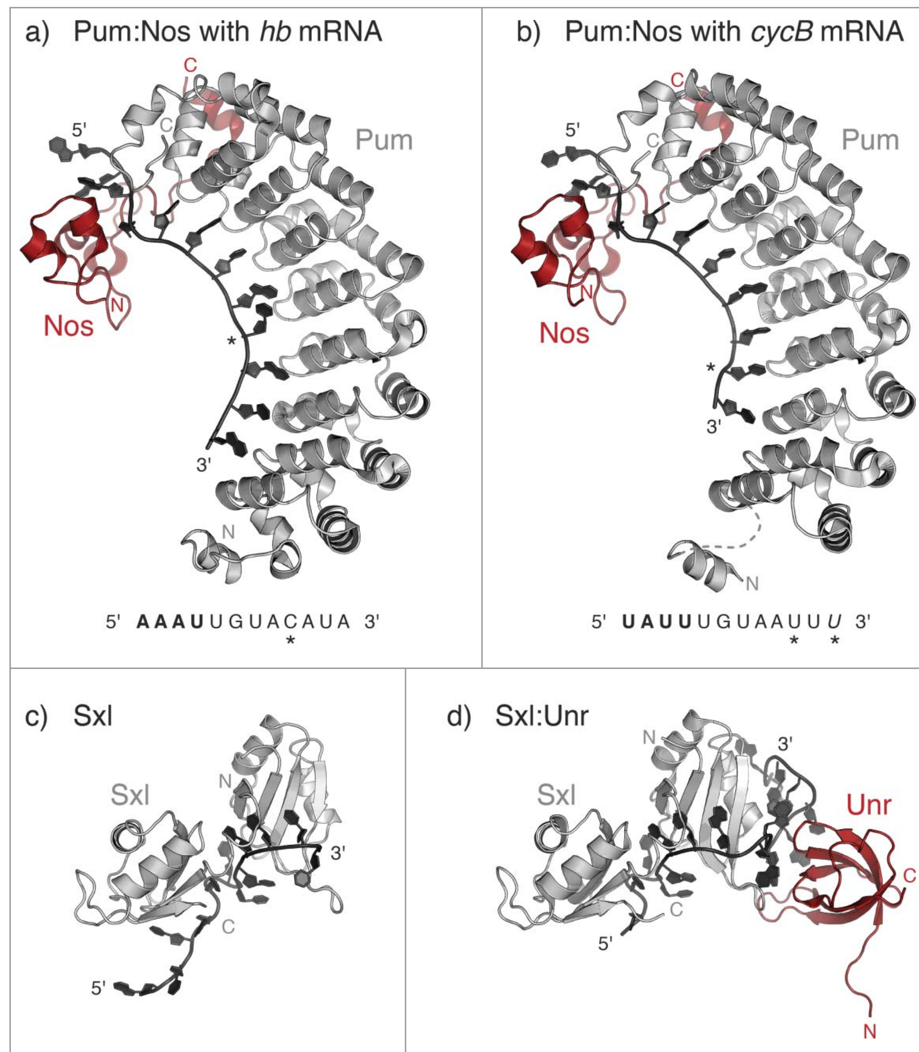

Figure 4. Examples of combinatorial control. (a-b) Crystal structure of the *Drosophila* Pum:Nos complex bound to the Pum Recognition Element (PRE) from *hunckback* (*hb*) (a) (PBD 5KL1) or *cyclinB* (*cycB*) mRNA (b) (PDB 5KL8[76]). The RNA sequence is written under the corresponding structure, with the nucleotides recognized by Nos in bold; asterisks indicate nucleotides that deviate from the PRE consensus. The U residue at the 3′ end of *cycB* PRE (in italic) is not visible in the structure. (c) Crystal structure of the two RRM domains of Sxl in complex with the Sxl-binding element from *transformer* (*tra*) mRNA (PDB 1B7F[130]). (d) Crystal structure of the ternary complex between Sxl, Unr and the RNA recognition element from *male-specific lethal 2* (*msl2*) mRNA (PDB 4QQB[78]). Unr binds RNA through its Cold Shock domain.

embryogenesis is also poorly understood. At the posterior pole, two Osk protein isoforms are translated from alternative start codons: Short Osk is necessary and sufficient to induce pole cell formation and posterior patterning in the embryo, while Long Osk is required for proper anchoring of both *osk* mRNA and Short Osk to the posterior cortex of the oocyte.[92-95] Short Osk recruits the DEAD box helicase Vasa through its N-terminal LOTUS domain to initiate germ plasm assembly.[94,96] In addition, short Osk associates with germ plasm localized mRNAs, including its own and *nos* mRNA.[96,97] Analogous to Egl and Exu, Osk binds RNA through an enzyme fold: the C-terminal OSK domain, which resembles a SGNH hydrolase, but lacks the catalytic residues.[96,97] The RNA sequence specificity of Osk has not been yet determined. In addition to the LOTUS and OSK domains, Long Osk contains an N-terminal extension, which is required, but not sufficient, for the posterior anchoring of *osk* mRNA.[95] Despite the presence of the LOTUS domain, Long Osk cannot recruit Vasa, suggesting that the N-terminal extension is somehow modulating the function of the other Osk domains.[92,94,96] Instead, Long Osk stimulates clathrin-mediated endocytosis and contributes to the organization of the actin cytoskeleton at the posterior of the oocyte, promoting its own and Short Osk maintenance at the posterior pole through a poorly understood mechanism.[98]

Translational repression and selective degradation

Translational repression mechanisms frequently target translation initiation, which is generally rate-limiting. During initiation, the scaffolding factor eIF4G interacts with the cap-binding protein eIF4E and the Poly(A)-Binding Protein (PABP), to connect 5′ and 3′ end of the mRNA in a closed loop conformation that is thought to stimulate translation; in addition, eIF4G interacts with eIF3, which in turn recruits the small ribosomal subunit (reviewed in Jackson et al., 2010).[99] The eIF4E:eIF4G interaction is targeted by a conserved class of translational repressors, the eIF4E binding proteins (4E-BPs) (reviewed in Topisirovic et al., 2011).[100] A member of this family is the *Drosophila* protein Cup, which is required during

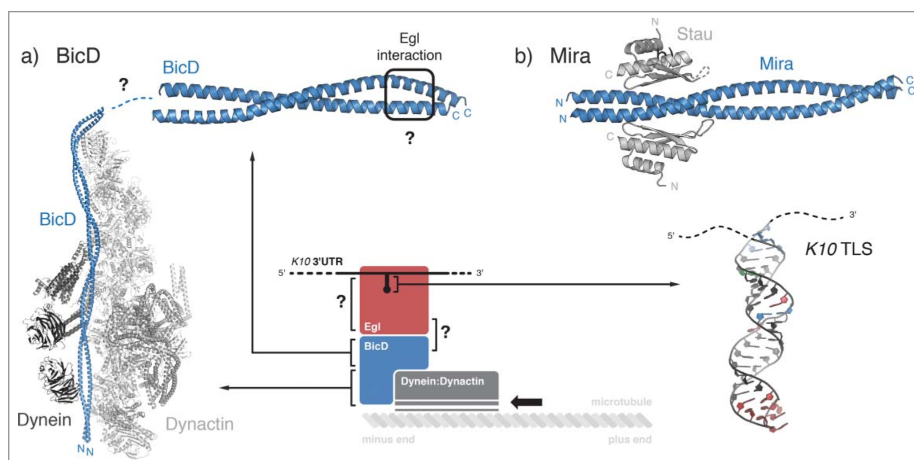

Figure 5. Transport and anchoring. (a) Schematic representation of Egl-BicD mediated transport of *K10* mRNA. Structural information is currently available for *K10* TLS (PDB 2KE6[33]); for the first and second coiled coil regions of BicD in complex with the Dynein:Dynactin molecular motor (PDB 5AFU[82]); and for the C-terminal coiled coil region of BicD, with the Egl-binding site defined by mutation analysis (PDB 4BL6[83]). Still unknown are the molecular details of Egl interaction with the RNA and with BicD, as well as the connection between BicD N-terminal and C-terminal coiled coil domains. (b) Crystal structure of a fragment of Mira coiled coil region bound to the fifth dsRBD (dsRBD5) of Stau (PDB 5CFF[91]). BicD and Mira both act as homodimers.

oogenesis and early development for repressing translation of the axis-determining factors *osk, nos*, and *gurken* (*grk*). Cup does not contact RNA, but is recruited to its mRNA targets by different RBPs (Bru in the case of *osk* and *grk*; Smg for *nos).*[101-104] The crystal structure of an N-terminal fragment of Cup in complex with eIF4E revealed how Cup interacts through two binding motifs (one canonical and one non-canonical), and engages the same surfaces of eIF4E responsible for interaction with eIF4G[102,104-106,107,108] (Fig. 6). In addition, Cup binding stabilizes eIF4E and increases its grip on the target mRNA, thus protecting it from decapping and degradation.[107,108] Alternatively, translation initiation can be inhibited by preventing the recruitment of the large ribosomal subunit, as described for ZBP1.[36]

Other proteins repress translation of their target mRNA by recruiting the CCR4:NOT deadenylation complex: examples include Cup, Bicaudal C (BicC), Smg, Pum and Nos.[109,47,108,110] For the recruitment of the CCR4:NOT complex, at least some of these proteins rely on short linear motifs embedded in peptide regions of predicted disorder (e.g., Nos[111]). Interestingly, many RNA- and DNA-binding proteins show a significant enrichment in low complexity regions when compared with the entire proteome.[112] In addition to providing quickly-evolving interaction surfaces, these regions have the potential for forming reversible amyloid-like fibers *in vitro*[113]; *in vivo*, the same sequences could mediate oligomerization and assembly of large particles that would reinforce translational repression by steric exclusion of the ribosomes, as proposed for *osk* mRNA.[39]

Indeed, to ensure efficient repression of the localizing mRNA, translation is usually inhibited at multiple steps. Complete repression of *nos* expression, for instance, requires both Cup-mediated inhibition at the initiation step[104] and Smg-mediated deadenylation.[109,114]

Translational activation

How mRNA recognition factors, translational repressors and localization machinery could dissociate from the localized mRNP is a critical but poorly understood step in the mRNA localization pathway. Release could be mediated by targeting the localizing factor for degradation when it is no longer needed, as has been shown for Mira.[88] Another mechanism involves dissociation of the RBP from the recognition element on the mRNA. For example, the majority of *nos* mRNA is

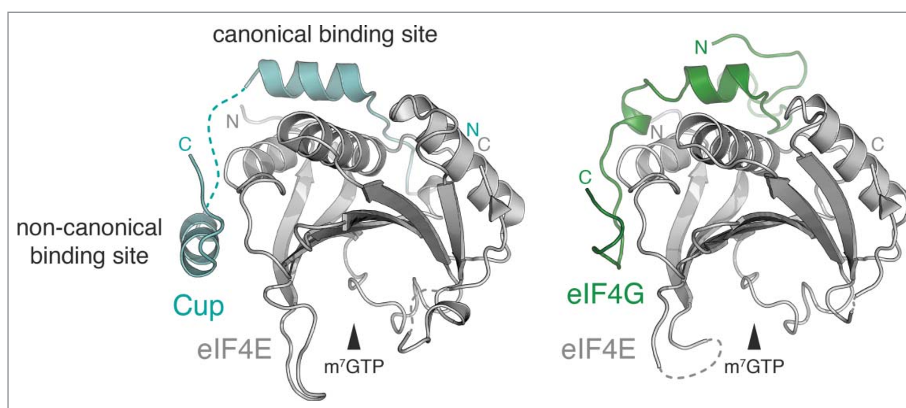

Figure 6. Translational repression. Crystal structure of *Drosophila* eIF4E in complex with a peptide from Cup (left) (PDB 4AXG[107]) or eIF4G (right) (PDB 5T47[131]).

evenly distributed throughout the *Drosophila* embryo and translationally repressed in a Smg-dependent manner.[104,109,114] However, at the posterior pole, Osk protein prevents Smg binding to *nos* mRNA, thereby allowing its translation.[114-116] The molecular details of this regulation are still unclear. Osk could bind Smg directly, and perhaps alter its affinity for RNA[115]; alternatively, Osk and Smg could compete for the same binding site on *nos* mRNA, as suggested by the discovery that Osk is also an RNA-binding protein.[96,97]

Post-translational modifications, such as phosphorylation, also seem to play an important role. The affinity of 4E-BPs for eIF4E, for example, is decreased by the phosphorylation of 4E-BPs at multiple sites.[117,118] Analogously, Src-dependent phosphorylation of ZBP1 relieves translational repression of *β-actin* mRNA by disrupting ZBP1:RNA interaction,[36] though the molecular details of this regulation are still unclear.

Future perspectives

Structural studies have greatly advanced our understanding of the mechanisms underlying mRNA localization. However, many issues remain. The challenge for the future is to understand the assembly and dynamics of the large multiprotein-RNA complexes involved in mRNA localization.

First, structural studies will have to tackle larger multimolecular assemblies. RBPs often contain multiple domains that combine protein- and RNA-binding activities, and, due to their complexity and flexibility, offer a challenge for structural studies. Moreover, many localizing mRNAs assemble in multimeric complexes, through both protein- (e.g., Exu, Bru, Osk, BicD, Mira) and RNA-mediated dimerization (e.g., *bcd*). The increasing number of examples in which combinations of structural motifs, protein domains and protein interaction partners are exploited to modulate RNA-binding affinity and specificity, however, prompts for an effort in the characterization of more physiologic complexes. It will be challenging to characterize stable assemblies and to purify them in suitable amounts for structural studies. Cryo-EM approaches hold promise to solve large assemblies at high resolution and with the need of less material. However, inherent flexibility and heterogeneous composition of the targets remains an issue across techniques when tackling large assemblies.

Localizing mRNP composition changes at the different steps of gene expression. Studies of single molecule dynamics are particularly promising to gain mechanistic insights of mRNA localization at high resolution. These studies provide a direct readout of the spatial and temporal details of localization. It will be particularly challenging to integrate information from these kinds of studies with the static information derived from structural studies. In this respect, structural snapshots of the same component/complex in different cellular states are very informative although technically very challenging.

A third avenue will be to integrate the wealth of genetic information with structural and single molecule studies. Genetic studies, especially in *Drosophila*, provide much data on the identity and hierarchy of the factors required for mRNA localization, and represent an excellent starting point for a more detailed biochemical characterization. Furthermore, novel genome editing techniques will facilitate the *in vivo* validation of functional hypotheses from structural data in reverse genetic approaches.

It will also be essential to chart the complement of RBPs and RNA target motives by large-scale studies. New methods of RNA-protein interaction[119,120] including CRAC,[121] CLIP,[122] PAR-CLIP,[123] iCLIP,[124] hiCLIP[125] provide a rich source of RBPs and RNA targets. Those will be the starting points for structural studies of complexes to understand RNA binding modes.

The variety of canonical and non-canonical RNA-binding domains used by RBPs, together with the divergence in RNA signal sequences, makes it difficult to derive rules for target mRNA selection. Bioinformatics predictions and studies on isolated RNA elements can provide valuable information. However, experimental validation of the protein:RNA interaction remains essential; this is especially true for cases in which the RNA secondary structure changes following post-transcriptional modifications of the nucleotide bases (e.g., N^6-methyladenosine-dependent RNA structural switches[126]), or interaction with the protein partner (e.g., structural rearrangements in the E3 localization element from yeast *ASH1* mRNA bound to the localization machinery[127]).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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