Published in final edited form as:
Cell Microbiol. 2014 April ; 16(4): 482–489. doi:10.1111/cmi.12268.

The emerging role of RNA-binding proteins in the life cycle of *Trypanosoma brucei*

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Summary

One of the key questions in understanding the biology of an organism is how to correlate cellular fate and function with gene expression patterns. This is particularly relevant for pathogenic organisms, like the parasitic protozoa *Trypanosoma brucei*, who often cycle between different hosts, thereby encountering vastly different environments. Survival in and adaptation to new surroundings requires activation of specific gene networks, which is most often achieved by regulatory mechanisms embedded in the transcriptional machinery. However, in *T. brucei* and related trypanosomatids these responses appear to be accomplished mainly by post-transcriptional mechanisms. Although an understanding of how this parasite modulates gene regulatory networks is in the early stages, RNA-binding proteins (RBPs) are beginning to take center stage. Here, we discuss recent progress in the identification of RBPs with crucial roles in different stages of the *T. brucei* life cycle, and in elucidating targets of RBPs.

Introduction

The protozoan parasite *Trypanosoma brucei*, the causative agent of African trypanosomiasis in humans (HAT) and nagana in animals, is transmitted by the tsetse fly (Glossina spp.), the blood-feeding dipteran vector. Like many parasites, *T. brucei* undergoes remarkable transformations during its life cycle, each adapting to distinct surroundings in the mammalian host or the insect vector (Vickerman *et al.*, 1988). Within the bloodstream of the mammal, the parasites exist as proliferative slender forms, which establish parasitaemia. The differentiation of slender to quiescent, stumpy bloodstream forms occurs in response to cell density (Vassella *et al.*, 1997) and proceeds gradually through transitional forms, referred to as intermediates. Stumpy trypanosomes are arrested in their cell cycle and are primed to respond to the environmental changes associated with the uptake by the tsetse fly (Matthews, 2011). In the insect midgut, stumpy forms differentiate into procyclic forms that are no longer infectious to mammals (Dyer *et al.*, 2013). Reacquisition of infectivity is achieved through a complex developmental program that culminates in the tsetse salivary glands with the generation of metacyclic forms, which again are non-dividing forms ready to cope with a change in their environment when they are transmitted to the mammalian host. Trypanosomes rapidly respond to transmission between hosts by a complex cellular differentiation that includes radical changes in surface protein expression, metabolism,

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organelle function, and cytoskeletal architecture. For example, throughout the life cycle, the trypanosome plasma membrane is covered by densely packed surface coats consisting of GPI-anchored proteins. In the bloodstream the variant surface glycoprotein (VSG) coat is the paradigm for antigenic variation (Horn *et al.*, 2010), in the tsetse midgut, trypanosomes express procyclins, a family of EP (Glu/Pro repeat-containing) and GPEET (Gly/Pro/Glu/Glu/Thr repeat-containing) proteins (Roditi *et al.*, 1987) and epimastigote forms express a family of proteins known as brucei alanine-rich proteins or BARPs (Urwyler *et al.*, 2007).

Whereas the *T. brucei* life cycle was first described early in the 20th century, we are just beginning to get insights into regulatory mechanisms operating at different life cycle stages. Traditionally, an analysis of circuits controlling developmental processes mainly concentrated on the transcriptional machinery. However, in recent years it has become increasingly evident that post-transcriptional processes play equally important roles in the output of gene products and in some organisms, like the trypanosomatids (including the genera *Trypanosoma* and *Leishmania*), the regulation of gene expression appears to have moved away almost entirely from transcriptional control to post-transcriptional regulatory mechanisms. The conceptual shift in trypanosomatids started with the realization that protein coding genes in these organisms are organized into long unidirectional clusters, where multiple genes are co-transcribed by RNA polymerase II (Pol II). In general, there is no apparent functional connection between neighboring genes or genes in an array. Polycistronic pre-mRNAs are processed to mature mRNAs by *trans*-splicing of the spliced leader (SL) at the 5′ end and 3′ end formation/polyadenylation (Preusser *et al.*, 2012). Current evidence is consistent with Pol II transcribing all protein coding genes at comparable rates, although this is inferred from limited experimental data. However, the final output of mature mRNAs and proteins can vary tremendously between genes, and even between adjacent genes. In addition, expression levels of the same gene differ dramatically between various developmental stages. This conundrum elevated post-transcriptional mechanisms to major players in the regulation of gene expression. These include, but are not limited to, differential pre-mRNA processing (efficiency of *trans*-splicing and polyadenylation, alternative *trans*-splicing and polyadenylation), mRNA transport and subcellular localization (efficiency of export from the nucleus, sequestration in a variety of cytoplasmic mRNP bodies/granules), mRNA stability, mRNA translation, protein stability and post-translational modifications. In this scenario, RNA-binding proteins (RBPs) are emerging as key factors. The evolving picture is that, as in other eukaryotes, trypanosome RBPs interact through *cis*-acting elements with any part of the pre-mRNA or the mature mRNA, untranslated regions (3′UTR or 5′UTR) or coding sequence (CDS), and are suspected to have dominant roles in controlling gene expression in trypanosomatids at all steps of mRNA metabolism. Only recently evidence has emerged to support the view that RBPs can drive profound changes in the gene expression profile in these parasites, and as a result, influence trypanosome development. Here we briefly review the *T. brucei* RBPs playing a role in gene expression and life-cycle progression. Our overview does not cover general factors involved in RNA degradation, pre-mRNA splicing, polyadenylation, export and translation, and for a more complete view on RNA metabolism in trypanosomes, the reader is referred to additional excellent recent reviews on the subject covered here (Kramer *et al.*, 2011, Clayton, 2013), mRNA splicing in trypanosomes (Gunzl, 2010, Michaeli, 2011, Preusser *et al.*, 2012), mRNA degradation and translation initiation in trypanosomes and leishmanias (Clayton *et al.*, 2007) and mitochondrial RNA processing in trypanosomes (Aphasizhev *et al.*, 2011).

Repertoire of RNA-binding proteins

The **RNA-Recognition Motif** (**RRM**) domain is one of the most commonly found protein domains in nature. The structure of this versatile module of ~90 amino acids (Fig. 1A)

consists of a four-stranded β-sheet packed against two α-helices (Nagai *et al.*, 1990). *T. brucei*, like all other eukaryotes, contains a large number of RRM-containing RBPs, around 70 (De Gaudenzi *et al.*, 2005, Wurst *et al.*, 2009). RRMs most often are involved in sequence-specific interaction with single-stranded RNA, but can also interact with DNA, as well as other proteins, including themselves.

The **Cys_3His Zinc finger** or **CCCH** domain represents a subclass of the large zinc finger family with a characteristic disk-like structure (Amann *et al.*, 2003)(Fig. 1B) and an apparent preference for binding to AU-rich RNA elements. The *T. brucei* genome encodes 48 proteins with this signature (Kramer *et al.*, 2010).

There are four *T. brucei* proteins with a recognizable **ALBA** (acetylation lowers binding affinity) domain. In addition, these proteins contain a C-terminal stretch of multiple RGG repeats. The monomer structure (Fig. 1C) consists of four stranded β-sheet and two α-helices (Wardleworth *et al.*, 2002). Proteins of this family have both DNA and RNA binding activity.

Pumilio domain RBPs

PUF proteins (*Drosophila* Pumilio and *C. elegans* Fem-3-binding factor BF are founding members of this family) were initially characterized in other eukaryotes as RBPs that can regulate the stability and the translation of their targets with binding sites in the 3′ UTR of the affected mRNAs. Some members have functions in pre-rRNA processing (Thomson *et al.*, 2007). The core of the pumilio RNA-binding domain (Fig. 1D) consists of α-helical tandem repeats (Edwards *et al.*, 2001, Wang *et al.*, 2001), typically eight, each interacting with one base of the RNA sequence recognized by the protein (Wang *et al.*, 2002). The RNA elements bound by PUF proteins usually contain the trinucleotide UGU (Wang *et al.*, 2002). *T. brucei* harbors 11 PUF protein genes (Luu *et al.*, 2006).

RBPs involved in the differentiation from bloodstream to procyclic forms

ZFP1 (Tb927.6.3490) and ZFP2 (Tb927.11.14950), two CCCH-family proteins, were the first trypanosome RBPs to be implicated in development and its regulation (Hendriks *et al.*, 2001). ZFP1 was transiently up-regulated during the differentiation from bloodstream to procyclic forms and also expressed at higher levels in established procyclic cells. ZFP2 was present at equal levels in bloodstream and procyclic cells. RNAi knockdown of ZFP2 inhibited differentiation to procyclic forms, as judged by reduced EP procyclin expression, impaired kinetoplast repositioning and inhibited morphological restructuring (Hendriks *et al.*, 2001). Additionally, ectopic expression of ZFP2 in procyclic forms resulted in gross morphological changes termed “nozzle” phenotype, the result of polar extension of the trypanosome cytoskeleton in cells which are early in the cell cycle. ZFP1 null bloodstream form cell lines were shown to be compromised in their ability to differentiate to procyclics, specifically in kinetoplast repositioning (Hendriks *et al.*, 2005). ZFP3 (Tb927.3.720) was identified by means of its similarity in primary structure to ZFP1 and ZFP2 (Paterou *et al.*, 2006). Ectopic ZFP3 expression in bloodstream *T. brucei* was shown to enhance the differentiation to procyclics. Expression in procyclics induced the “nozzle” phenotype previously observed for ZFP2, supporting the conclusion that these RBPs function in the same differentiation pathway. Protein-protein interactions ZFP1-ZFP2 and ZFP1-ZFP3 were demonstrated with a yeast two-hybrid system and all three proteins could be co-immunoprecipitated from cell extracts (Paterou *et al.*, 2006). ZFP3 was also shown to be enriched on polysomes in procyclic cells, but not in bloodstream cells, and this enrichment correlated with the “nozzle” phenotype and enhancement of differentiation. Later studies (Walrad *et al.*, 2012) demonstrated that ZFP3 associates with a subset of mRNAs that was enriched in stumpy bloodstream form trypanosomes, the abundance of these transcripts was

modulated by ZFP3 levels and was mediated by sequences in the 3′UTRs of the mRNA targets.

ZC3H18 (Tb927.7.2140), a CCCH-family protein with two characteristic domains, was shown to be required for differentiation of bloodstream trypanosomes to procyclic forms (Benz *et al.*, 2011), but its mode of action needs to be investigated.

RBP10 modulates mRNAs in bloodstream-form trypanosomes

RBP10 (Tb927.8.2780), a small RRM-containing protein was the first example of an RBP with profound life-cycle stage-specific effects on the global trypanosome transcriptome, and its developmental regulation (Wurst *et al.*, 2012). RBP10 was found to localize primarily in the cytoplasm and its expression was shown to be up-regulated in bloodstream-form trypanosomes. The protein was also detected to be differentially phosphorylated (Urbaniak *et al.*, 2012) with the modification found mostly in the bloodstream form. RNAi knockdown of RBP10 in bloodstream trypanosomes resulted in the down-regulation of a large number of mRNAs normally found elevated in bloodstream forms, and conversely, overexpression of the protein in procyclics led to an increase of many bloodstream-form specific mRNAs (Wurst *et al.*, 2012). Thus, RBP10 was able to promote a bloodstream-form transcriptome pattern in *T. brucei*. Genes involved in sugar transport and metabolism were among the most affected by RBP10 and flagellum and cytoskeleton mRNAs were also modulated broadly. Finally, forced expression of RBP10 in bloodstream forms inhibited their differentiation to procyclics. At present it is unclear whether RBP10 interacts with mRNAs, however the RBP10 effects on mRNA metabolism were clearly shown to depend on the 3′UTRs of the affected transcripts (Wurst *et al.*, 2012).

RBPs during trypanosome development in the tsetse fly

A second example of an RRM protein with a remarkable role in regulating trypanosome development is RBP6 (Tb927.3.2930). RBP6 was found to be highly enriched at the mRNA level in trypanosomes inhabiting the proventriculus of infected flies, when compared to procyclics from the midgut (Kolev *et al.*, 2012). Inducible overexpression of RBP6 in procyclics, in an effort to mimic events in the proventriculus, resulted in progression through the developmental program of the parasite. Epimastigotes and, within a week of RBP6 expression, metacyclic trypomastigotes appeared in culture in the absence of any other developmental triggers. The metacyclic cells produced *in vitro* by inducible RBP6 expression were shown to express the VSG coat and importantly, the activated VSG genes possessed a metacyclic-type VSG Pol I promoter and were found in monocistronic transcription units (Kolev *et al.*, 2012). More recently, it was suggested that RBP6 is a remote ELAV-like *T. brucei* homolog that binds *in vitro* to an AU-rich element (AUUUAUU) present in the 3′UTRs of mRNAs (Najafabadi *et al.*, 2013). Interestingly, expression of RBP6 in procyclic cells was found to down-regulate mRNAs containing the identified sequence signature mRNAs (Najafabadi *et al.*, 2013), an opposite effect in comparison to available data for ELAV family proteins from other species. Identifying the precise *in vivo* RNA-binding sites for RBP6 will undoubtedly clarify the repertoire of RBP6 targets and shed more light on its mode of action. The combined examples of RBP10 and RBP6 as potent triggers of developmental changes are a strong indication for the critical role of RBPs as post-transcriptional regulators of gene expression in *T. brucei* and other trypanosomatids.

Recently, the four *T. brucei* ALBA proteins were characterized (Mani *et al.*, 2011, Subota *et al.*, 2011). ALBA3 and ALBA4 (Tb927.4.2040/Tb927.4.2030) are primarily cytoplasmic proteins found to be expressed throughout the *T. brucei* life cycle, except in developmental

stages found in the proventriculus of tsetse representing the transition from procyclic to epimastigote forms (Subota *et al.*, 2011). Nutritional stress, a likely condition in the fly proventriculus, caused ALBA3/4 to localize to stress granules together with the helicase DHH1. Knockdown by RNAi of ALBA3/4 in procyclic trypanosomes grown in culture, mimicking the down-regulation of the proteins in the proventriculus, resulted in cellular morphology resembling the development in the fly proventriculus, specifically, elongation of the cell body and repositioning of the nucleus and the kinetoplast in a epimastigote configuration. Overexpression of these proteins in trypanosomes in the fly impaired the normal differentiation taking place in the proventriculus. These results represent yet another striking example of perturbation of *T. brucei* development by modulating the expression of proteins with RNA-binding potential (Subota *et al.*, 2011). ALBA1/ALBA2 (Tb927.11.4460/Tb927.11.4450) are also primarily cytoplasmic (Mani *et al.*, 2011). They were identified as proteins interacting with regulatory elements in the GPEET procyclin mRNA 3′UTR, and shown to form complexes with ALBA3 and ALBA4, suggestive of multiple homodimers/heterodimers possibilities between the *T. brucei* ALBA family members. Like ALBA3/4, ALBA1/2 are also recruited to stress mRNP granules upon starvation, a likely indication that they may be involved in translational control, supported by their partial co-migration with polysomes during density gradient centrifugation and co-purification with translation initiation factors and poly(A)-binding proteins (Mani *et al.*, 2011).

Differential regulation of mRNA metabolism

The *T. brucei* homolog of hnRNP F/H (Tb927.2.3880) is a member of the RRM superfamily and was shown to play a major role in the differential regulation of mRNA metabolism (Gupta *et al.*, 2013). This RBP is highly up-regulated in bloodstream-form trypanosomes and shows primarily nuclear localization, but is also present in the cytoplasm. Just like in other eukaryotes, where hnRNP F/H proteins regulate alternative splicing, the trypanosome homolog regulates the efficiency of *trans*-splicing and acts as a repressor. Additionally, the protein has a role in controlling differential mRNA stability in the bloodstream and procyclic life-cycle stages. The protein preferentially contacts its RNA substrates at an AAGAA sequence motif, found enriched in both the 3′UTR (for mRNA stability control) and upstream of the CDS (for effects on *trans*-splicing). HnRNP F/H is the first example of a differentially expressed splicing regulator in trypanosomes (Gupta *et al.*, 2013).

RBPs with housekeeping functions

An RRM protein contacting its targets in the CDS is RBP42 (Tb927.6.4440). By using the powerful HITS-CLIP approach, it was shown that *in vivo* RBP42 binding sites cluster in the CDS of mRNAs, many of them coding for proteins involved in cellular energy metabolism (Das *et al.*, 2012). RBP42 is a cytoplasmic protein that is associated with polysomes.

ZC3H11 (Tb927.5.810), a CCCH-family protein, is a phosphorylated cytoplasmic protein up-regulated upon heat shock and was shown to preferentially bind and stabilize mRNAs for a group of chaperones required for protein refolding, thus regulating the heat shock response in *T. brucei* (Droll *et al.*, 2013). The interaction of ZC3H11 and mRNA was shown to be mediated by AUU repeats in the 3′UTRs, and the differential regulation of these mRNAs is dependent on these motifs.

Cell cycle and rRNA processing

PUF9 (Tb927.1.2600) was shown to be up-regulated in the S-phase of the cell cycle and inducible PUF9 RNAi knockdown resulted in accumulation of cells in G2/M (Archer *et al.*, 2009). The protein was found localized primarily to the cytoplasm. Four specific mRNA

targets were identified by PUF9 co-immunoprecipitation and it was demonstrated that PUF9 stabilizes 3 of these messages, leading to their up-regulation in S-phase of the cell cycle. This stimulatory effect of a trypanosome PUF protein on the expression of its targets is a deviation from the more typical repressive role assigned to these proteins. Nevertheless, the sequence motif 5′-UUGUACC-3′, containing the signature UGU recognized by many PUF proteins was found overrepresented in the 3′ UTRs of the identified targets. Additionally, a correlation between the presence of the motif, expression of PUF9 and cell-cycle changes in abundance of the target mRNAs strongly supported the case for stimulatory role of PUF9 in mRNA metabolism, likely by modulating RNA stability (Archer *et al.*, 2009).

Another cytoplasmically localized protein of this family, PUF1 (Tb927.10.4430), was shown to interact with the expression site associated gene 8 (ESAG8) protein, which is enriched in the nucleolus, and to positively affect the levels of both ESAG8 mRNA and ESAG8 protein (Hoek *et al.*, 2002). Overexpression of a HA-tagged PUF1 in bloodstream-form trypanosomes dramatically reduced their infectivity in mice. PUF1 has also been reported to associate with transcripts from repeat elements INGI and DIRE, and ribosomal RNA (Luu *et al.*, 2006). PUF7 (Tb927.11.14960) localizes to the nucleolus, associates with a protein termed nuclear cyclophilin 1, and has an effect on pre-rRNA processing (Droll *et al.*, 2010). A remarkable connection between pre-rRNA processing and regulation of the developmentally regulated Pol I-transcribed GPEET mRNAs was recently demonstrated, and two Pumilo domain proteins, PUF7 and PUF10 were implicated in this regulatory process (Schumann Burkard *et al.*, 2013). By screening a genomic RNAi library for negative regulators of GPEET expression, a protein termed NRG1 was identified, and its binding partners included PUF7 and PUF10. Knockdown of each of these genes resulted in higher propensity for GPEET expression, lower levels of 5.8S rRNA and higher levels of rRNA precursors. The links between Pol I transcription, pre-rRNA processing and Pol I-transcribed mRNAs metabolism will likely continue to surface in the future, since shared protein factors and close proximity of nuclear compartments, where rRNA and surface coat mRNAs are produced, possibly dictate close connections in regulatory mechanisms. It will be of great interest to elucidate how changes in rRNA production rates during differentiation from dividing to quiescent cells prior to a new cycle of host invasion affect the production and stability of Pol I-transcribed mRNAs coding for surface proteins.

RBPs awaiting functional assignment

UBP1 (Tb927.11.500) and UBP2 (Tb927.11.510) are two closely related abundant RRM proteins (Hartmann *et al.*, 2007). They were shown to localize primarily to the cytoplasm, but were not excluded from the nucleus. Modulating the expression levels of UBP1/2 affected the abundance of CFB1 mRNAs *via* an element in the 3′UTRs of these messages (Hartmann *et al.*, 2007). An interesting feature of the UBP1 and UBP2 genes is the presence of a very long 3′UTR in the corresponding mRNAs (discussed later).

PUF5 (Tb927.7.4730) is localized primarily in the cytoplasm and makes contacts with cellular RNA of unknown nature (Jha *et al.*, 2013). ZC3H12 (Tb927.5.1570) and ZC3H13 (Tb927.5.1580) are examples of two phosphorylated cytoplasmic zinc finger proteins, but their function is unclear (Ouna *et al.*, 2012). ZC3H20 (Tb927.7.2660) was demonstrated to bind to the 3′UTRs and stabilize at least two developmentally regulated mRNAs (Ling *et al.*, 2011).

Conclusions

RBPs have been universally shown to have a combinatorial mode of action, i.e. many RBPs are bound to the same mRNA and some may have opposing roles in RNA metabolism.

Additionally, most RBPs have multiple mRNA targets. The wide-spread, overlapping and often different effects of RBPs on the transcriptome present a great challenge in delineating the function of trypanosome RBPs in regulating gene expression and development. Future priorities should include precise cataloging of RNA targets and definition of preferred *in vivo* binding sites and distinguishing between direct and indirect effects on target RNA metabolism. Most importantly, we should strive to identify the molecular mechanisms driving the differential expression or differential action of RBPs in particular life-cycle stages of parasite development. There is a distinct possibility that expression of RBPs themselves is controlled by RBPs, and illuminating the cellular processes and signals that mediate sensing changes in the parasite environment (host identity and available nutrients) and signal transduction to the level of RBP expression holds the key to understanding the network of regulatory events that determine the gene expression program in different developmental stages. Interestingly, the 3′UTRs of several of the RBPs discussed here (Fig. 2) are well above the median length of 388-400 nucleotides determined in transcriptome-wide studies (Kolev *et al.*, 2010, Siegel *et al.*, 2010). If we consider 3′UTRs as the most commonly utilized platform for RNP regulatory complexes assembly, then the size of the 3′UTR could be an indication for the level of intricacy in the post-transcriptional control of expression for a particular gene. In longer 3′UTRs many more sequence and/or structure elements are available for binding to different RBPs and the number of possible RBP combinations associated with an mRNA increases. Thus, there is the potential for a larger number of signaling pathways involving RNA-binding components (proteins, regulatory RNAs, and even metabolites) to exert effect(s) on the expression of an RBP. Many other predicted RBPs in the trypanosome genome contain much longer than average untranslated regions in their mRNAs, suggesting that we have only scratched the surface of the RBP toolbox that *T. brucei* uses to modulate and fine-tune gene expression post-transcriptionally.

Acknowledgments

Work in the authors laboratory is supported by Public Health Service grants AI28798 and AI56333 to E.U. and AI043594 to C.T. from the National Institute of Allergy and Infectious Diseases. The funding agency had no role in the writing and the decision to publish the article. We apologize to all those colleagues whose relevant work could not be cited due to space limitations.

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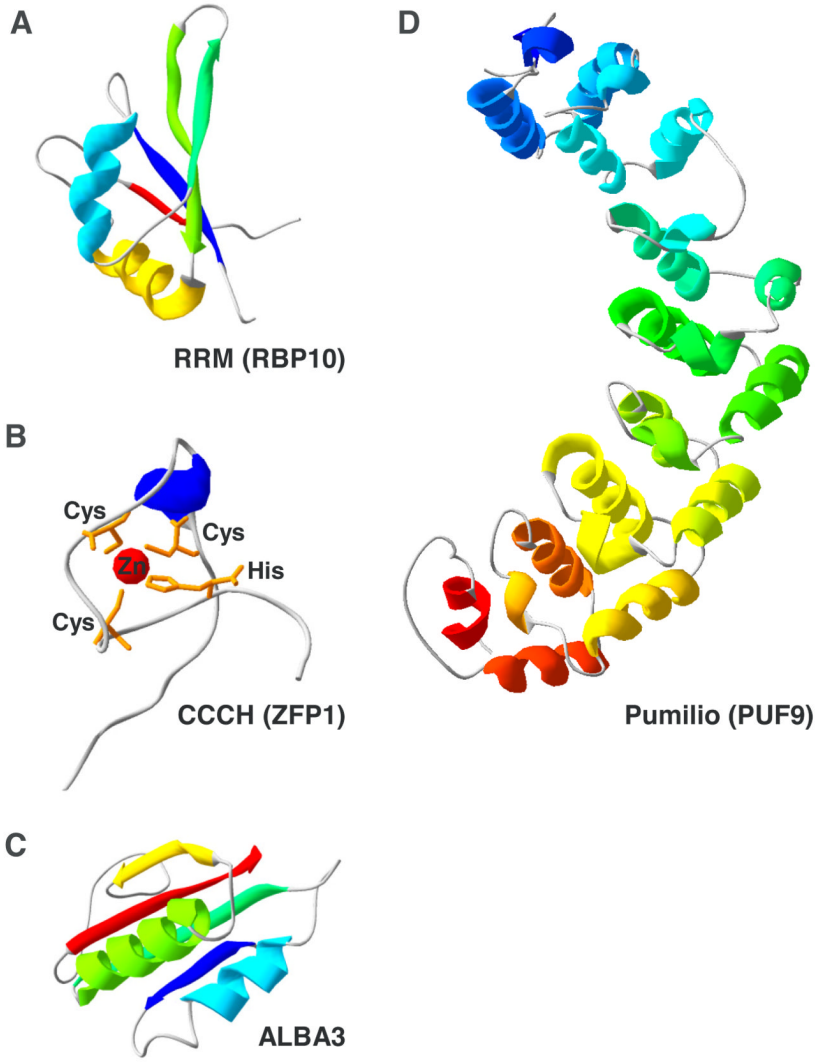

Fig. 1.

Tertiary structure models using the alignment interface of SWISS-MODEL (Bordoli *et al.*, 2009) for the RRM domain of RBP10 (**A**), the Cys3His zinc finger of ZFP1 (**B**), the nucleic acid binding domain of ALBA3 (**C**), and the Pumilio domain of PUF9 (**D**).

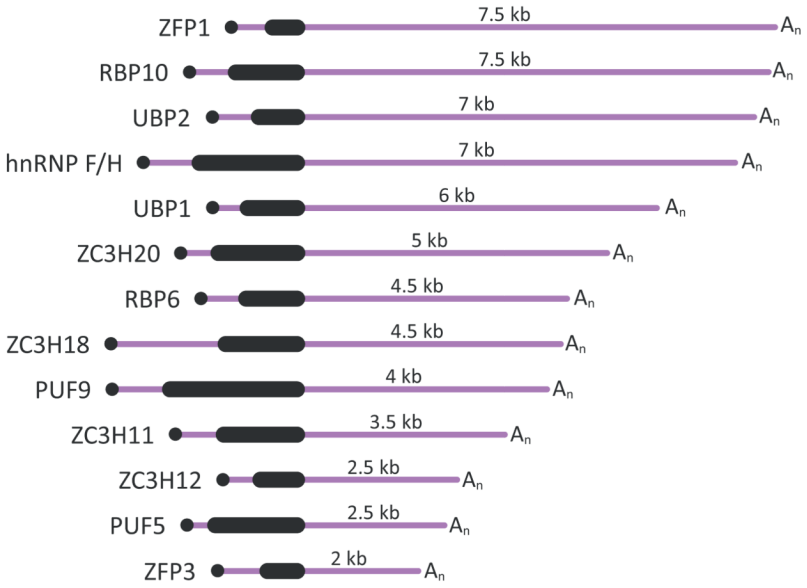

Fig. 2.

Schematic representation of the mRNAs coding for RNA-binding proteins discussed in this review. The drawings are according to the predicted sizes of the 3′UTRs based on high throughput sequencing data (Kolev *et al.*, 2010, Siegel *et al.*, 2010).The numbers listed are rounded to the closest 0.5 kb.