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Joining the dots: Protein-RNA interactions mediating local mRNA translation in neurons

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Abstract

Establishing and maintaining the complex network of connections required for neuronal communication requires the transport and *in situ* translation of large groups of mRNAs to create local proteomes. In this Review, we discuss the regulation of local mRNA translation in neurons and the RNA-binding proteins that recognise RNA zipcode elements and connect the mRNAs to the cellular transport networks, as well as regulate their translation control. mRNA recognition by the regulatory proteins is mediated by the combinatorial action of multiple RNA binding domains. This increases the specificity and affinity of the interaction, while allowing the protein to recognise a diverse set of targets and mediate a range of mechanisms for translational regulation. The structural and molecular understanding of the interactions can be used together with novel microscopy and transcriptome-wide data to build a mechanistic framework for the regulation of local mRNA translation.

Introduction

The transport and local translation of mRNAs is a key cellular process that regulates gene expression in space and time. The localization of an mRNA to a sub-cellular domain allows the synthesis of the encoded protein *in situ*, avoiding unwanted ectopic protein expression. Also, synthesis of the protein at its site of action facilitates a faster regulation of protein concentration in response to a signal. Finally, transporting an mRNA rather than the encoded protein is efficient and removes the need for

protein tagging, as the required identifiers can be embedded in the non-coding regions of the mRNA[1][2]. In Metazoan, local mRNA translation is required for cellular specialisation and to respond to extracellular messages, which is essential for organismal development and function. Early studies in the fly *D. melanogaster* have played an important role in our understanding of mRNA localisation and translation during development, showing that the spatial distribution of a set of mRNAs follows a precise temporal sequence and it is essential for the correct development and patterning of the embryo[3]. Later work showed that mRNA localisation is a prevalent phenomenon in development: this work includes an imaging study that revealed that ~70% of mRNAs are differentially distributed in the fly embryo[4]. Importantly, a crucial role for mRNA local translation in development has been shown in a range of different organisms in both vertebrates and invertebrates[5]. In mammalian neurons, sequencing analysis indicates that a large set of cellular mRNAs can be preferentially localized in axons and dendrites, where they encode a substantial share of the local proteome to create biochemically distinct subcellular compartments[6][7].

In the developing mammalian brain neurons must establish a complex network of connections in a highly regulated fashion. These connections are largely away from the cell body and are continuously re-shaped during the lifetime to encode information. Establishing, maintaining, and evolving the connections of a neuron requires the synthesis of a specific proteome at different cellular locations. The local neuronal proteomes are established by translating mRNAs *in situ* in response to signals encoded by, for example, a gradient of morphogen or communication across a synapsis[8]. These local mRNA translation events regulate neurite growth, axonal pathfinding, and synaptic maturation in brain development[8][9]. They are essential for the lifelong differentiation of synapses and for the process of long term potentiation and depression in response to a stimulus[8]. They are also essential for the maintenance and repair of axons in the adult[9].

Neuronal mRNA transport and the associated translational control requires the coupling of the mRNAs to the cellular transport and translation machineries. This coupling is performed by *trans*-acting RNA binding proteins, and the selectivity of the mechanism is obtained via the proteins' recognition of sequence and/or structure-specific signals present within the target mRNAs. Consistent with the key role played by the RNA-binding proteins regulating local mRNA translation, their mis-expression or dysfunction is linked to severe neurodevelopmental and neurological disorders[10][11][12]. These include Fragile X Syndrome (FXS), autistic spectrum disorders (ASD), Amyotrophic lateral Sclerosis (ALS), spinal muscular atrophy (SMA) and fronto-temporal dementia (FTD), among many[10].

Below, we discuss briefly the recent evidence for the widespread and regulated local mRNA translation in neurites and the different processes involved. We then review our current understanding of how multi-domain RNA-binding proteins recognise and select their target mRNAs in mammalian neurons and how this recognition relates to mRNA transport and translational control.

The local translation of mRNAs in mammalian neurons establishes local proteomes in the axonal and dendritic compartments.

In the last five years, a number of studies have taken advantage of high resolution fluorescent *in situ* hybridization (FISH) and next-generation sequencing technologies to show that a very large share of the neuronal population of mRNAs is differentially localized in neurites. In 2012, Cajigas and co-workers reported that the dendritic and axonal mRNA population from a rat hippocampal region is enriched in >2500 mRNAs codifying for translational regulators, ribosomal proteins, receptor proteins, scaffolding components, signalling proteins, transporting and others[6]. Interestingly, the direct visualisation of a smaller number (71) of chosen mRNAs using high resolution FISH indicated that different mRNAs have a different distribution along the dendrites, which underlies the complexity of mRNA localisation[6]. The large size and diversity of the pool of neurite-enriched mRNAs has been confirmed by several recent studies performed both in hippocampal brain sections[13][14] and in cells[15][16][17]. These analysis indicate that 1000-3000 mRNAs are differentially distributed between the neuronal soma and neurites. Importantly, a study in mouse showed that a few thousands axonally-localized mRNAs are actively translated in the retinal ganglion cells, both during development and in the mature animal[7]. It also showed that this set is actively regulated during development, with only a quarter of mRNAs being translated at every stage[7]. Independently, a study in the dendrites of pyramidal neurons from mouse hippocampus[14] reported on a similar-sized set of actively translating mRNAs which was regulated in response to a conditioning experience. Finally, a recent proteomic study directly confirmed that the majority of the dendritic proteome is synthesized from locally translated mRNAs[16]. Overall, these studies showed that the proteome of dendrites and axons can be regulated according to behavioural and developmental needs by the localisation and regulated *in situ* translation of the relevant mRNAs.

Local mRNA translation is regulated by RNA-binding proteins.

The transport and translational control processes that underlie the local translation of an mRNA are mediated by RNA-binding proteins that recognise, in *trans,* structure and/or sequence elements on the mRNA molecule (the so-called 'zipcodes') and associate, either directly or via protein adaptors, with molecular motors and with components of the translation machinery. In neurons, active long range transport of mRNA is mediated by kinesin and dynein motors moving along the cell's microtubule network (Figure 1). The chain of interactions linking the motors to the transported mRNAs have been characterised most extensively in *Drosophila*. These interactions, which are partially conserved in vertebrates have been recently reviewed,[18] and are not discussed in detail here. In mammalian neurons, a number of proteins have been shown to be essential in the formation of mammalian protein-RNA granules and to their transport in neurites. Examples include the Fragile X mental retardation protein (FMRP)[11], the Zipcode Binding Protein 1 (ZBP1, also called IMP1 or IGF2BP1)[19][20][21] and Staufen[22][23]. In addition, it has been shown that, similar to *Drosophila*, kinesin and dynein motors mediate mRNA transport in mammalian neurons and the actual movement of mRNAs and proteins has been described in detail in a number of pioneering studies. However, we have only rudimentary information on the molecular connections between the RNA binding proteins and the motors[18]. Probably the best characterized of these interactions is the one between the RNA-binding protein ZBP1 and the kinesin KIF11. Here biochemical and functional data have recently identified a direct contact between ZBP1 N-terminal RRM domains and KIF11 C-terminus[24].

Importantly, the RNA-binding proteins required for mRNA local translation in mammalian neurites also mediate the translational control of the transported mRNAs (Figure 1). Translational repression of the mRNAs during transport is needed to prevent ectopic expression (although recent data highlights that, at least for some mRNAs, translation can take place while the mRNA is moving along the dendrite[25]) and can be achieved via general translation regulation pathways[1]. A first example of these mechanisms is the blocking of translation initiation by the recruitment of protein effectors that sequester the general translation initiation factor eIF4E - the so-called eIF4E binding proteins (4E-BPs). This is a well-studied mechanism and examples of 4E-BPs recruitment by RNA-binding proteins include the recruitment of CYFIP1 by FMRP[26], the recruitment of Neuroguidin by Cytoplamic polyadenylation element binding protein 1 (CPEB1)[27] and the interaction of Pumilio 2 with 4E-T[28]. A second well-characterized process regulating translation initiation is the shortening of the polyA tail of the mRNA, which prevents binding of the polyA binding proteins and efficient assembly of the initiation complex. In the cytoplasm, the length of the polyA tail can be regulated by the protein CPEB1. CPEB1 binds at the immediate 5' of the polyA and associates with both a non-canonical polyA polymerase and the de-adenylase PARN whose activity creates a polyA of the required length[29]. Other RNA binding proteins regulate local translation via specific mechanisms, which however are still being explored at the molecular level. Examples include the block of translational elongation by the protein FMRP via its direct interaction with ribosomes[30] and the packaging of the β-actin mRNA into protein-RNA particles by the protein ZBP1 to impede ribosomal access[31][32].

A crucial step in the translational control of the localized mRNAs is the relief of translational repression in response to a signal that leads to local protein synthesis. A common mechanism to relieve translational repression is the phosphorylation of the regulatory RNA–binding proteins. A first example is the phosphorylation of ZBP1 Y396 by the kinase Src in response to a BDNF signal. ZBP1 Y396 phosphorylation impairs the interaction with the β-actin mRNA - whose transport and translation is important for dendritic morphology[19] and branching[33] - and leads to the unpacking of the mRNA-containing protein-RNA particle. This in turn, allows ribosome assembly and β-actin mRNA translation[32][31]. Also, the phosphorylation of CPEB1 by the kinase Aurora A leads to mRNA polyadenylation and to de-repression of translation[29]. Translation can also be activated by the de-phosphorylation of the protein regulator. The de-phosphorylation of FMRP S499 by the phosphatase PP2A leads to the release of the ribosome and to translational activation[34][35][36]. It is worth highlighting that translational repression can be released by the direct modification of post-translational regulation of general regulatory factors. mTOR dependent phosphorylation of 4E-BPs represent a common mechanism for eIF4E release and translational activation[37].

The mRNA regulation discussed above requires the formation of multi-component protein-RNA particles (RNPs), which in neurons are also defined neuronal granules. Neuronal granules can contain RNA-binding proteins, molecular motors, translational regulators and ribosomes, or ribosomal subunits, in addition to mRNAs. The packaging of these components into a protein-RNA particle is mediated by RNA-binding proteins that make both specific and less-specific interaction, the latter via low sequence complexity regions. While the physical details of how low sequence complexity regions mediate a process of reversible aggregation, it is clear that proteins such as Fused in sarcoma (FUS) and others play an important role in the assembly of protein-RNA particles, and in mRNA transport and translational control[38].

RNA-binding proteins coordinate the different molecular processes which are required to regulate local protein synthesis. These proteins also control the selectivity of regulation via the recognition of the mRNA targets. Below we review our understanding of protein–RNA recognition in a number of key regulators and discuss open questions and future challenges.

The combinatorial use of RNA binding domains underpins a diversity of protein-RNA recognition modes and of regulatory mechanisms.

mRNA recognition by RNA-binding proteins is mediated by conserved RNA binding domains (RBDs) or motifs, which are often found in multiple copies and/or in different combinations within the same protein[39] (exemplified in Figure 2). These RBDs have different degrees of affinity and specificity for the RNA target. Some, for example the α-helical Pumilio (PUM) repeat[40], recognise uninterrupted sequences of several nucleotides with a high degree of specificity and affinity. However, in the majority of cases a strong and selective binding is obtained by the combinatorial recognition of the RNA by multiple RBDs. These RBDs have intermediate-to-low affinity and specificity and include single stranded RNA recognition domains, such as the common KH[41], RRM[42] and ZnF[43][44][45] domains as well as domains that recognise the RNA double helical structure, such as the dsRBM domain[46][47]. In addition, the proteins include linear low sequence complexity motifs that fold upon binding the RNA, such as the RGG box[48] and non-canonical RNA-binding domains[49][50]. As expected from the multiple RNA binding domains present in individual proteins and from the moderate affinity of most RBDs in isolation, RNA recognition is often combinatorial, with several domains participating in each protein—RNA interaction. The role of the individual domains may depend on which target is bound[51]. The target-dependent role of individual domains in RNA binding, and the combinatorial mode of recognition, expand the capability of the protein to recognize different RNAs, while retaining specificity. It also facilitates the regulation of these dynamic interactions[51]. Below we discuss how RNA-binding proteins with different domain compositions, binding modes, and mechanism of actions, are able to recognise a diverse ensemble of targets, and how this is linked to protein function.

FMRP is an RNA-binding protein that plays an important role in the regulation of local translation in neurons, and down-regulation or dysfunction of FMRP is the causing factor for FXS, the most common monogenic cause of mental retardation and a leading cause of autism[11]. In physiological conditions, FMRP recognise a large ensemble of mRNA targets. Separate microarray[52] and crosslinking immunoprecipitation, coupled with high throughput sequencing (HITS-CLIP)[30] analysis in mouse brain cells have shown that FMRP binds and translationally regulates hundreds of neuronal mRNAs, many of which encode proteins related to synaptic morphology and function[52][30]. A key question to understand the action of FMRP at the molecular level is which mRNAs are targeted by FMRP and how[11]. FMRP contains four putative RNA-binding domains, including three atypical KH domains, (KH0, KH1 and KH2)[53][54] and one RGG box (Figures 2 and 3). The non-canonical KH0 domain, which is packed against the N-terminal domain of the protein[54] does not contain the conserved GxxG loop – which is a hallmark of KH domain-RNA interactions. The KH1 and KH2 domains form a single structural unit with an atypical inter-domain orientation and interface. Further, KH1 has a classical KH fold, but no positively charged residues in the GxxG loop – which are loosely correlated with the domain's RNA binding affinity[55]. KH2 has a 60-amino acid expansion in

the 'variable loop', a structural element that is also engaged in RNA recognition. These variations in the structural elements mediating canonical KH–RNA recognition imply that mRNA binding by the FMRP KH domains cannot be modelled based on our existing understanding of KH-RNA interactions. Systematic evolution of ligands by exponential enrichment (SELEX) and biochemical experiments revealed that FMRP RGG box interacts with high affinity with a G-quartet (GQ) structure. A GQ-forming sequence[56] is present in many of the targets identified in the microarray study, which is consistent with the GQ playing a general role in FMRP-target recognition. In addition, G-quartets have been shown to mediate FMRP recognition in different mRNAs[57][58][59][60][61][62], including FMRP mRNA itself[61]. However, identifying which elements of the GQs are recognised by FMRP (and therefore which G-quartets are recognised by FMRP) has proven challenging. Interestingly, the recent structure(s) of the SELEX-derived GQ in complex with FMRP RGG box has shown that FMRP interacts at the junction between the GQ and a short SELEX-derived stem[54][63] (Figure 3). This implies that the specificity in a FMRP-GQ interaction may be at least partially encoded by the sequences flanking the GQ[64], which may help define FMRP RGG box targets *in vivo*. The analysis of FMRP HITS-CLIP data from rodent brain has showed that the protein interacts most frequently with the coding sequence (CDS) of the target mRNAs rather than with their 3'UTR. Further, the interaction takes place across the bound CDS(s) rather than being focused on specific hotspots. Such a distribution of binding sites has been linked to the function of FMRP during the process of translational elongation, which involves reversibly stalling ribosome progression along the mRNA. It is worth pointing out that, despite the broad distribution of binding sites FMRP binds selectively a subset of mRNA and the question remains of how this subset is selected. Motif analysis of a later CLIP (PAR-CLIP) study in HEK93 cells indicated that the FMRP binding clusters are enriched in several short linear motifs (GGA, GAC) rather than in the originally identified GQ motif. Follow up mutational analysis indicated that recognition of the linear GGA/GAC sequences requires the stability and/or functionality of FMRP KH domains, and also that multiple repeats of the linear motifs are required to obtain high affinity binding. While the work of the past few years has clarified that the different domains of the protein recognize different RNA sequences and structures (including recognition elements comprising multiple short stems[65][66] and a SELEX-derived loop-loop pseudo-knot[67] that are not discussed here in detail), it is unclear whether and how these individual recognition event may combine to select the RNA target. As discussed above, FMRP has been proposed to regulate translational elongation by binding reversibly to the ribosome. A recent 12.8 Å resolution CryoEM structure of *Drosophila* FMRP bound to a ribosome has been recently solved and has suggested that the FMRP RGG box may interact with GQ elements on the trapped mRNA, while the KH domains may interact with the ribosomal protein (L5) and possibly with ribosomal RNAs[68].

ZBP1 – ZBP1 (also called IGF2 mRNA binding protein 1; IMP1 or IGF2BP1) plays an important role in organismal development[69], and ZBP1 mis-expression leads to impaired embryonic development and prenatal or neonatal death. In neurons, ZBP1 regulates axonal growth and branching and synaptic morphology[19][20][70]. The different functions of ZBP1 are underpinned by its regulation of the transport, translation and degradation of a diverse set of neuronal mRNAs. The best studied target of ZBP1 is the mRNA encoding the protein β-actin, whose concentration is important to regulate cytoskeleton organisation. ZBP1 associates with β-actin mRNA in the perinuclear region and mediates its transport to the final destination in a translationally repressed form. There, phosphorylation of ZBP1 by Src in response to a signal mediates mRNA release and translation, as discussed above. Importantly, microscopy studies have suggested that ZBP1 binding to β-actin is a

key step of β-actin mRNA packaging in RNPs and that this packaging is necessary for both transport and to limit the access of the ribosome, i.e. for translational repression[28][32]. The study of the ZBP1-β-actin mRNA system using microscopy has provided unique insights into mRNA transport and translational repression and understanding the ZBP1-RNA interaction is important to understand RNA re-modelling and potentially, to gain an insight in mRNA packaging. However, β-actin is one of many mRNA targets recognized by ZBP1 and understanding the ZBP1-RNA interaction is important to rationalize target recognition. ZBP1 contains six putative RNA-binding domains - two RNA recognition motifs (RRMs) and four canonical K-homology (KH) domains (Figures 2 and 3) - that are organized in three two-domain units. Recognition of different mRNA targets relies on the binding of either two (KH3-KH4) or four (KH1-KH2-KH3-KH4) of the KH domains, depending on the target[71][72][73], while whether the RRM domains play a role in RNA binding is unclear, although they possess a low but detectable RNA binding capability when in isolation (in *Xenopus*)[74]. ZBP1 recognition of β-actin mRNAs is mediated by the recognition of two specific sequences by KH3 and KH4 (ACA and CGGAC respectively) within the β-actin mRNA Zipcode[72][75]. The two RNA sequences bind on opposite sides of the KH3KH4 di-domain[72][75][76] (Figure 3), and biophysical and functional data indicate that these interactions are coupled[72][75]. Further, binding to the di-domain creates a loop in the RNA, re-modelling its structure[72][75][76]. Kinetic data and a biophysical model based on known cellular concentrations of IMP1 protein and β -actin mRNA explains that the interaction is driven by the protein, but not the mRNA, concentration[75]. This is consistent with the *in vivo* scenario whereby the steep increase in IMP1 concentration during brain development regulates IMP1 interaction with the β-actin mRNA. The model is also consistent with a set of fluorescence correlation spectroscopy data reporting on the local protein concentration and RNA binding in mouse fibroblasts and neurons[32].

While the ZBP1 β-actin mRNA recognition mode, which is shared by other neuronal mRNA targets, has been studied *in vitro* and *in vivo*, our understanding of global ZBP1-RNA interactions is more limited. PAR-CLIP, iCLIP and eCLIP transcriptome-wide studies of IMP1-RNA interaction in human ES cells and immortalized cell lines have identified that targets are enriched in short CA or CAU sequences[77][78][79], although a recent study has reported an enrichment for the GGAC sequence in CLIP data, which is linked to m6A-methylation of this sequence[80]. Deconvoluting the information present in these studies to define different RNA binding modes is inherently difficult and the lack of information on ZBP1 KH1 and KH2 domain-RNA binding adds to these difficulties and has so far prevented us from rationalizing the contributions of the individual domains to the ZBP1-RNA interactions.

CPEB1 – CPEB1 is a general regulator of translation both during embryonic development and in adults[81]. In mammalian neurons, CPEB1 mediates the transport of selected mRNAs to individual synapses to regulate synaptic differentiation upon stimulation. Consistent with the role of CPEB1 in synaptic plasticity, CPEB1 knock-out leads to a deficit in synaptic long term potentiation and memory defects. CPEB1 function in mRNA transport and translational regulation requires the assembly of a multi-component protein-RNA complex that includes Kinesin and Dynein motors, a non-canonical polyA polymerase, a deadenylase (PARN), the 4E-BP Neurogenin (in neurons) and scaffolding proteins. The two molecular motors mediate the mRNA transport in neurites, while the PARN de-adenylase ensures a short polyA is maintained and translation is repressed[29][82]. Phosphorylation by the kinase Aurora A leads to a re-arrangement of the complex involving the loss of the PARN de-adenylase and the disruption of the interaction between the 4E-BP Neurogenin (in neurons) and eIF4E, leading to local translation at the synapse[81]. CPEB1 associates with a set of mRNAs carrying a Cytoplasmic Polyadenylation Element (CPE) UUUUUAU sequence which represents the functional site of CPEB1 and is positioned 3' of the mRNA polyadenylation signal[81]. In mammalian neurons, well characterized targets include *GluN2A*, an mRNA which encodes an important NMDA receptor, and *CamKII* which encodes a kinase targeting Calmodulin. While, as far as we are aware, CLIP-type studies of CPEB1 in neuronal cells have not been published, a transcriptomic CLIP-type study in

Drosophila cells has reported that that the *Drosophila* homologue of CPEB1, Orb, interacts with >3000 mRNAs. Orb binding sites are located mostly in the mRNA 3'UTR consistently with the reported function of the protein in the control of polyadenylation and translational initiation. Also consistent with the function of the protein in cytoplasmic polyadenyation, the most enriched sequence reported by this CLIP study (UUUUA) recapitulates the CPE RNA element[83].

CPEB1 recognition of the CPE signal has been recently described both at the molecular and structural level. CPEB1 contains four putative RNA binding domains, two RRM domains and two Zinc Fingers (the so-called ZZ domain) at its carboxy-terminal end[81][84] (Figure 2 and 4). A structure of the two RRM domains in complex with the CPE explains that the specific recognition of the UUUUA/C sequence is mediated by the two RRM domains, that, upon interacting with the RNA arrange in a V-shape to capture the five recognised nucleotides[84] (Figure 4). Interestingly, the structure also suggest how the ZZ and N-terminal domains would position with respect to the RRM, providing an insight in how the CBEP1 complex is arranged on the mRNA molecule (Figure 4). However, despite this structural information, a question still exist on whether other RNA determinants of CPEB1 binding exists in addition to the CPE and whether the ZZ domain plays a role in RNA target selection[84].

Staufen2 - Staufen plays an key role in mRNA localisation in the antero-posterior patterning of the *Drosophila* oocyte[22], but Staufen and its paralogues are also important in neurogenesis and the development of the nervous system both in fly[3] and in mammals[85][86]. In the mature nervous system, Staufen proteins regulate dendritic development and synaptic plasticity (reviewed in [87]). Staufen is important in the assembly of protein-RNA particles and in mRNA transport and translational repression. In the current model of regulation, particle assembly and translational repression start in the nucleus and the Staufen protein accompanies the bound mRNA to the synapse. Two Staufen paralogues exist in mammals. Staufen1 is widely expressed in the organism, while Staufen2 is neuron-specific. Both Staufen1 and 2 are involved in the localisation of neurite-related mRNAs but are thought to have a non-redundant function. The reduced expression of the neuron-specific Staufen2, which we focus on, alters synaptic plasticity and memory[86][88], which is not surprising as the targets of these protein include CAMKII mRNA.

On the contrary to ZBP1 and CPEB1, Staufen recognise dsRNA elements. The protein contains four complete double stranded RNA-binding domains or motifs (dsRBD) (Figure 2 and 4) and dsRBD3 and 4 are thought to mediate RNA binding. dsRBD domains recognizes the geometry of the A-form RNA helix but tolerate some deviations including some terminal end structures, as originally shown for the third dsRBD of the *Drosophila* Staufen protein[46][89] (Figure 4). A dsRBD recognition site can span 14-16 base pairs in a perfect A-form helix[90] and such a structure is extremely rare in mRNAs. Recognition of non-canonical helical structures is arguably important to expand the number of RNAs that are bound by this domain. Indeed, a hiCLIP study mapping Staufen1 interaction with double stranded RNA helices in highly proliferating cells indicated that the protein interacts with a large number of sites containing helices of variable length and that can also include irregularities. dsRBD can also make contact with individual nucleobases in the RNA minor groove[46][89][90][91], but Staufen does not recognise specific RNA sequences[92]. Finally, a very recent iCLIP study in mammalian brain indicated that Staufen2 interacts with a large ensemble of targets: more than 300 mRNAs 3'UTRs were reported to cross-link to the protein, many of which are related to neuronal function[93]. These targets include the *CAMKII* and *Calm3* mRNAs, that are recognised by Staufen2 via structured elements in a retained intron within the mRNA[93][94]. While we possess a general understanding of dsRBD-RNA recognition, how Staufen2 (and Staufen1) select the mRNA targets is

still being investigated. It is however worth mentioning that RNA recognition is likely to be mediated by multiple dsRBDs, as individual dsRBDs bind to RNA with Kds around micromolar, and many dsRBD containing proteins (e.g. ADAR, PKR, DRGC8) interact with RNA using two dsRBDs. Also, additional proteins may be involved in Staufen selection of the cellular targets.

This brief overview of RNA recognition by the FMRP, ZBP1, CPEB1 and Staufen2 proteins highlights the differences in domain composition and in the RNA elements recognised by the proteins. These differences are associated to distinct regulatory mechanisms e.g. repression of translational elongation versus translation initiation, indicating that the RNA binding mode of these proteins reflects their underlying functional complexity. Regardless, RNA binding by the different proteins also present important similarities. The proteins have all been reported to bind large sets of target mRNAs with the combinatorial action of multiple RNA binding domains. Interestingly, this recognition mode is also observed in other well-studied regulatory proteins, such as hnRNPA2, Syncrip, Marta2/KSRP, TDP43 and MBNL2 (Figure 5). Importantly, for a number of isolated domains, and in some cases di-domain units, we possess a detailed understanding of RNA recognition, such that it is possible to rationalize the specific recognition of nucleobases and sequences that we know to be essential for recognition and function *in vivo*. Further and importantly our mechanistic understanding of combinatorial recognition is still tentative and in particular it is difficult to translate this mechanistic understanding from the *in vitro* system to the cellular environment.

Protein-RNA regulation networks

The local translation of individual mRNAs is regulated by multiple RNA binding proteins. For example, CamKII mRNA is regulated by the CPEB1[95], TDP-43[96] and FMRP[97][98] proteins, while the regulation of β-actin mRNAs is mediated by the ZBP1, TDP43[96] and Staufen2[99] proteins, and Map1B mRNA by the Marta2[100] and KSRP[100] proteins. Underlying these regulatory inputs is the ability of individual mRNAs to bind multiple protein regulators which recognise sequence and structure-based protein recognition motifs[101][102]. These motifs are mainly located in the mRNA 3'UTR, which is longer and highly regulated in the mammalian brain[103]. In turn, individual proteins regulate multiple mRNAs. The Staufen, ZBP1, FMRP and CPEB1 proteins each bind large sets of targets as discussed above, and many of these targets are reported to be co-regulated, for example between the CPEB1 and FMRP proteins[11]. Arguably, co-regulation of neuronal mRNA is unlikely to be limited to a few important cases, but rather is likely to be pervasive.

The multiple protein-RNA interactions create a complex network that regulates the axon and/or dendrite-enriched pool of mRNAs. mRNAs co-regulation raises the question as to what extent the recognition of RNA Zipcodes depends on specific interactions between different RNA binding proteins. The RNA binding proteins regulating local mRNA translation include both RBDs and protein interacting domains, as well as domains that can bind to both protein and RNA. How RNA binding proteins regulating mRNA local translations in mammalian neurons recognise the mRNA targets in a coordinated fashion is still largely to be investigated at the molecular and structural level. However, in *Drosophila* the structures of a few protein-protein-RNA complexes have exemplified how specific protein–protein interactions may expand RNA recognition [18]. For example, in Pumilio (Pum)-Nanos (Nos) recognition of *hunchback* and *CyclinB* mRNAs, binding of Nos increases the affinity of Pum for the RNA and extends the RNA recognition surface[104]. Interesting this interaction is part of a

network that include a third protein, Brat, that together with Pum acts as a translational repressor[105] and recognise the target ssRNA via the non-canonical NHL domain[106]. A second example is the one of the interaction between the Sex lethal (Sxl) and Upstream of N-Ras (Unr) proteins where the two protein create a joint recognition surface for the large 3' section of the target motif[107].

Perspectives and future work

In the last five years, contributions from different areas of research have revealed the complexity of local mRNA translation in neurons. A combination of novel cross-linking techniques and molecular biology tools, second generation sequencing and bioinformatics analysis have allowed to map the interaction of many of the proteins regulating local mRNA translation at the transcriptome level[108][109][77][110]. In many of the studies using CLIP-type methods, binding has been correlated to functional information to describe the action of the RNA-binding proteins at the transcriptome level. Further, a number of recent versions of the CLIP technique (e.g. hiCLIP[92]) define binding sites in relation to RNA structures and modifications, directly connecting protein binding to RNA regulation. This has extended the capability of the method(s) to describe functional RNA recognition and in future developments we can expect will help define the connections that have been reported between the processes of mRNA transport and translation that we discuss here and the mechanisms that create alternative 3'UTR and gene sequences by alternative polyadenylation, intron retention or mRNA modification (reviewed in[111]).

In microscopy, advances in molecular labels, instrumentation and data processing have allowed to investigate mRNA transport and translation in neurons at the single molecule level but also obtain semi-quantitative information on the local concentration, interactions and motions of the protein and RNA molecules[112][32]. This information is important to establish mechanistic models to describe protein-RNA interactions in the cell. In a further development, the use of novel methods for combinatorial data acquisition and processing have allowed to simultaneously examine the precise locations of hundreds of different RNAs in the cell[113]. While the potential of the technique is still largely unexplored, the promise is to further the integration of microscopy data with the transcriptome-wide analysis of the interactions.

Despite the advances discussed above, our molecular understanding of the protein-RNA interactions regulating local mRNA translation is still, in many cases, limited. Structural and biophysical information on protein-RNA recognition are available for a number of one and two-domain RNA binding units (Figures 3, 4 and 5), but our mechanistic insight into the combinatorial interactions is still rudimentary. Building this insight will require data on the structure of the larger complexes where RNA is recognised by the multi-domain RNA binding units or, when required, by multiple proteins. It will also require capturing the motions of the protein and RNA molecules, which represent an essential component of recognition in protein-RNA interactions[114]. Finally, it will require kinetic data that describe the contribution of the domains to the interaction. The integration of different structural biology methods (NMR, X-ray, possibly cryoEM, depending on size and flexibility) together with small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS)[115][116] and recently implemented biophysical tools such as Biolayer

Interferometry[117][118], provide a set of techniques to meet these important challenges and rationalize microscopy and transcriptome-wide data on multi-component protein-RNA interactions.

Figure Legends

Figure 1: mRNA localisation and translation in neurons

Cartoon of a neuron with mRNA and regulatory proteins as discussed in this. The mRNA molecule (black line) is transcribed in the nucleus and immediately bound by several RNA binding proteins (coloured ovals). The complex is then exported to the cytoplasm where a cytoplasmic ribonucleoparticle (RNP), or neuronal granule, of different composition is formed. Motor proteins (green) mediate the transport of neuronal granules along microtubules (blue). The mRNA is translationally repressed until a signal releases the RBP-mediated translational block, and the ribosome complex (grey) translates the mRNA into protein (red). The 'sushi belt' model[119], where the mRNA is shuttling along the dendrites until it is recruited by an excited synapse (lightning bolt/yellow star) is represented by a circular arrow.

Figure 2: Domain organization of representative neuronal RNA binding proteins

RNA-binding proteins regulating local mRNA translation have multiple, different, RNA-binding domains. Domain types are listed at the bottom and right of the Figure, with known RNA binding domains are highlighted within a purple box. Abbreviations are: RRM – RNA recognition motif, KH – K-Homology domain, ZnF – Zinc finger domain, dsRBD – double stranded RNA binding domain, Puf – Pumilio domain, NURR - N-terminal unit for RNA recognition, and NHL (NCL-1 -HT2A - LIN-41) domain. MBNL1 contains two ZnF domains that are fused into a single ZZ domain, represented by two overlapping yellow circles. PUM2 contains a large N-terminal region and is not represented in scale compared to the other RNA binding domains, denoted as //. The NHL domain, here represented within in the axonal regulator TRIM2[120], is composed by multiple repeats of a β-sheet that however do not form structurally independent units. It is therefore represented as an elongated shape. Unstructured RGG box domains are represented as black waves. Note the central part of the sequence of the 5th dsRBD of Staufen2 is missing. Known protein-protein interaction domains are not represented except for the Agenet-like domains of FMRP, which are unlikely to bind RNA but they are structurally joined with KH0 and represented here for consistency with Figure 3. Finally canonical and non-canonical domains of the same class (e.g. RRM, KH) have been represented with the same logo.

Figure 3: Structural perspective on RNA-binding by the ZBP1 and FMRP proteins

Left: protein structures are displayed as a cartoon and coloured grey. Where available, the structure of the protein in complex with RNA is shown. In those structure only the nucleotides bound by the protein are displayed. The RNA phosphate backbone is coloured in blue and the RNA bases in yellow. Domains for which structural information has not been published are represented as coloured

shapes as in figure 2. Black dashed lines indicate two domains are connected and are not a physical representation of the protein linker. The Agenet-like domains of FMRP are unlikely to bind RNA, but they are structurally joined with KH0 and are therefore represented here. In the construct of FMRP KH12 the variable loop of KH2, between the β2- and β'-strands, has been shortened to a length found in most KH domains (4-7 nt), which is represented by *. The Sc1 G-quartet whose structure is represented here is a SELEX-derived RNA molecule, while the sequences bound by IMP1 are derived by the β-actin Zipcode. The RNA nucleotides bound by the protein are annotated below the figure. Please note the structural representation on the left has been obtained by superimposing the structures of the KH3-RNA bound and KH4-RNA bound KH34 complexes as described in[75]. Right: zoom in the protein-RNA contacts present in the two structures. Sidechains of amino acids that are involved in RNA recognition are displayed and coloured by atom type (Carbon is here blue). For ZBP1, the two central nucleotides, which are recognised with a higher specificity, are displayed. The key H-bonds are highlighted (purple dashed line). The bases of the Sc1 FMRP SELEX-derived RNA target are coloured in dark grey (G-quartet) and light grey (helix) with the bases that make contact with the RGG box coloured by atom type (Carbon is here blue). Hydrogen bonds are displayed. PDB codes for the represented structure are: FMRP: Agenet-like12+KH0: AQVZ; KH12: 2QND; RGG Box: 5DEA. IMP1: KH3: 2N8M; KH4: 2N8L.

Figure 4: Structural perspective of RNA binding by the CPEB1 and Staufen2 proteins

Left: structural representation and colour coding as for figure 3. The bases interacting with CPEB1 RRM1 are highlighted by a double blue underline. # highlights that the interactions between the CPEB1 RRM12 and ZZ di-domain have been modelled. The hairpin whose structure is represented is a model system for Staufen dsRBD3-RNA recognition, rather than a physiological target. Right: Zoom in showing protein-RNA recognition as in figure 3. Sidechains of amino acids that are involved in RNA recognition are displayed and coloured by atom type. The interaction of Staufen2 dsRBD3 with RNA hairpin is mediated by two protein loops and the first alpha helix of the protein, and by moieties in the RNA major groove and the two flanking RNA minor grooves. PDB codes for the represented structures are: CPEB1 RRM12: 2MMK; ZZ: 2MKE. Staufen dsRBD3: 1EKZ.

Figure 5: Structures of the RNA-binding domains of HuD, TDP43, hnRNPA2, KSRP/Marta2, MBNL1, FUS Syncrip and PUM2 proteins

Structural representation and colour coding as for figure 3. Please note in HuD the longer chain between RRM2 and RRM3 is scaled down, represented as //. # highlights that the interactions between the CPEB1 RRM12 and ZZ di-domain have been modelled. The interacting RNA sequences are displayed below the structure and underlined, bases making contact with two domains have therefore a double underline. Bases displayed in black do not make contact with the protein domain. PDB codes for the represented structures are: HuD: 1FXL; TDP-43: 4BS2; hnRNPA2: 5HO4; KSRP: KH1: 2OPU; KH2: 2OPV; KH3: 4B8T; KH4, 2HH2; KH23: 2JVZ; MBNL1: ZnF12: 5U9B; ZnF34: 3D2S; FUS: RRM1, 2LA6; Syncrip: 6ES4; PUM2: 3Q0Q.

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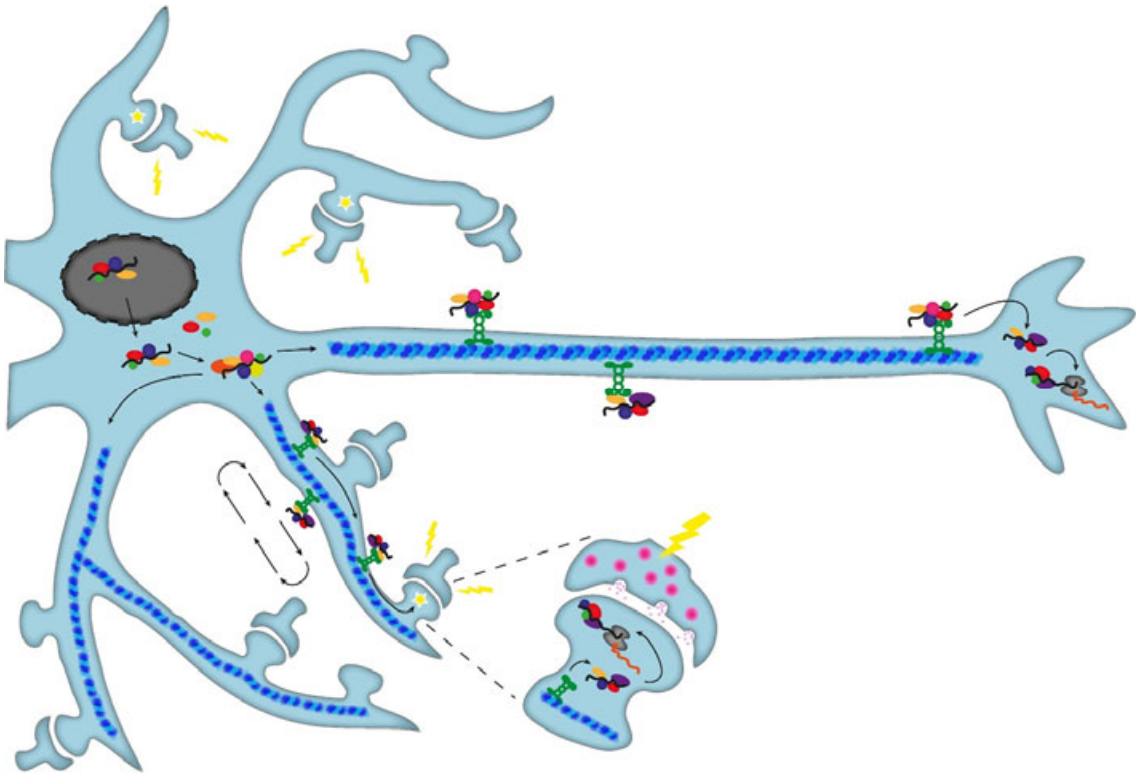

Figure 1

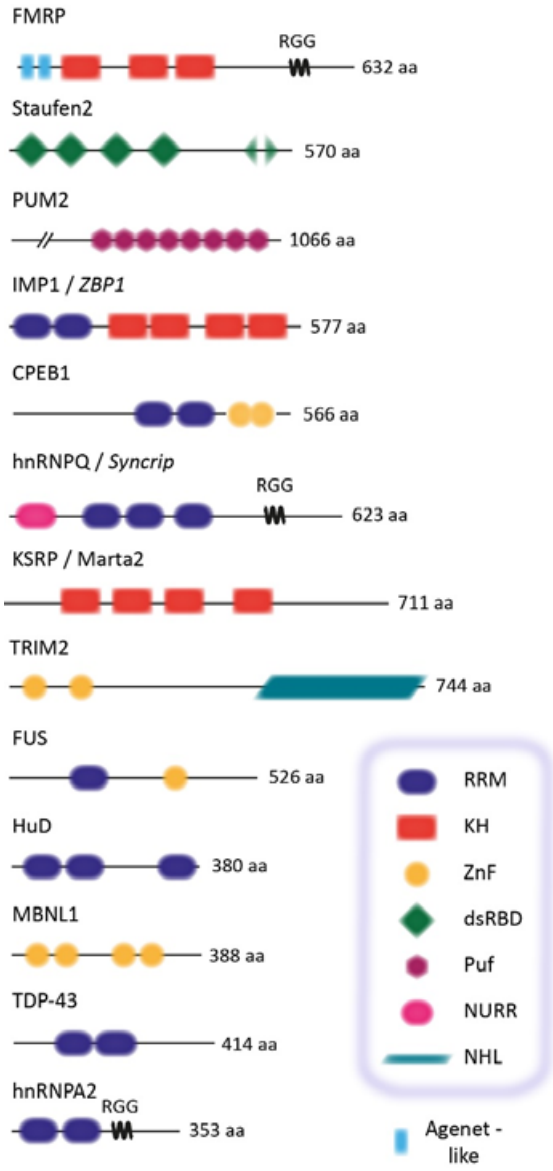

Figure 2

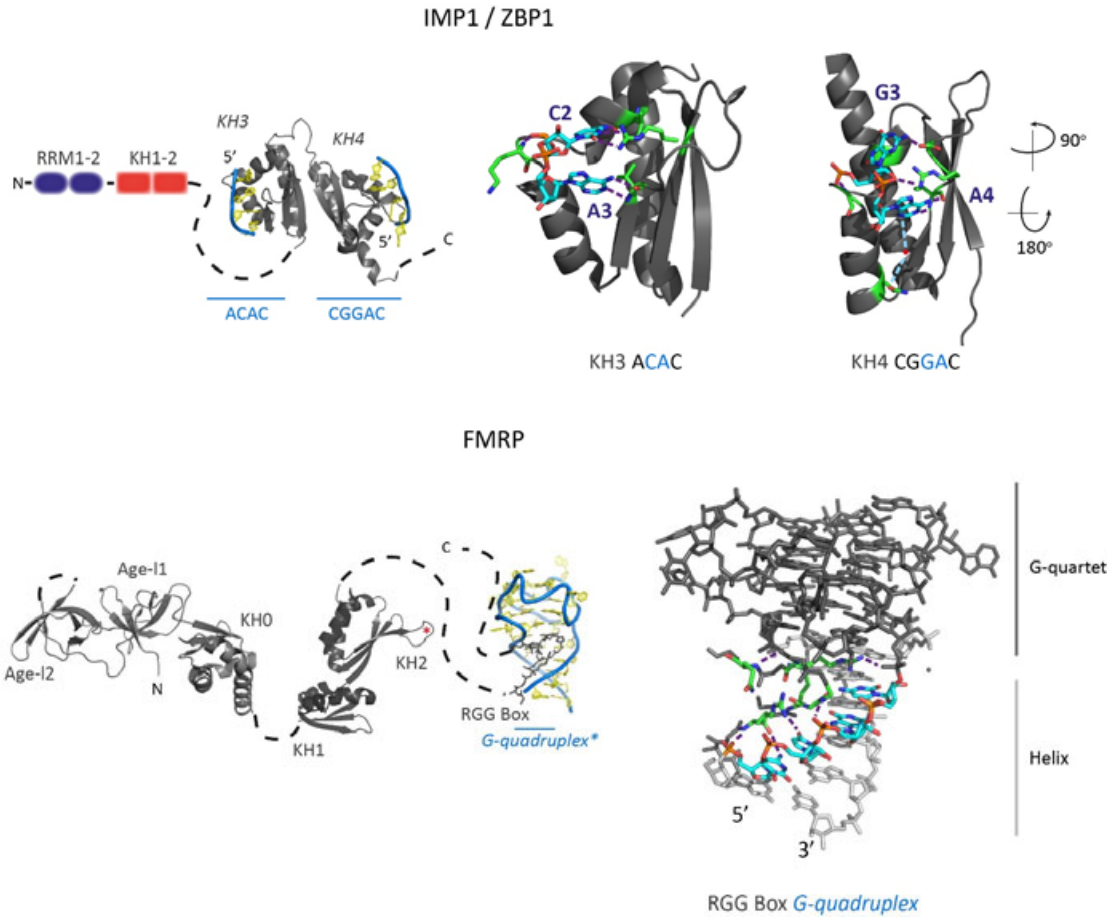

Figure 3

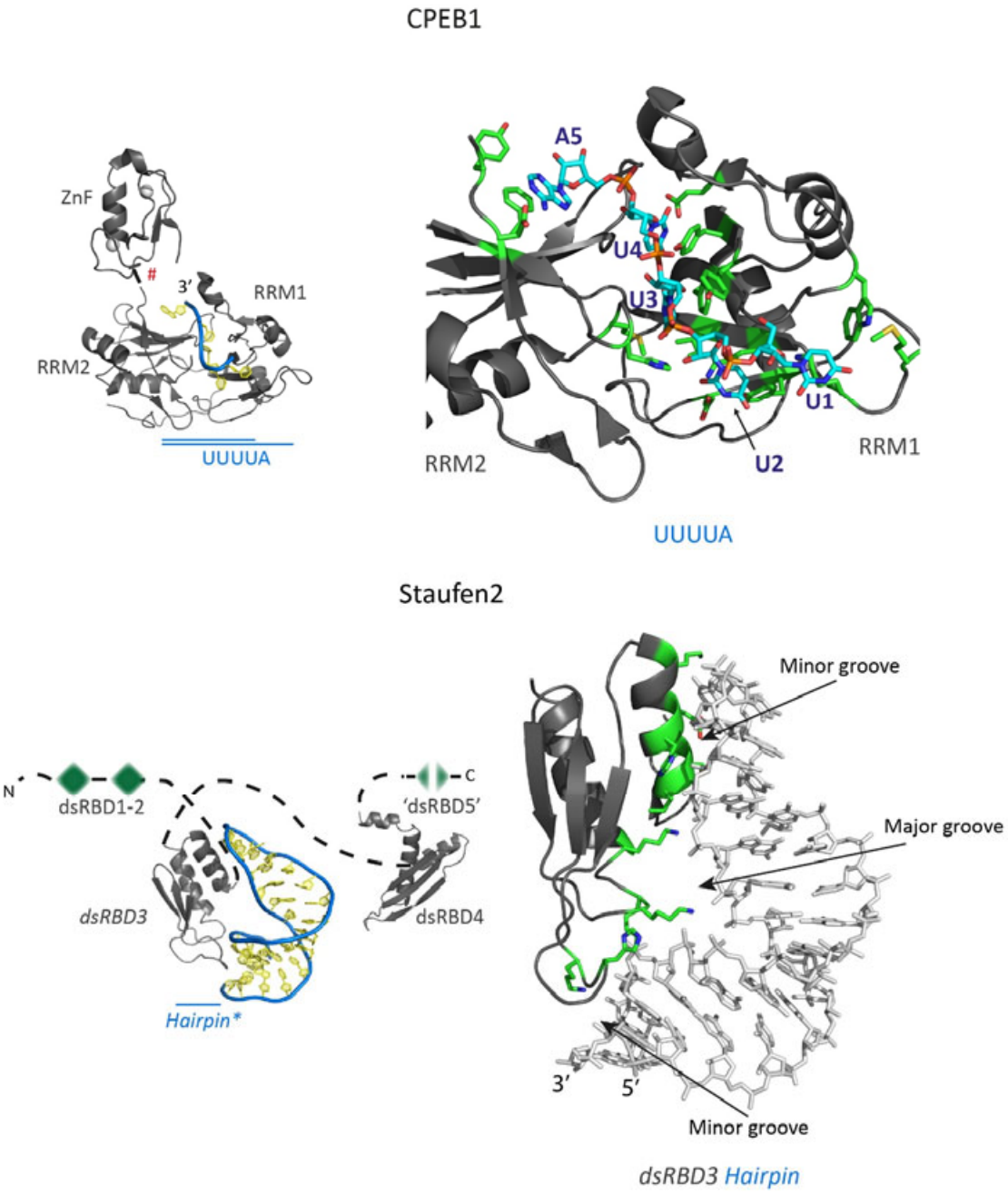

Figure 4

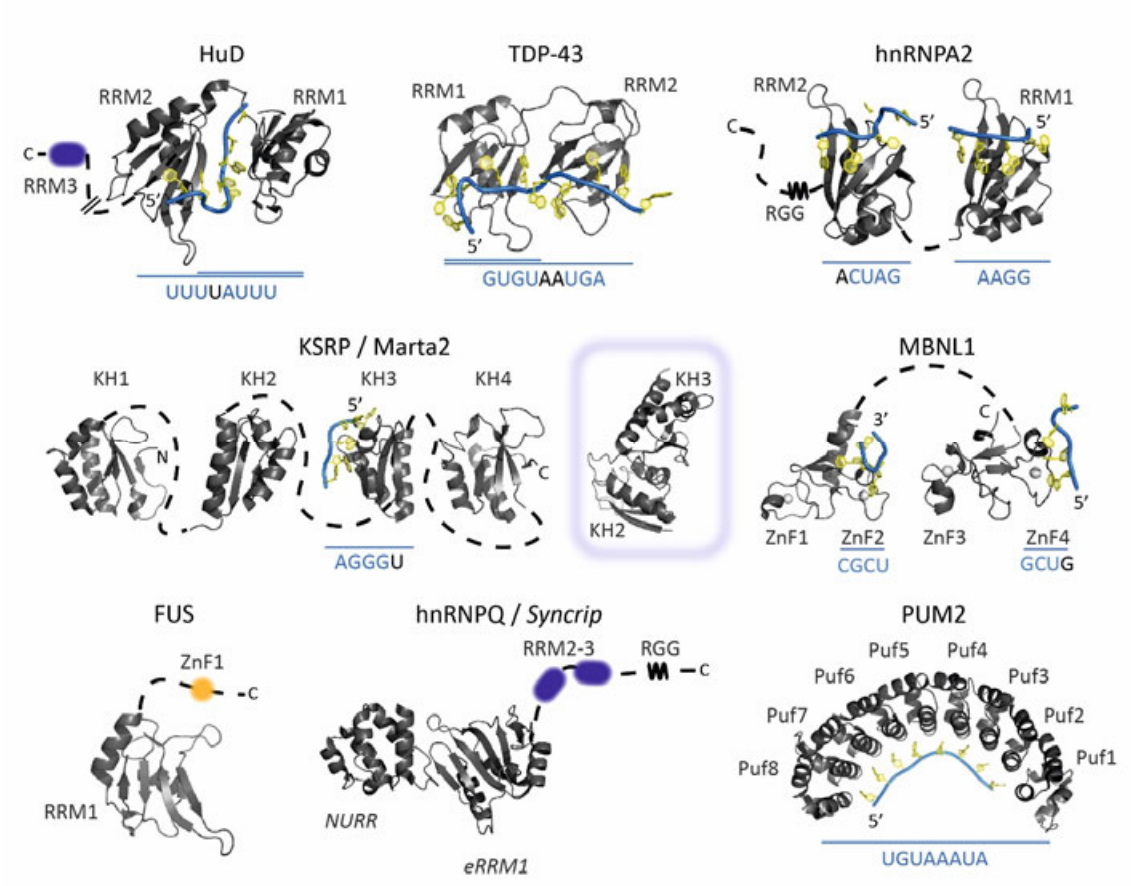

Figure 5