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Modulation of Gene Expression by RNA Binding Proteins: mRNA Stability and Translation

Kotb Abdelmohsen

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1. Introduction

Regulation of gene expression is an essential process through which mammalian cells counter the changes in their microenvironment. These changes drive cells to respond to different stimuli that trigger cellular re-programming towards proliferation, differentiation, development, apoptosis, senescence, carcinogenesis, etc. Once mRNAs are transcribed they are subjected to posttranscriptional events that regulate mRNA metabolism including stability and translation. These two processes normally dictate the protein levels encoded by mRNA. RNA-binding proteins (RPBs) that bind mature mRNA sequences normally have an important regulatory effect on the mRNA.

RBPs are also named mRNA turnover and translation regulator RBPs (TTR-RBPs) since they are capable of regulating both mRNA stability and translation. This family includes numerous members such as Hu proteins [HuA (HuR), HuB, HuC, and HuD] known to bind AU-rich sequences in the 3′ untranslated region (3′UTR) to enhance mRNA translation or to increase its stability [1, 2]. Other RBPs such as T-cell intracellular antigen 1 (TIA-1), TIA-1-related (TIAR), tristetraprolin (TTP), fragile X mental retardation protein (FMRP), polypyrimidine tract-binding protein (PTB), CUG triple repeats RNA-binding protein (CUGBP), nucleolin, and heterogeneous nuclear ribonucleoproteins (hnRNP) A1, A2, and C1/C2 have been shown to influence mRNA stability or translation through interaction with the 3′UTR, CR or the 5′UTR [3-9].

Hu proteins are among the RBPs that are well characterized. While HuR is ubiquitously expressed, HuB, HuC and HuD are primarily neuronal [10]. HuR, also known as embryonic lethal, abnormal vision, Drosophila-like 1 (ELAV L1), binds mRNAs bearing AU- and U-rich sequences, which are considered binding signatures, or RNA-recognition motifs (RRMs)

found in numerous mRNAs [11]. HuR binds target mRNA to enhance its stability and/or translation. These targets are involved in several processes such as cell growth, survival, proliferation, stress response, senescence and carcinogenesis [2, 12-17].

The RBP AU-binding factor 1 (AUF1), also known as heterogeneous nuclear ribonucleoprotein D (hnRNPD), is known to bind AU-rich sequences mostly in the 3'UTR of target mRNA. AUF1 belongs to a large family that includes several hnRNPs such as hnRNNP A, B, C, D, E, F, H, I, K, L, M, Q, R and U. AUF1 promotes the degradation of several target transcripts. However, it was also found to enhance the stability and translation of some mRNAs [18-21]. AUF1 is alternatively spliced and all known four isoforms (p37, p40, p42, p45) bind mRNAs but with different binding affinities [22]. AUF1 is thought to recruit mRNAs to the exosome and proteasome for degradation [23, 24]. Target mRNAs are implicated in several processes such as cell cycle, stress response, apoptosis, and carcinogenesis [21].

TIA-1 and TIAR RBPs bind AU/U-rich sequences in the 3'UTR of target transcripts and suppress mRNA translation [25]. However, they can also modulate translation through 5' terminal oligopyrimidine tracts (5'TOP) in response to changes in the cellular environment [26]. Under stress conditions, these proteins are thought to halt mRNA translation in RNA-protein aggregations known as stress granules [27].

The RBP nuclear factor 90 (NF90) interacts with many RNAs bearing AU-rich sequences. NF90 normally suppresses the translation of target mRNAs involved in cell cycle, translation, proliferation and cell division [28]. Although FMRP is expressed in several tissues, it has an essential role in neuronal and intellectual development. FMRP regulates stability and translation of several genes involved in synaptic plasticity [29]. Mutation of FMRP normally results in the inability to inhibit translation and can lead to fragile X syndrome, mental retardation, premature ovarian failure, autism and Parkinson's disease. FMRP is found in RNA granules associated with ribosomal RNA (rRNA) in dendrites [30, 31]. TTP, a zinc finger protein, binds AU-rich sequences in mRNA transcripts to promote their decay. Target mRNAs are involved in cell cycle, inflammation, and carcinogenesis [32]. Nucleolin interacts with mRNAs bearing AU-rich or G-rich sequences to regulate mRNA stability and/or translation. Target transcripts are involved in several processes such as cell cycle, cell morphology, development, growth, proliferation, and carcinogenesis [3]. KH-type splicing regulatory protein (KSRP) RBP binds AU-rich sequences of target transcripts promoting mRNA decay. These targets encode cytokines, chemokines, transcription factors, proto-oncogenes, and cell-cycle regulators [33].

If the RBP functions to promote mRNA degradation such as AUF1 or TTP, then the mRNA half-life is shortened and therefore protein levels will be subsequently low. On the other hand, if the RBP functions to promote mRNA stability such as HuR, then the RBP will subsequently increase protein levels by extending the mRNA half-life. Similarly, if the RBP modulates mRNA translation, protein levels will be influenced accordingly. Figure 1 summarizes these general effects of RBPs as posttranscriptional gene regulators.

This chapter will focus on the effects of RBPs on mRNA stability and translation. It is believed that two or more RBPs may have a functional interplay among themselves and with small RNA molecules known as microRNAs, through binding to the same mRNA. Examples of stabilized and destabilized genes will be indicated, as well as translationally enhanced or suppressed mRNAs and the involvement of encoded proteins in the cellular process.
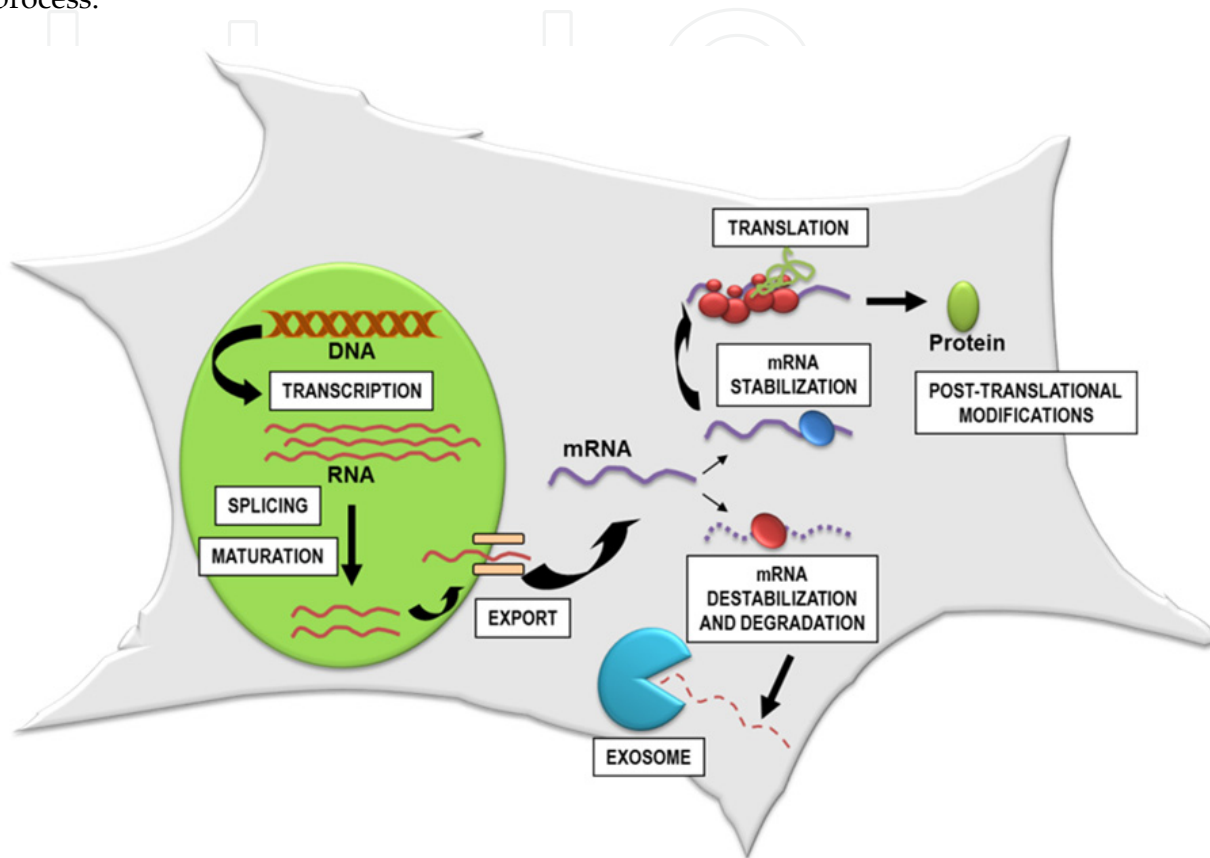

Figure 1. Regulation of mRNA stability and translation by RNA-binding proteins (RBPs). They mainly influence the fates of target mRNAs at the post-transcriptional levels. In the cytoplasm, stabilized mRNAs are protected from degradation leading to more protein levels. Destabilized mRNAs are driven to degradation machinery leading to lower protein levels. RBPs can also influence the abundance of mRNAs in the translation machinery (polysomes).

2. Methodology

In this section the methodology of investigating binding of RBPs to target mRNAs as well as the effects on mRNA stability and translation will be explained.

2.1. mRNA-ribonucleoprotein immunoprecipitation (mRNP-IP)

PAS beads from Sigma (P-3391) (or preswollen beads from Sigma) can be used to coat the IgG control antibody or the specific antibody recognizing the RBP. Mix 10 µg antibody, 60 µl volume beads and 200 µl of NT-2 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 0.05% Nonidet P-40). Rotate overnight at 4°C.
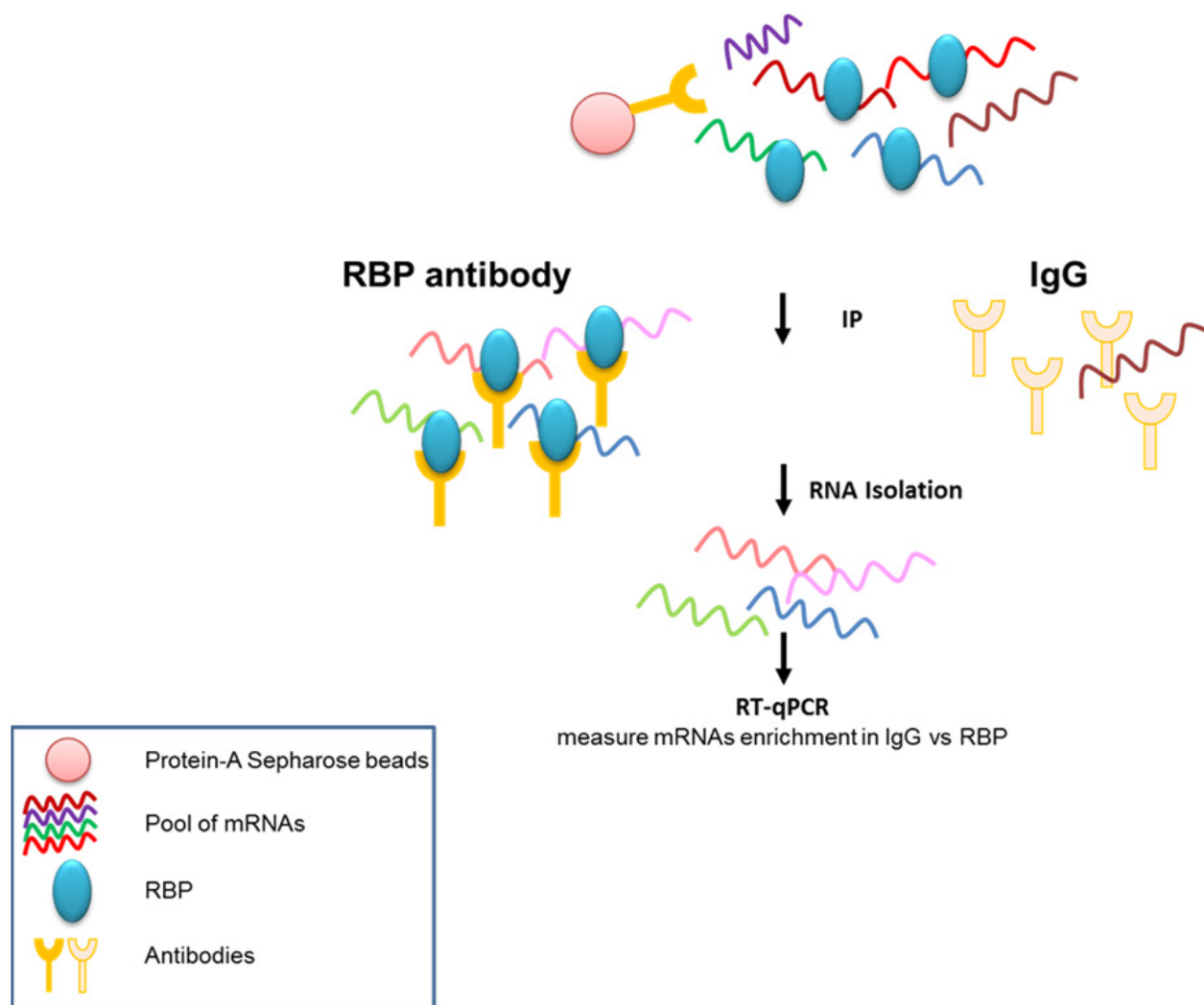
Figure 2. Schematic illustration of ribonucleoprotein immunoprecipitation (see text).

Harvest and lyse tissue culture cells in ice-cold lysis buffer supplemented with RNAse inhibitors and protease inhibitors for 10 minutes on ice. Lysis buffer is prepared by mixing 100 mM KCl, 5 mM MgCl2, 10 mM Hepes, pH 7.0, 0.5% Nonidet P-40, and 1 mM Dithiothrectol (DTT) added at the time of use. Spin 30 min at 14,000 rpm (20,000 x g) /4°C and transfer supernatant to the fresh tubes. Wash the pre-coated beads two times with NT2 buffer and add equal amounts of lysates to each antibody. Rotate at 4°C for one to two hours. Wash the beads Five times with 1 ml aliquots of ice-cold NT-2 buffer (5000 g, five minutes). Add 100 µl NT2 buffer having five µl DNase I (2 U/µl) and inculate at 37°C for 5-10 minutes. Add 1 ml NT2 buffer, spin at 5000 g for 5 minutes, and discard supernatant. Then, add the 5 µl of Proteinase K (10mg/ml), 1 µl 10% SDS and 100 µl NT-2 and incubate with shaking at 55°C for 15-30 minutes. Spin at 5000 g for 5 minutes to collect supernatant which contains the RNA. To the supernatants add 300 µl of the lower layer of acid phenol-CHCl3 (Ambion) and vortex for one minute at room temperature. Spin at room temperature for one minute (14,000 rpm). Collect the upper layer, add 25 µl sodium acetate pH 5.2, 625 µl 100% ethanol and 5 µl glycoblue, mix well and store at 20°C overnight.

Spin at 14,000 rpm at 4°C for 30 minutes and discard supernatant. Wash the pellet with 1 ml 70% ethanol and spin at 14.000 rpm at 4°C for two minutes and then air dry pellet at room temperature for five minutes. Resuspend the pellet in 20-40 μl of RNAse-free water. This RNA can be used as any other RNA for real time PCR analysis or microarrays. If the gene of interest is enriched in RBP-IP which is twofold or higher compared to IgG-IP, then this gene and the RBP do interact. Figure 2 represents a schematic of mRNA-Ribonucleoprotein immunoprecipitation. For experimental examples see references [11, 34, 35].

2.2. Assessing the half-life ($t_{1/2}$) of target mRNAs

RBPs normally influence either mRNA stability or translation. Downregulation or overexpression of the RBP is helpful to determine whether the RBP affects mRNA stability. This can be achieved by transfection of either siRNA to downregulate, or a construct to overexpress, RBP of interest. Transfected cells are then treated with actinomycin D (2 μg/ml) to inhibit transcription. Cells can be harvested every hour for about six hours followed by isolation of RNA and real time PCR to measure the levels of genes of interest. The ribosomal RNA 18S is normally used for normalization. It is also recommended to measure the levels of a housekeeping gene or a gene that is not targeted by the RBP of interest. If the RBP influences mRNA stability, an increase or a decrease in the $t_{1/2}$ will be observed and can be calculated using this assay [11, 34, 35].

2.3. Evaluation of mRNA translation by polysome fractions

Prepare sucrose gradient solutions; 10-50% sucrose, 300 mM NaCl, 15mM MgCl$_2$, 15 mM Tris-Cl 7.5, 0.1 mg/ml cycloheximide and 1mg/ml Heparin. Layer the gradient with 2 ml of each solution starting with 10% (top) and ending with 50% (bottom). Be careful not to introduce any air bubbles into the gradient. Leave gradients at 4°C overnight to allow the step gradient to linearize. Lyse cells as described above (lysis buffer) and place cell lysates slowly at the top of the gradient. Use ultra-centrifugation spin for three hours at 35000 rpm at 4°C (SW41 rotor). Collect polysome fractions of 1ml each. An example of a polysome profile from HeLa cells is shown in Figure 3. It is important to note that the polysome profile might differ between cell lines and tissues. To analyze the distribution of a particular mRNA in the polysome profile, RNA is isolated from each fraction using Trizol followed by real time PCR and distribution can then be calculated. It is important to note that the sum of the mRNA distribution profile must be 100%. If the RBP affects mRNA translation, one or two observations can be found in the mRNA distribution profile. Significant changes in the amounts of mRNA in heavy translated fraction and a shift in the mRNA distribution profile either towards light or heavy fractions. These examples are illustrated in Figure 3 and reference [35].

3. Hu family

3.1. HuR

As mentioned above, the Hu family includes four RBPs, among them HuR which is ubiquitously expressed. HuR protein recognizes mRNAs through three RNA-recognition

motifs [36]. It binds AU-rich sequences present in the 3'UTR or 5'UTR [11, 13]. The interaction of HuR with target mRNA is modulated by HuR phosphorylation through the checkpoint kinase Chk2 [34, 35]. This kinase also regulates HuR levels under heat shock conditions through ubiquitin-proteasome pathway [37]. Protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) p38 also modulate the association of HuR with target mRNA [38-40]. Although HuR is predominantly nuclear, it is capable of shuttling to the cytoplasm through its nucleoyctoplasmic shuttling sequence (HNS) and transport machinery including chromosome region maintenance 1(CRM1), transportins 1 and 2, and importin-1α [41-44]. In addition, under stress conditions such as arsenite or heat shock, HuR aggregates in the cytoplasm with RNAs, known as RNA granules or stress granules, where mRNAs are stored or translationally suppressed [37, 45]. Levels of HuR are regulated by microRNA miR-519 which suppresses HuR mRNA translation [46]. Thus, these and other factors may influence the levels of HuR as a whole or its abundance in the cytoplasm and subsequently affect target mRNAs.
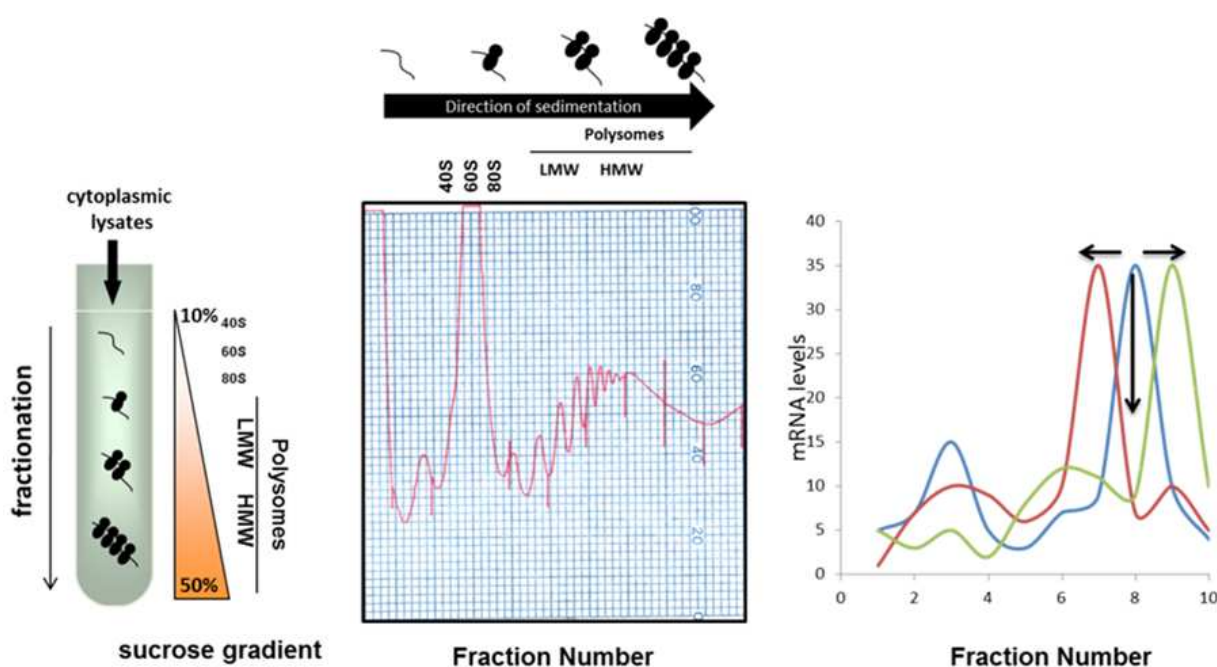

Figure 3. Schematic representations of translation assay (polysome profiling) to study the influence of RNA-binding proteins (RPBs) on mRNA translation (see text). Left; sucrose gradient, middle; example of a polysome profile using HeLa cells and right; examples of changes in mRNA distribution in polysome profile. Blue represents control, red represents an example of reduced mRNA translation and green represents an example of increased mRNA translation.

3.2. Stabilized HuR targets

Several target mRNAs are stabilized by HuR including cyclins A2, B1, E1, and D1 and p21. Proteins encoded by these mRNAs are involved in cell cycle, proliferation and cell survival. HuR also regulates the stability of genes such as silent mating type information regulation 2 homolog 1 (SIRT1), B-cell lymphoma 2 (BCL-2), epidermal growth factor (EGF), eukaryotic translation initiation factor 4E (eIF4E), and prothymosin α (ProTα) mRNAs.

These genes are involved in cell survival and proliferation. HuR promotes carcinogenesis by stabilizing mRNAs that encode for proteins such as Snail, matrix metallopeptidase 9 (MMP-9), urokinase (uPA) and urokinase receptor (uPAR), which enhance tumor invasion. Other genes such as vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) are also stabilized by HuR and involved in angiogenesis. Recent high-throughput analysis of HuR targets using photoactivatable ribonucleoside crosslinking and immunoprecipitation (PAR-CLIP) revealed the number of HuR binding sites per transcript, levels of binding, and degree of HuR-dependent RNA stabilization. Interestingly, HuR was found to bind pre-mRNAs and non-coding RNAs suggesting that HuR may integrate processing and stability [47].

These data suggest that HuR has a protective effect on its target mRNAs. While mRNAs can be recruited to the degradation machinery such as exosome and processing (P) bodies, HuR might be competing for binding to these labile mRNAs preventing or slowing down the degradation process. Since HuR is mostly present in the nucleus and PAR-CLIP identified pre-mRNAs bound to HuR, it is likely that HuR co-transcriptionally binds nuclear RNA substrates. This may also imply that HuR may play roles in splicing or maturation of these RNAs.

3.3. Translationally regulated HuR targets

HuR binds the 3′UTR of several mRNAs to enhance translation. For example, HuR enhances prothymosin α (ProTα), B-cell leukemia (BCL-2), and cyclin A2 mRNA translation. These genes are involved in cell cycle, proliferation, and cell survival [1]. While HuR binds the 3′UTR of wingless-type MMTV integration site family, member 5A (Wnt5a) mRNA to suppress translation, it binds the 3′UTR *of* thrombospondin 1 (TSP1) and vascular endothelial growth factor (VEGF) mRNAs to enhance its translation which are involved in carcinogenesis. In addition HuR binds 5′UTR of p27 and suppresses translation; p27 is involved in cell cycle, proliferation and cell survival. HuR also binds the 5′ UTR of the hypoxia-inducible factor 1, alpha (HIF-1α) mRNA and promotes translation. This regulatory effect involves internal ribosome entry site (IRES) present in the 5′UTR of HIF-1α mRNA [48]. These data suggest that HuR can enhance mRNA translation through binding to the 3′UTR or 5′UTR. Future studies are required to investigate the roles of HuR coordinated with IRES located in the 5′UTRs to initiate mRNA translation.

3.4. HuD

Among Hu family proteins, HuD is also well studied. This RBP was initially described as neuronal specific [13]; however recent studies have shown HuD to be expressed in other cells such as pancreatic β cells [49, 50]. HuD is essential for neuronal development, identity, and differentiation through stabilization of mRNAs encoding proteins involved in these processes such as growth associated protein 43 (GAP-43), p21, acetylcholinesterase (AchE), and other targets [51-54]. In addition, HuD is involved in Parkinson's and Alzheimer's diseases and highly expressed in neuroblastomas [55-58]. Recent studies showed that HuD

is regulated by the microRNA miR-375 which suppressed neuritis outgrowth [49]. In pancreatic β cells HuD was also found to bind the 5'UTR of preproinsulin mRNA and negatively regulate mRNA translation [50]. These findings suggest that HuD is expressed in other tissues than neuronal tissues and might have important regulatory functions yet to be investigated.

4. hnRNP family

These RBPs include several members such as hnRNP C, hnRNP D, and hnRNP K. hnRNP D, also known as AUF1, destabilizes mRNAs through binding to the 3'UTR. However some studies have indicated that AUF1 may also stabilize mRNAs and enhance mRNA translation. AUF1 post-transcriptionally regulate the expression of several genes involved in cancer and inflammation [21]. AUF1 is expressed as four isoforms due to alternative splicing of exons 2 and 7. The encoded isoforms are $p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$, and $p45^{AUF1}$ according to their molecular masses. AUF1 isoforms contain two RNA recognition motifs (RRMs) that mediate binding to mRNA transcripts [21].

4.1. Influence of AUF1 on target mRNA

Unlike HuR, AUF1 promotes the degradation of the vast majority of its known targets through the recruitment of the mRNA to the exosome and the proteasome [23, 24], for example: cell cycle related genes such as cyclin D1, p21, p27 and p16INK4a [59-62], apoptosis regulators such as B cell leukemia (BCL-2) and growth arrest and DNA-damage-inducible protein alpha (GADD45α) [63, 64]; inflammatory related genes such as granulocyte-macrophage colony-stimulating factor (GM-CSF): and interleukin 6 (IL6) and human inducible nitric oxide synthase (NOS) [65-67] and DNA replication and repair related genes such as thymidylate synthase (TYMS), jun D proto-oncogene (JUND and c-fos (FOS) [68-70]. However, AUF1 can promote the stability and translation of some target transcripts. For example, AUF1 enhances c-MYC mRNA translation and stabilizes interleukin 1β (IL1B) mRNA in LPS stimulated cells [18, 71].

5. Other RBPs

In addition to the abovementioned RBPs, several others are known to post-transcriptionally regulate gene expression in similar fashions. For instance, TTP is known to promote the decay of mRNAs containing ARE sequences including several genes involved in cancer and inflammation such as IL-2, IL-3, IL-6, IL-10, and IL-12 [72-76].

Nucleolin post-transcriptionally modulates the fates of target mRNAs that bear AU-rich and/or G-rich sequences. Nucleolin targets include several genes involved in cellular processes such as proliferation, cell survival, and cell cycle as well as in diseases such as cancer and Alzheimer [3, 77]. For example, nucleolin binds the 3'UTR of BCL-2, GADD45A, gastrin (GAST) and β-globin enhancing mRNA stability. However, nucleolin binds the 3'UTR of APP mRNA, promoting its decay [78-83]. While nucleolin enhances the translation

of mRNAs encoding for matrix metallopeptidase 9 (MMP9), AKT1 and cyclin I (CCNI) through binding to the 3'UTR, it suppresses translation of genes encoding for the tumor protein p53 (TP53) and prostaglandin endoperoxide H synthase-1 (PGHS-1) through binding to the 5'UTR [3, 84, 85].

Thus RBPs play an essential role in post-transcriptional gene regulation through binding to different regions of numerous mRNAs encoding for proteins involved in almost all cellular processes impacting cell fates in response to physiological and environmental stimuli.

6. RBPs interplay

Different RBPs are capable of binding to the same RNA inferring diverse effects on the fates of target transcripts. For instance HuR, AUF1, and nucleolin bind BCL-2 mRNA. While nucleolin and HuR promote the stability, AUF1 enhances the degradation of BCL-2 mRNA [63, 86-89]. This implies that HuR and nucleolin have a cooperative effect which is antagonized by AUF1.

Another example is illustrated in the case of GADD45A mRNA. While nucleolin stabilizes GADD45A mRNA, it seems that this effect might be antagonized by AUF1 which promotes its decay and TIAR which suppresses translation [64, 83].

These examples indicate that at least two or more RBPs can bind to the same mRNA molecule in a functional interplay to cooperatively or competitively regulate the fates of target mRNAs, translation and/or stability.

7. Interplay with microRNAs

MicroRNAs (miRNAs) are short RNA molecules, about 22 nt long, that regulate gene expression through RNA-induced silencing complex (RISC) [90, 91]. Targeted mRNAs are silenced either through degradation or translation suppression. RBPs RBPs jointly with miRNAs regulate the fates of mRNAs. While RBPs have diverse effects on target mRNAs, miRNAs only promote mRNA degradation or suppress its translation. In some cases miRNAs and RBPs cooperatively regulate the mRNA to a certain fate. For example, HuR was found to recruit let-7 to suppress c-MYC mRNA translation [92]. HuR competes with miR-494 and miR-548c-3p for the regulation of nucleolin and TOP2A mRNA respectively [93, 94]. These and several other examples indicate that RBPs are capable of competing or cooperating with other RNA binding factors such as miRNA to regulate the expression of target genes.

8. Concluding remarks

RBPs are involved in many cellular processes and pathological conditions such as cancer. Indeed, RBP such as HuR is highly expressed in cancer tissues and is believed to enhance tumorigenesis [2]. AUF1 is also involved in cancer progression through the modulation of neoplastic gene regulatory pathways [21]. Nucleolin is involved in cancer and Alzheimer's disease, while TTP is involved in cancer and inflammation [32].

Thus RBPs may influence not only gene expression but also diseases and disease-progression. Differential expression or subcellular localization of RBPs in diseases could be useful diagnostic markers and targeted for therapy. Indeed inhibitors of HuR and nucleolin have been reported to influence tumorigenesis in vitro, but their therapeutic usefulness in organisms remains untested [95-100].

In the past decade we have gained useful and specific knowledge about RBPs. We have advanced from <RNA binding> to <sequences specific binding> to <binding signatures/motifs> of RBPs. Nonetheless, it is important to identify the complete set of target RNAs which includes coding and non-coding RNAs in different cellular compartments. For example, the use of techniques such as photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) can provide a more global spectrum of RNA binding activities of RBPs [47, 101]. In addition, predominantly nuclear or nucleolar RBPs can be also studied by this technology to uncover their binding activities to other RNA species such as microRNAs, long non-coding RNAs, or even nuclear specific RNAs.

This will advance our knowledge in assessing other functions of RBPs in the regulation of gene expression.

Author details

Kotb Abdelmohsen
Laboratory of Molecular Biology and Immunology, NIA-IRP, NIH, Baltimore, MD, USA

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