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# AUF1 regulation of coding and noncoding RNA

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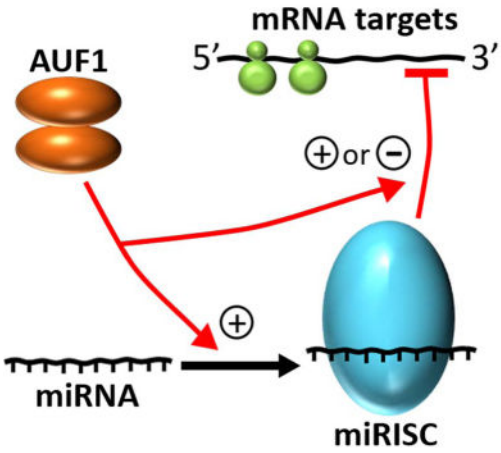
## Abstract

AUF1 is a family of four RNA-binding proteins generated by alternative pre-mRNA splicing, with canonical roles in controlling the stability and/or translation of mRNA targets based on recognition of AU-rich sequences within mRNA 3′ untranslated regions. However, recent studies identifying AUF1 target sites across the transcriptome have revealed that these canonical functions are but a subset of its roles in posttranscriptional regulation of gene expression. In this review, we describe recent developments in our understanding of the RNA-binding properties of AUF1 together with their biochemical implications and roles in directing mRNA decay and translation. This is then followed by a survey of newly discovered activities for AUF1 proteins in control of miRNA synthesis and function, including miRNA assembly into miRISC complexes, miRISC targeting to mRNA substrates, interplay with an expanding network of other cellular RNA-binding proteins, and reciprocal regulatory relationships between miRNA and AUF1 synthesis. Finally, we discuss recently reported relationships between AUF1 and long noncoding RNAs and regulatory roles on viral RNA substrates. Cumulatively, these findings have significantly expanded our appreciation of the scope and diversity of AUF1 functions in the cell, and are prompting an exciting array of new questions moving forward.

## Graphical Abstract

**Conflicts of interest**
The authors declare no conflicts of interest for this article.




AUF1 enhances loading of select miRNAs into RISC complexes but can also regulate miRISC access to mRNA substrates.

## Introduction

In eukaryotes, gene expression occurs via a highly orchestrated series of compartmentalized steps. For protein-coding genes, transcription by RNA polymerase II generates a pre-mRNA that is subsequently processed and exported to the cytoplasm, allowing it to then be translated by ribosomes to yield its encoded protein product. However, each step in gene expression is also subject to regulatory control. Following transcription, most gene regulatory processes involve selective recognition and manipulation of RNA targets. In the nucleus, functional consequences of these interactions may include altering the position or location of pre-mRNA splicing or 3′-cleavage and polyadenylation events, targeted nuclear RNA decay, or modulating the efficiency with which an mRNA is exported to the cytoplasm. RNA-targeted interactions in the cytoplasm can regulate mRNA decay kinetics and subcellular localization, while also influencing the rates of ribosome engagement and translation.

Traditionally, mechanisms regulating gene expression at post-transcriptional levels were considered principally in terms of selective transcript recognition by RNA-binding proteins (RBPs). In eukaryotes a notable exception is nuclear splicing, where core spliceosome ribonucleoprotein complexes are recruited to 5′-splice and branch point sites via base pair complementarity to small nuclear RNA subunits.[1] Nevertheless, other nuclear and cytoplasmic RNA-targeted mechanisms controlling mRNA transport, localization, degradation, and recruitment to ribosomes have canonically been defined in terms of site-specific RBP recognition.[2,3] However, the discovery of microRNA (miRNA) and other endogenous non-coding RNA species revealed critical deficiencies in that paradigm, and it is now well-established that post-transcriptional control of gene expression involves targeted recognition of substrate mRNAs by both protein and RNA molecules acting in *trans*. Recently, advances in transcriptome-wide surveys of RBP and miRNA targeting events have demonstrated that many mRNAs are recognized by a plurality of *trans*-acting proteins and/or RNAs,[4,5] raising the prospect that combinatorial and/or competitive relationships may exist

between diverse RNA-binding partners. Furthermore, newly discovered substrate targeting and metabolic relationships between RBPs and *trans*-acting RNAs also indicate that extensive cross-talk may exist between these post-transcriptional gene regulatory modalities.[6,7]

In recent years, a paradigm for interrelationships between RBP and miRNA-directed control of gene expression has emerged about a family of RBPs collectively termed ARE/poly(U)-binding/degradation factor 1 (AUF1). In this review, we discuss the canonical functions of AUF1 in control of mRNA decay and translation via recognition of specific sequence elements in mRNA 3′-untranslated regions (3′UTRs), then extend these models of post-transcriptional gene regulation by AUF1 to include: (i) reciprocal regulatory relationships between AUF1 and miRNA synthesis, (ii) roles for AUF1 in several facets of miRNA metabolism and targeting, and (iii) a new frontier involving AUF1 interactions with long non-coding RNA (lncRNA) and viral RNA substrates. Cumulatively, these diverse functions of AUF1 suggest that its role extends far beyond directly targeting specific mRNA substrates for degradation, but instead may serve as a key integrator of post-transcriptional control, impacting expression of genes both directly and indirectly by coordinating multiple RNA-targeted regulatory pathways.

## THE AUF1 PROTEIN FAMILY

The AUF1 (also known as hnRNP D) family of proteins encompasses four members generated by alternative splicing of a common pre-mRNA.[8] Differences in protein size and amino acid composition result from exclusion or inclusion of exon 2 and/or 7 in the mature mRNA, encoding a 19-amino acid insert near the N-terminus and a 49-amino acid sequence near the C-terminus, respectively. The resulting four protein isoforms are named by their apparent molecular weights and designated $p37^{AUF1}$ (contains neither exon 2 nor 7-encoded domains), $p40^{AUF1}$ (contains exon 2-encoded domain), $p42^{AUF1}$ (contains exon 7-encoded domain) and $p45^{AUF1}$ (contains both exon 2 and exon 7-encoded domains) (Fig. 1). Despite the selective inclusion of distinct protein domains resulting from differential splicing events, all four isoforms of AUF1 share some common structural elements, including two tandem, non-identical RNA Recognition Motif (RRM) domains containing canonical RNP-1 and RNP-2 sequence motifs, as well as an 8-amino acid glutamine-rich sequence located C-terminal to RRM2.[8,9] Also, all isoforms form stable dimers in solution.[10]

The various AUF1 isoforms can localize to both nuclear and cytoplasmic compartments, although not equally as $p42^{AUF1}$ and $p45^{AUF1}$ are typically enriched in nuclei while the smaller isoforms are generally more uniformly distributed throughout the cell.[9,11–13] While there remains little consensus on the mechanisms controlling the subcellular localization of AUF1 isoforms, several possibilities have been proposed. One factor that may direct AUF1 protein localization is a 19-amino acid sequence located near the C-terminus of all four isoforms that binds the nuclear transport factor transportin 1.[14] However, another model proposes that sequences encoded by exon 7 interrupt a nuclear import activity found in the smaller isoforms ($p37^{AUF1}$ and $p40^{AUF1}$); although such an arrangement would require a distinct nuclear targeting mechanism to minimize cytoplasmic accumulation of $p42^{AUF1}$ and $p45^{AUF1}$.[15] An alternative strategy that may contribute to the subcellular distribution of

AUF1 isoforms is selective interaction with specific nuclear (scaffold attachment factor-β) or cytoplasmic (14-3-3σ) factors.[12,16] The potential for AUF1 proteins to form heterodimeric structures in solution [15] further amplifies the complexity of possible localization mechanisms. Finally, activation of select cellular signaling pathways[17,18] and cellular stresses including those resulting from viral infection can re-localize AUF1 proteins.[19–21] In the case of coxsackievirus B3, cytoplasmic enrichment of AUF1 appears to be linked to deposition in nascent stress granules.[22]

## RNA recognition by AUF1

Early biochemical analyses of AUF1, generally using the p37$^{AUF1}$ isoform as a model, demonstrated direct, robust, and specific binding to U-rich RNA targets derived from the AU-rich elements (AREs) responsible for rapid decay of many mRNAs.[23,24] AREs are potent, *cis*-acting determinants of mRNA stability and translational efficiency found in the 3′UTRs of many labile mRNAs, including those encoding cytokines, oncoproteins, inflammatory receptors and G protein-coupled receptors.[25–27] The canonical ARE sequence is distinguished by the presence of AUUUA pentamers, frequently in multiple and/or overlapping arrangements, within a U-rich region that may span in size from 40 to 150 nucleotides.[25] Besides AUF1, these sequences provide binding sites for a diverse array of factors collectively termed “ARE-binding proteins”. Depending on the specific factor recruited, the functional consequences of protein binding may stabilize or destabilize the mRNA,[18,28–33] or alternatively alter its translational potential.[34–36]

AUF1 binding to ARE-like substrates has been rigorously characterized at the biochemical level, from the perspectives of both protein and RNA sequence requirements. Studies using protein truncation mutants revealed that the RRM domains are necessary but not sufficient for high affinity RNA binding, requiring contributions from N- and C-terminal protein domains to maximize the stability of resulting ribonucleoprotein (RNP) complexes.[37] Furthermore, AUF1 isoforms show different affinities for RNA targets. Specifically, p37$^{AUF1}$ and p42$^{AUF1}$, which lack the exon 2-encoded domain, show higher affinity for model ARE substrates relative to isoforms that contain the exon 2-encoded domain (p40$^{AUF1}$ and p45$^{AUF1}$).[8,10] On extended RNA substrates, initial AUF1 dimer binding events can be followed by subsequent rounds of protein recruitment to yield oligomeric AUF1 complexes (Fig. 2).[23,37] The proclivity of AUF1 dimers to form larger multi-subunit structures on RNA targets is also isoform-dependent, with those containing exon 7-encoded sequences (p42$^{AUF1}$ and p45$^{AUF1}$) forming the most stable oligomeric complexes.[10]

The precise RNA requirements for AUF1 binding have been more elusive, and accumulated data suggest a degree of promiscuity among RNA target sites. Using ARE-based RNA substrates, biochemical approaches have shown that the RNA footprint necessary for maximal p37$^{AUF1}$ or p42$^{AUF1}$ binding affinity is approximately 30 nucleotides,[10] surprisingly large for proteins that exist as dimers of 70 to 80 kDa in size. However, binding is not exclusive for canonical ARE domains, since AUF1 RNPs will form on comparably sized polyuridylate substrates with no energetic penalty,[23,38] in contrast to more stringent ARE-binding factors like tristetraprolin.[39] AUF1 recruitment is also weakened by localized folding of RNA targets, consistent with prominent roles for direct protein contacts with

unpaired bases.[40] Not all RNA contacts are base specific, however. Analyses of p37$^{AUF1}$ binding to modified RNA substrates revealed that a contiguous AU-rich domain of as few as 15 nucleotides was sufficient to nucleate assembly of a high affinity ($K_d$ < 10 nM) RNP, provided that it included a 3′-purine residue and at least 19 nucleotides 5′ of the U-rich sequence (Fig. 3). While interactions with the upstream domain only contribute a net enhancement of 0.5 – 1 kcal/mol to RNP complex stability and are largely independent of RNA sequence, these contacts are required for AUF1 to remodel local RNA structure.[33] AUF1 substrate requirements resolved biochemically have also been supported by recent screens for cellular RNA targets. A predicted AUF1-binding motif elucidated by microarray analysis of RNP-immunoprecipitation reactions contained 79% A/U content across 29–39 nucleotides, although one caveat was that the motif did not explicitly register to sequences of known AUF1 target mRNAs.[41] More recent photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) analyses of AUF1 binding sites indicated that RNA targets were not limited to the canonical AU-rich sequences, but also showed an abundance of U- and GU-rich motifs[36] consistent with the relaxed stringency suggested by biochemical studies. However, a paradoxical result from the PAR-CLIP screens was the identification of several miRNA partners for AUF1 which, measuring as small as 20 nt in length, are far shorter than the minimal ARE-based substrates required for high affinity RNP formation. Subsequent experiments revealed that one GU-rich miRNA, let-7b, bound p37$^{AUF1}$ directly *in vitro* with a $K_d$ of 6 nM,[42] far superior to the $K_d$ = 26 nM observed for p37$^{AUF1}$ binding to a comparably sized AU-rich RNA substrate.[10] How AUF1 is able to form such stable RNP complexes with let-7b and possibly other miRNA targets is unknown, but it is likely that distinct sequence and/or structural determinants within these substrates present optimal contacts for protein recognition. Unfortunately, the current dearth of crystallographic or NMR-based structural information on AUF1 RNP complexes remains a major impediment to resolving the biochemical basis for substrate selectivity by these proteins.

Formation of AUF1 RNPs can also have dramatic but very specific consequences on the local structure of RNA targets.[10,43] Association of an initial AUF1 dimer induces a condensed conformation on RNA substrates, detected by shortening of the distance between their 5′- and 3′-termini in solution (Fig. 2). While this behavior is observed for all AUF1 isoforms, the structural consequences of subsequent binding events vary depending on the presence of the protein domain encoded by exon 7. As such, oligomeric structures formed by the smaller isoforms maintain RNA substrates in condensed states, while p42$^{AUF1}$ and p45$^{AUF1}$ induce extended RNA conformations as successive protein dimers are recruited.[10] Current understanding of relationships between AUF1 binding and local RNA conformations is further complicated by observations that they are also influenced by protein phosphorylation events.[44] While the global significance of RNA conformational changes induced by AUF1 remains unclear, an appealing hypothesis is that they may obstruct or enhance access for other *trans*-acting proteins or RNAs that target nearby sites on mRNA substrates. This possibility will be discussed further in the context of miRNA recruitment (below).

Modulation of AUF1-directed RNA folding by protein phosphorylation is likely only one example of an ever growing list of AUF1 functions that might be regulated by

posttranslational modifications, which conceivably could have major impacts on AUF1 subcellular localization, RNA-binding activity, protein turnover, and engagement with other protein factors. Three distinct phosphorylation sites have been identified within the exon 2-encoded domain, at Ser83, Ser87, and Thr91.[44,45] This domain is limited to the $p40^{AUF1}$ and $p45^{AUF1}$ isoforms, yet other forms of AUF1 have also been identified as phosphoproteins in cells,[46,47] indicating that the catalog of phosphorylation sites remains incomplete. Furthermore, AUF1 modifications are not limited to phosphorylation, as AUF1 is also subject to proline isomerization by Pin1, which inhibits AUF1 binding to and destabilization of GM-CSF mRNA,[48] arginine methylation within its C-terminal RGG motifs,[49] and ubiquitination.[50] This range of posttranslational modifications is not unique to AUF1, as other ARE-binding factors including HuR can exist in similarly diverse populations of covalently modified forms.[51]

## AUF1 functions in control of mRNA turnover and translation

The major mRNA decay pathway in eukaryotes is initiated by 3′→5′ excision of the poly(A) tail, principally through the Ccr4/Not and Caf1/Not deadenylase complexes.[52] The decaying mRNA is subsequently shuttled to P-bodies which contain 5′-decapping factors and 5′→3′ and 3′→5′ exonucleases.[53] Deadenylation is normally rate-limiting and AREs generally promote mRNA decay by accelerating deadenylation.[54] Numerous studies have associated AUF1 binding to specific mRNA substrates with accelerating their degradation; these are reviewed extensively elsewhere.[53–55] This canonical function was recently supported by observations that expression of a sizeable fraction of AUF1-targeted mRNAs identified by PAR-CLIP were inversely related to AUF1 levels.[36] Similarly, in AUF1 knockout mice TNFα and IL-1β mRNAs were dramatically induced in macrophages following even modest endotoxin challenge owing to defects in the degradation of these transcripts.[56]

The mechanism(s) by which AUF1 binding to an ARE enhances mRNA decay remain obscure, but some details have emerged. AUF1 proteins appear to possess no intrinsic nucleolytic function but rather are believed to recruit various components of the mRNA decay machinery to ARE-containing transcripts. Ancillary factors identified in AUF1 complexes include eukaryotic translation initiation factor 4G (eIF4G), poly(A)-binding protein (PABP), and heat shock proteins Hsp70, Hsc70 and Hsp27.[57–59] Although specific roles for these co-purifying factors in AUF1-enhanced mRNA degradation are largely unknown, Hsp27 recruitment appears to exert a destabilizing function since ARE-containing transcripts are stabilized when Hsp27 expression is silenced.[59] By contrast, Hsp70 can form direct high affinity complexes with AREs that stabilizes mRNA targets,[60] suggesting that it might co-purify with AUF1 by virtue of binding at adjacent sites on RNA substrates rather than as part of an AUF1-initiated *trans*-factor assembly. Finally, some evidence has been presented that AUF1 might accelerate mRNA degradation by recruiting the exosome 3′-exonuclease complex[61] or even targeting mRNAs to the proteasome.[57] However, as neither of these activities has been shown to enhance the rate-limiting step of mRNA decay (*i.e.*, deadenylation), it appears likely that a distinct mechanism linking AUF1 to the recruitment or activation of specific deadenylase complexes remains to be discovered, possibly analogous to the Ccr4/Not-recruiting function of tristetraprolin.[62] Another intriguing

possibility is that assembly of AUF1-initiated *trans*-acting complexes might enhance accessibility of deadenylases to the poly(A) tail by altering the stability or dynamics of PABP:poly(A) complexes.

While most examples to date support an mRNA-destabilizing role for AUF1, isolated cases have emerged contradicting that paradigm. For example, AUF1 binding is associated with stabilization of some transcripts. In a breast fibroblast cell model, *SDF-1*, *α-SMA*, *TGF-β1*, and *IL-6* mRNAs were collectively stabilized by AUF1 as part of an IL-6/STAT3/NF-κB-driven pro-inflammatory positive feedback loop.[63,64] Similarly, AUF1 silencing suppressed expression of a cohort of AUF1-targeted mRNAs identified by PAR-CLIP. These included mRNAs encoding several proteins associated with chromosome maintenance such as HP1α, centromere protein D, and DNAJ,[36] although possible AUF1-dependent effects on transcription[65,66] cannot yet be discounted. Other examples have indicated a role for AUF1 in regulating translation. AUF1 promotes translation of *MYC* mRNA by competing with the translational suppressor TIAR for a common binding site.[34] AUF1 also enhances translation of the transforming growth factor-β-activated kinase TAK1, which maintains the NF-κB activation circuit in lipopolysaccharide-activated monocytes.[67] Analyzing ribosome profiles of AUF1-binding mRNAs identified over 100 transcripts where ribosome occupancy was substantially increased or decreased when AUF1 was depleted.[36] In 70% of these cases, no accompanying change in mRNA steady-state level was observed, indicating that modulating translation efficiency is not a rare consequence of AUF1 binding, nor need it be coupled to alterations in mRNA decay kinetics.

The examples listed above highlight a major question currently dogging the field: how can AUF1 exert such distinct functional roles on different RNA targets? An appealing model is that the consequences of AUF1 binding may be highly context-dependent. Conceivably, this could be influenced by: (i) RNA sequence, (ii) local RNA structural environment, (iii) protein post-translational modifications, (iv) proximal or cooperative binding with other RBPs or miRNAs, or (v) competition with other *trans*-factors. These features might also be impacted by the specific AUF1 isoform involved. For example, the PAR-CLIP datasets indicate significant overlap among RNA targets associated with each AUF1 isoform, but also substantial isoform-specific contacts.[36] Other reports also highlight isoform-dependent differences in AUF1 function. Early studies indicated that a reporter mRNA containing the ARE from *GM-CSF* mRNA was only destabilized robustly by overexpressed $p37^{AUF1}$,[31] while either $p37^{AUF1}$ or $p42^{AUF1}$ could destabilize a reporter containing the 3′UTR of *IL-6* mRNA.[68] Furthermore, more recent work indicates AUF1 isoform-dependent roles in inhibiting mRNA decay. For example, *IL-10* mRNA was only stabilized in lipopolysaccharide-stimulated monocytes if $p40^{AUF1}$ was present,[69] while stabilization of estrogen receptor α mRNA in uterine tissue was specific for the $p45^{AUF1}$ isoform.[70] Isoform-selective functions of AUF1 may reflect differences in RNA-binding specificity, a hypothesis supported by PAR-CLIP data showing that the various isoforms bind overlapping but distinct subpopulations of cellular RNA targets, frequently varying by over 50% in pairwise comparisons.[36] However, isoform-specific variations in RNA-binding affinity, oligomerization potential, and consequences on local RNA structure may also contribute to differential regulatory outcomes (described above). Finally, current data do not assess potential roles for AUF1 heterodimers or RNA-mediated hetero-oligomeric complexes on

the functional consequences of AUF1:RNA interactions. While the isoform composition of AUF1 dimers and higher-order assemblies can conceivably be regulated by relative expression levels and co-localization in specific cellular compartments, another intriguing possibility is that RNA-induced hetero-oligomers might be targeted to specific mRNA substrates by tandemly arranged RNA binding sites for distinct protein isoforms.

## MODULATION OF miRNA FUNCTION BY AUF1

miRNAs are small (generally 21–24 nt) RNAs with diverse roles as *trans*-regulators of gene expression. They are produced from hairpin-shaped precursors[71] that may synthesized within a dedicated primary transcript (pri-miRNA) or as a byproduct of other RNA processing events (reviewed in Refs. [7,72,73]). Pri-miRNAs are transcribed in the nucleus, where double-stranded hairpin precursor-miRNAs (pre-miRNA) are then excised by the microprocessor complex which includes the RNase III enzyme Drosha and DGCR8, an essential co-factor. Once transported to the cytoplasm via exportin 5 and Ran GTPase, the pre-miRNA is further processed by Dicer, another RNase III protein, to yield its mature duplex miRNA form. The guide strand of the miRNA is then assembled with a member of the Argonaute (AGO) family of proteins to generate the RNA-induced silencing complex (RISC).[74] This miRNA-loaded RISC complex (miRISC) is then targeted to specific mRNAs by base pair complementarity with the miRNA, frequently emphasizing the miRNA seed region, spanning nucleotides 2–7 from the 5′-end.[75] Canonical consequences of miRISC recruitment are mRNA degradation and/or translational suppression, although a growing body of evidence also suggests nuclear roles for miRISC complexes in regulating transcription.[76]

### AUF1 regulation of miRISC recruitment

Since the post-transcriptional gene regulatory effects of miRNAs require association with cognate mRNA targets, cellular mechanisms that alter miRNA:mRNA recognition could presumably manipulate this process. In recent years, collaboration and/or competition amongst RBPs and miRNAs has emerged as a critical factor in the regulated expression of many genes, and general principles are thoroughly reviewed elsewhere.[5,6,77] In 2013, AUF1 was added to the growing number of RBPs that could influence miRISC binding to mRNA targets. These observations were prompted initially by *in vitro* screens for AUF1-binding mRNAs, when a subpopulation of candidates validated to bind AUF1 in cells was noted to be enriched in transcripts known to be regulated by miRNAs.[78] The hypothesis that functional relationships might exist between AUF1 and miRISC recruitment (Fig. 4, **arrow A)** were supported by similar observations involving the mRNA-stabilizing, ARE-binding protein HuR. For example, HuR binding to *MYC* mRNA is required for let-7-directed repression of *MYC* translation,[79] while binding to lincRNA-p21 promotes destabilization of this transcript, again involving let-7 recruitment.[80] Conversely, HuR can inhibit miRNA-mediated suppression of gene expression in other contexts.[81,82] Additional support for crosstalk between AUF1 and miRISC recruitment was given by observations that ARE-like sequences are enriched near functional miRNA binding sites in mRNA 3′UTRs.[83] To determine whether AUF1 impacted miRISC recruitment or function on select AUF1-binding mRNAs, interactions between these transcripts and AUF1 or AGO2, the sole AGO family

member with endonuclease activity,[84] were measured in cells where one or both *trans*-factors were depleted. Intriguingly, AUF1 and AGO2 recruitment to *MYC* mRNA were mutually inhibitory, supporting a competitive relationship, while binding of each protein enhanced recruitment of the other on *HOXB8* mRNA.[78] Furthermore, for several mRNA targets rapid decay was only observed when both AUF1 and AGO2 were present, while in other cases functional outcomes were independent of each other. Subsequently, collaboration between AUF1 and miRISC-directed control of gene expression has been identified in other systems, including suppression of DNA methyltransferase 1 (DNMT1) expression in endometrial stromal cells under hypoxic stress. Here, destabilization of *DNMT1* mRNA required coordinated recruitment of miR-148b and AUF1 to proximal sites within the mRNA 3′UTR.[85]

While these examples highlight positive and negative functional relationships between AUF1 and miRISC recruitment and function, the biochemical mechanisms by which these events are coupled remain unknown although several possibilities may be proposed. First, miRISC recruitment could be enhanced (or potentially even inhibited) by direct contact between AUF1 and AGO2 or other RISC components. Some support for this model may be interpreted from observations that AUF1 and AGO2 can be co-immunoprecipitated, and that co-purification is enriched under hypoxic stress concomitant with *DNMT1* mRNA destabilization,[85] although the possibility that these proteins are tethered by common RNA molecules cannot yet be excluded. Alternatively, reciprocal inhibition of AUF1 and AGO2 recruitment, as observed for *MYC* mRNA,[78] could be mediated by competition between AUF1 and miRISC for cognate binding sites. Finally, the ability of AUF1 proteins to modify the local RNA structural landscape (described above) may contribute to allosteric relationships with miRISC recruitment, by exposing or occluding nearby target sites.

## AUF1 as part of a global network of RBPs that can regulate miRISC function

The examples above demonstrate potential competition or collaboration between AUF1 and miRISC function, but it must also be remembered that AUF1 is only one of 25 or more proteins that bind AREs and related sequences.[86] Furthermore, substantial evidence for coincident or competitive RNA-binding events involving multiple proteins has been accumulating over the past decade. Considering just AUF1 and HuR, for example, array analyses of co-purifying transcripts identified many common targets, including a subset that could bind AUF1 and HuR simultaneously[87] although spatial relationships between protein binding events on individual RNA molecules could not be rigorously resolved. However, a subsequent in-cell FRET study detected both proteins in homo- and hetero-oligomeric complexes in both nuclei and cytoplasmic compartments, supporting a model whereby they may bind in close proximity on common mRNA targets.[88] Other examples have identified competitive relationships between AUF1 and HuR in mRNA recognition. *In vitro* experiments showed identical RNA footprints for both AUF1 and HuR binding to androgen receptor mRNA.[89] Both proteins also compete for a binding site within the 3′UTR of *JUND* mRNA, although this equilibrium is shifted in favor of HuR by the presence of polyamines.[90] However, forthcoming biochemical and functional analyses of HuR and AUF1 binding events will be greatly aided by the availability of PAR-CLIP datasets for both proteins,[36,91] which have provided a wealth of new model transcripts upon which their

interrelationships may be interrogated. Comparisons of RNA targets recognized by each factor have revealed 6550 overlapping sites to date (Fig. 5) mapping predominantly to introns and 3′UTRs, and representing 7% of all HuR targets but 23% of all AUF1 hits.[36] Notably, HuR targeted 82% of all AUF1-binding domains within mRNA 3′UTRs, particularly at U/AU-rich motifs, suggesting that it is in these regions where combinatorial or competitive roles of AUF1 and HuR are most likely to be manifested.

To this point we have considered regulatory relationships between AUF1 and miRISC complexes, but also widespread competition (and possible collaboration) for AUF1 and HuR binding to common RNA sequences. Combining these themes together with known relationships between HuR and miRISC (described above) augur that miRISC function can be intimately entwined with a spectrum of RBP:mRNA interactions. A recent example of such multifactorial control was observed for bone morphogenetic protein 2 (*BMP2*) mRNA, which contains a 3′UTR ARE-like sequence targeted by HuR, AUF1, and miR-633. In a pre-osteoblast cell model, HuR inhibited the suppressive role of miR-633 at this element, likely by competing for binding to the target site.[92] However, an elegant series of mRNA point mutations showed that AUF1 recruitment was required for miR-directed gene suppression at this site, since abrogating AUF1 binding or miR-633 base pair potential increased reporter expression, but combining these mutations had no further effect. This example shows how competition between AUF1 and HuR can direct the activity of a specific miRNA.

The regulatory potential of multifactorial RBP:miRNA networks could conceptually be profound, and manipulable by a variety of conditions. For example, alterations in the levels of individual RBPs based on tissue specificity, developmental stage, or acute changes resulting from signaling events would be expected to alter this equilibrium, potentially enhancing or restricting miRISC access. Returning to the *DNMT1* mRNA example, enhanced decay owing to AUF1-coupled miR-148b recruitment under hypoxic stress was accompanied by a modest decrease in HuR expression, which likely contributed to diminution of HuR binding to mRNA target site and concomitant enhancement of AUF1 access.[85] Beyond RBP availability, RNA-centric features could also conceivably influence the equilibrium between RBPs and miRISC at specific sites, including the relative affinity and positioning of diverse *trans*-factors on common mRNA targets (influenced by both primary and higher-order RNA structural considerations), as well as the potential effects of RNA structural remodeling by associated *trans*-factors. In the case of AUF1 at least, the complexity of this last regulatory mode is already amplified by its protein concentration-, isoform-, and phosphorylation-dependence (Fig. 2). Cumulatively, these concepts may provide a means by which gene-specific consequences of RBP and miRNA functions may be manifested. We anticipate that forthcoming studies will determine which, if any, of these models is correct, and will doubtless include novel mechanisms by which RBPs and miRNA can reciprocally influence their activities.

## AUF1 binding to miRNAs

Beyond modulating miRISC recruitment to mRNA target sites, new evidence that AUF1 can bind some miRNAs directly indicates additional roles for this protein in miRNA biology.

PAR-CLIP analyses from human embryonic kidney cells identified 14 distinct miRNA targets of AUF1 in this cell model, including 4 members of the let-7 family (let-7a-2, -7b, -7f-2, and -7i).[36] Subsequent biochemical assays verified that all AUF1 isoforms directly interacted with let-7b and miR-21, both of which include UU- and UG-rich sequences,[42] and in the case of let-7b demonstrated surprisingly strong affinity given the short length (22 nt) of the miRNA substrate (described above). Several functional consequences could be envisioned for RBP binding to specific miRNAs, including sequestering them from assembly into miRISC complexes or altering miRNA turnover kinetics. However, in the case of AUF1, focusing principally on binding to let-7b, a different role was identified. Silencing AUF1 expression in cells had no effect on levels of let-7b or other tested miRNAs,[42] indicating that AUF1 binding was unlikely to impact their turnover. However, in the absence of AUF1, let-7b loading onto AGO2 was dramatically inhibited (Fig. 4, **arrow B)**, which in turn abrogated its ability to accelerate decay of let-7b-targeted mRNAs. The mechanism whereby AUF1 enhances let-7b loading onto AGO2 is unknown, but *in vitro* experiments indicate that no additional co-factors are required. Furthermore, it is unlikely to exclusively involve AUF1:AGO2 contacts, since AGO2 loading of miR-10 (not an AUF1 target) was unaffected by AUF1. Rather, the loading mechanism appears to be coupled to AUF1:miRNA contacts, since in addition to let-7b, AGO2 association with the weak AUF1-binding miRNAs miR-21 and miR-130b was also reduced when AUF1 was silenced.[42] Regardless of mechanism, these data also implicate AUF1 as a major regulator of the let-7-driven gene silencing program, which, given the diverse roles of let-7 family members in development and suppression of oncogenic phenotypes,[93,94] suggest a novel mechanism for some of AUF1's anti-proliferative properties (reviewed in Ref. [95]). Finally, direct interactions with miRNAs might also be subject to competitive and/or combinatorial control by RBPs. For example, HuR also binds select miRNA targets[91], and like AUF1, can form very stable complexes ($K_d$ = 1.4 nM) with let-7b.[96]

## Reciprocal relationships between AUF1 and miRNA synthesis

In addition to regulating the assembly and substrate targeting of select miRISC complexes, AUF1 is also a general regulator of miRNA synthesis. This role is mediated via AUF1's canonical mRNA-destabilizing function, by targeting and accelerating decay of *DICER* mRNA (Fig. 4, **arrow C)** through interactions at multiple sites within its coding region and 3′UTR.[97] In AUF1-overexpressing cells, the resulting decrease in DICER protein levels reduced accumulation of several miRNA species, including miR-24, miR-130a, miR-203 and let-7. A physiological application of this mechanism was demonstrated across an array of healthy and tumor tissues, where increased expression of AUF1 in many tumors was accompanied by diminution of DICER levels.[97] A subsequent study demonstrated that AUF1-directed suppression of DICER could also be manipulated pharmacologically.[98] The hypoglycemic agent metformin induced nuclear retention of AUF1, particularly the larger isoforms, via an AMP kinase-dependent mechanism. This relocalization inhibited AUF1 binding to cytoplasmic *DICER* mRNA, resulting in increased production of DICER protein and concomitant enhancement in the synthesis of several microRNAs, including a subset associated with senescence and aging (miR-20a, miR-34a, miR-130a, miR-125b and let-7c).[98]

Reciprocally, AUF1 itself is suppressed by miRNA-directed events (Fig. 4, **arrow D)**, consistent with a model of interrelated regulation of RBP and miRNA production recently reviewed by Ciafre and Galardi.[99] The tumor suppressor microRNAs miR-141 and miR-146b-5p interact with distinct regions within the 3′UTR of *AUF1* mRNA and target the transcript for degradation.[100] Curiously, the impact of AUF1 suppression by these miRNAs on inhibiting osteosarcoma cell proliferation and migration is based on accelerated decay of mRNA substrates that AUF1 normally stabilizes in this cell model: *PDK1*, which encodes an AKT-activating kinase,[101] and *ZEB1*, a transcription factor that drives the epithelial-mesenchymal transition.[102] This experimentally defined example of miRNA regulation of AUF1 was previously predicted in a computational study that highlighted an abnormally high concentration of potential miRNA target sites among mRNAs encoding ARE-binding proteins.[103] In the case of AUF1, this model would appear to present a potential feed-forward cycle, where elevated miRNA levels suppress AUF1 production, which in turn enhances DICER synthesis leading to production of more miRNAs. As new studies emerge, it will be very interesting to see how this circuit is applied and controlled within the global networks regulating miRNA synthesis and function.

## AUF1 INTERACTIONS WITH OTHER NON-CODING AND VIRAL RNAS

Beyond mRNA and miRNA targets, PAR-CLIP analyses identified over 1700 binding sites for AUF1 proteins within endogenous lncRNAs, including contacts at multiple distinct locations within the *NEAT1*, *MALAT1*, and *XIST* transcripts.[36] LncRNAs are defined as RNA molecules longer than 200 nucleotides that lack protein coding roles. Like mRNAs, most but not all lncRNAs are transcribed by RNA polymerase II and subject to subsequent processing steps including splicing, 5′-capping, and 3′-polyadenylation.[104] The lncRNA field is currently expanding at an exponential pace as novel examples and functions continue to be defined. However, in general lncRNAs typically play regulatory roles involving one or more of three mechanisms: as molecular decoys, guides, and/or scaffolds, which are mediated by their abilities to interact variously with DNA, RNA, and/or protein targets. In the nucleus, these functions allow lncRNAs to selectively promote or prevent assembly and/or targeting of diverse multi-subunit complexes including transcriptional regulators and chromatin-modifying activities.[105,106] Additional roles influencing pre-mRNA splicing have also been detected.[107] In the cytoplasm, lncRNAs perform a host of different tasks, including modulating mRNA turnover and translation kinetics as well as facilitating protein-targeted functions such as post-translational modification and degradation.[108] Regulatory control of mRNA targets may involve sequestering miRNAs and/or RBPs from the transcript (termed “sponging”) or recruiting specific *trans*-factors. Recent data suggest that many cytoplasmic lncRNAs also associate with ribosomes, interactions that may contribute to their translational regulatory roles[109] or control their decay kinetics.[110]

One AUF1-targeted lncRNA identified by PAR-CLIP is *NEAT1*, a principally nuclear RNA that plays a structural role in the formation of nuclear paraspeckles and a regulatory role in the export of specific mRNA substrates.[111,112] Binding by AUF1 suppresses *NEAT1* levels by accelerating its degradation, leading to enhanced nuclear retention of *NEAT1*-regulated transcripts.[36] However, accelerated decay is not a uniform consequence for lncRNAs associated with nuclear AUF1, since levels of *MALAT1* and *XIST* were not sensitive to

AUF1 concentration, despite robust association with this protein. Future studies will have to define whether AUF1 binding imposes distinct functional consequences on these alternative lncRNA targets. A second example of an AUF1-regulated lncRNA is *linc-RoR*, best known for maintaining the dedifferentiated state of stem cells by sequestering specific miRNAs to prevent suppression of key pluripotency factors Oct4, Nanog, and Sox2.[113] The miRNA sponge activity of *linc-RoR* is also responsible for its promotion of tumor development and metastatic pathways in breast cancer.[114,115] By contrast to its roles as a miRNA sponge, *linc-RoR* binding to AUF1 appears to function as a protein sponge, sequestering AUF1 from access to canonical mRNA substrates such as *MYC* mRNA.[116] Accordingly, suppressing *linc-RoR* levels concomitantly decreases *MYC* expression by liberating AUF1, which in turn binds and accelerates degradation of the *MYC* transcript. Although specific roles for AUF1:lncRNA interactions are currently limited to these few examples, the diversity of lncRNA targets recently revealed by AUF1 PAR-CLIP presages a significant expansion in our understanding of the interrelationships between AUF1 and lncRNA expression and function in the coming years.

A final class of AUF1 targets that have recently been described are viral RNAs. A growing number of cases have been reported where AUF1 binding to non-coding viral RNA sequences is associated with positive or negative consequences for the virus. For example, AUF1 inhibits the replication cycle of picornaviruses such as poliovirus and human rhinovirus, involving recruitment to a distinct structured element in the 5′UTR of the viral RNA.[117,118] While AUF1 binding appears to function by preventing downstream translation of this RNA, viruses overcome this obstacle by proteolytically cleaving AUF1 using virally-encoded proteinases. AUF1 also inhibits translation and replication of the picornavirus enterovirus 71 (EV71) by a similar mechanism, involving direct binding to a specific structural feature (stem-loop 2) within its internal ribosome entry site (IRES).[20] Curiously, AUF1 binding to stem-loop 2 is in competition with the RBPs HuR and Ago2, whose association promotes translation from the IRES and subsequent viral replication.[119] This model is complicated further by observations that the EV71 IRES is also targeted by DICER, which generates at least four small RNAs, termed viral small RNAs (vsRNAs), from this substrate. One such product (vsRNA1) is derived from stem-loop 2 sequence, and paradoxically enhances recruitment of AUF1, HuR, and Ago2 to the stem-loop 2 binding site.[120] While the precise mechanisms by which AUF1 inhibits picornaviral translation remain unknown, it is conceivable that a major contributor is AUF1's ability to dynamically compete with antagonistic factors for common binding sites, analogous to an emerging paradigm in ARE-directed control of cellular mRNA decay and translation. However, a newly discovered role for AUF1 in replication of West Nile virus (WNV) appears to function through a distinct mechanism. Like other flaviviruses, WNV RNA requires a large-scale conformational transition in order to initiate the replication process.[121] A recent report demonstrates that $p45^{AUF1}$ can enhance this structural transition and activate viral RNA synthesis.[122] This AUF1 isoform appears to bind at multiple locations within the WNV RNA, but with highest affinity at an AU-rich region in the 3′UTR, a domain that is required for viral RNA replication in cells. The presentation of this RNA chaperone-like activity initiated by contact with an AU-rich sequence would appear to be consistent with the

intrinsic local RNA remodeling activity of AUF1 described above, which is readily observable on AU-rich RNA substrates and also presents isoform-specific features.[10]

## Conclusion

The AUF1 family of RNA-binding proteins constitute four isoforms that canonically function to positively or negatively regulate mRNA decay and/or translation by binding AREs and related sequences within target transcripts. The biochemical mechanisms that mediate and discriminate each function remain largely unknown, but are likely coupled to mRNA and protein context-specific features which may include mRNA target sequence or structure, selectivity for ancillary factors, and competition or collaboration with other *trans*-factors including RBPs and miRISC complexes that bind proximal sites on the mRNA substrate. However, PAR-CLIP analyses of RNA-binding sites for AUF1 across the transcriptome have added exciting new functions to this protein family, particularly with respect to interactions with miRNAs and lncRNAs. Notably, recent studies identified several mechanisms linking AUF1 to control of gene expression directed by these *trans*-acting RNAs. First, AUF1 binding to select miRNAs enhances their assembly into AGO2 complexes. Second, AUF1 binding to mRNA targets can enhance or inhibit miRISC recruitment and function at nearby or overlapping target sites, roles that may be coupled to AUF1's RNA structural remodeling activity but are also influenced by the actions of other cellular RBPs including HuR. Third, AUF1 can limit global miRNA production by suppressing DICER expression, while reciprocally being regulated itself by select miRNAs. Finally, AUF1 can also form functional complexes with some lncRNAs and viral RNAs. These interactions have already led to a variety of observed outcomes including control of lncRNA degradation, AUF1 sequestration, and viral RNA translation, although this list is likely to grow as new noncoding RNA targets of AUF1 are investigated.

Moving forward, work in the field will continue to elucidate and refine the mechanisms by which AUF1 impacts posttranscriptional gene regulatory events. Key questions that remain include: (i) what signals, cellular conditions, RNA determinants, and/or binding partners direct the various functional outcomes resulting from AUF1 binding to mRNA (and now noncoding RNA) targets? (ii) how does AUF1 enhance miRNA loading onto AGO2? and (iii) what biochemical mechanisms mediate competitive or cooperative binding and/or functional relationships between AUF1, other RBPs, and miRISC complexes on mRNA substrates? Addressing these emerging questions will significantly enhance our understanding of the dynamic and complex network of *trans*-factors responsible for controlling gene expression at posttranscriptional levels, and the multi-faceted roles of AUF1 in these processes.

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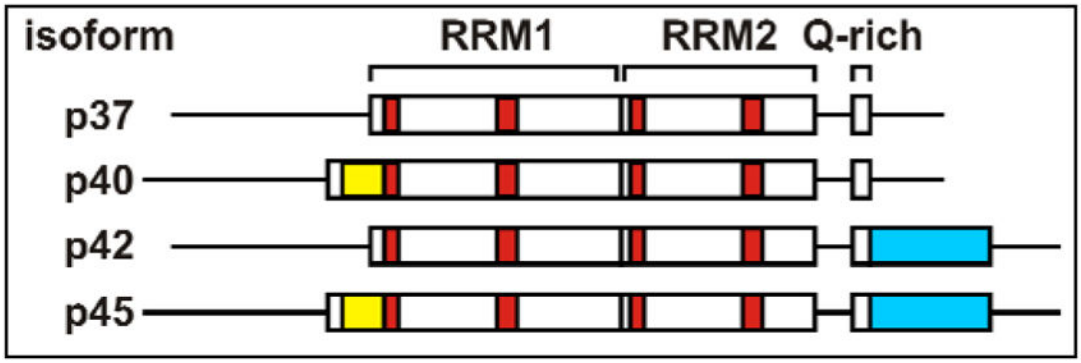


**Fig. 1.**

Domain organization of AUF1 isoforms. All contain tandem RRM domains, each with characteristic RNP-2 and RNP-1 sequence motifs (red boxes), followed by a short glutamine-rich domain. Isoform-specific sequences encoded by alternatively spliced exons 2 (yellow) and 7 (blue) are indicated.

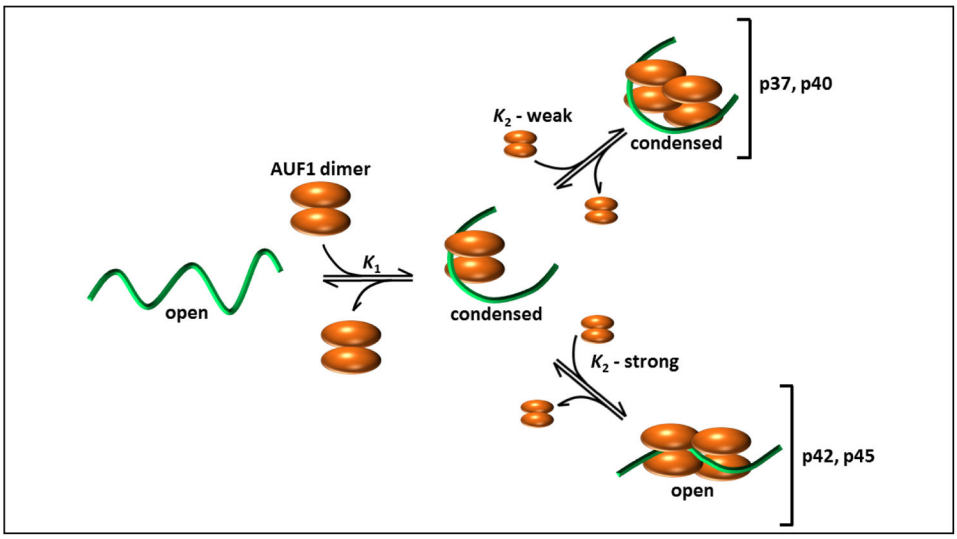


**Fig. 2.**

Assembly of AUF1 RNPs. Initial binding between an AUF1 dimer (orange) and an RNA substrate (green) generates an RNP complex with a $P_2R$ stoichiometry concomitant with adoption of a locally condensed RNA structure (center). Recruitment of a subsequent $p37^{AUF1}$ or $p40^{AUF1}$ dimer (top right) occurs with low affinity and maintains the condensed RNA fold. However, subsequent dimer binding events on $p42^{AUF1}$ or $p45^{AUF1}$ RNPs (bottom right) occur with high affinity and induce extended RNA conformations. Figure adapted from Ref. [55]

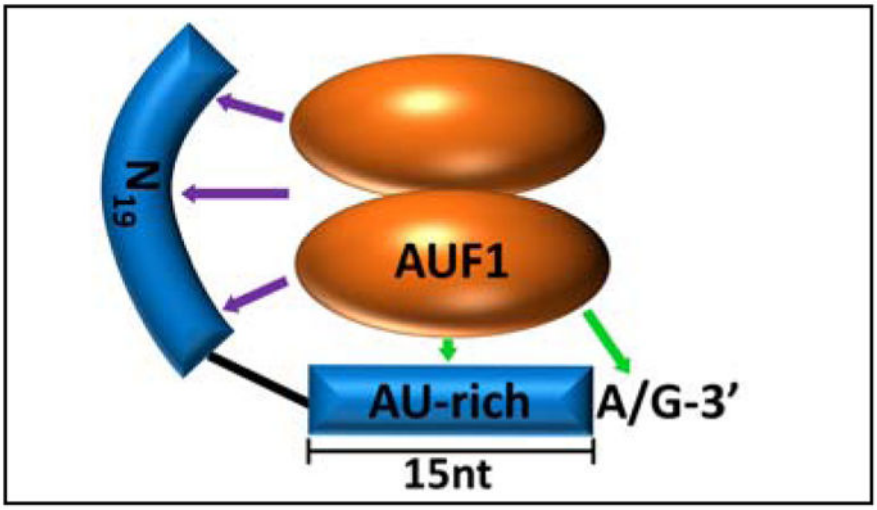


**Fig. 3.**

RNA domains contributing to high affinity binding of $p37^{AUF1}$ to RNA substrates. Green arrows denote base-specific contacts to AU-rich RNA sequences, while purple arrows show nonspecific contacts upstream of the nucleating U-rich domain. Protein contacts with the 5′-domain are also required for AUF1-induced remodeling of local RNA structure. Figure adapted from Ref. [33]

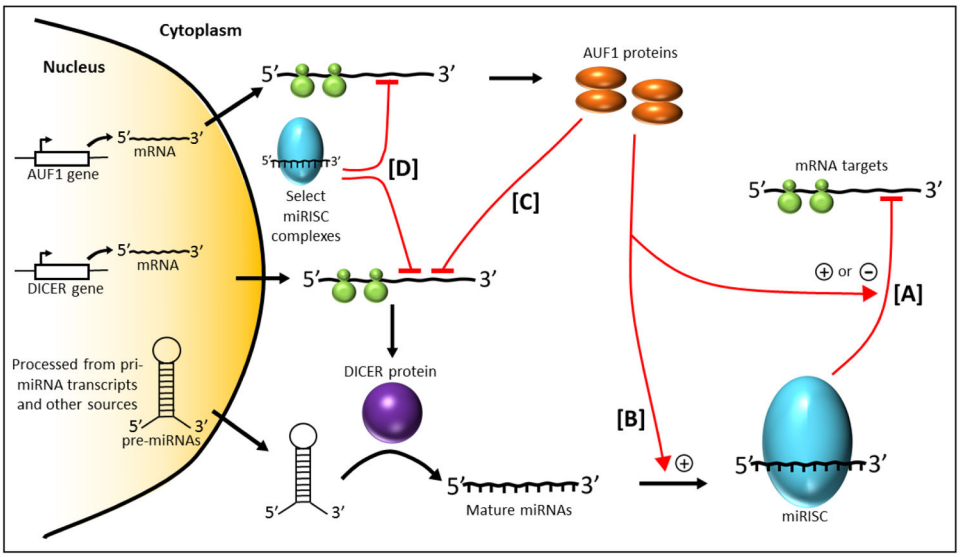


**Fig. 4.**

Functional and regulatory interrelationships between AUF1 and miRNAs. Major pathways described in the text are indicated by red arrows: [A] positive and negative consequences of AUF1 on miRISC recruitment to specific target sites on mRNA substrates, [B] AUF1-enhanced miRNA loading into RISC complexes, [C] suppression of miRNA synthesis by AUF1-targeted degradation of DICER mRNA, and [D] miRNA-directed control of AUF1 expression.

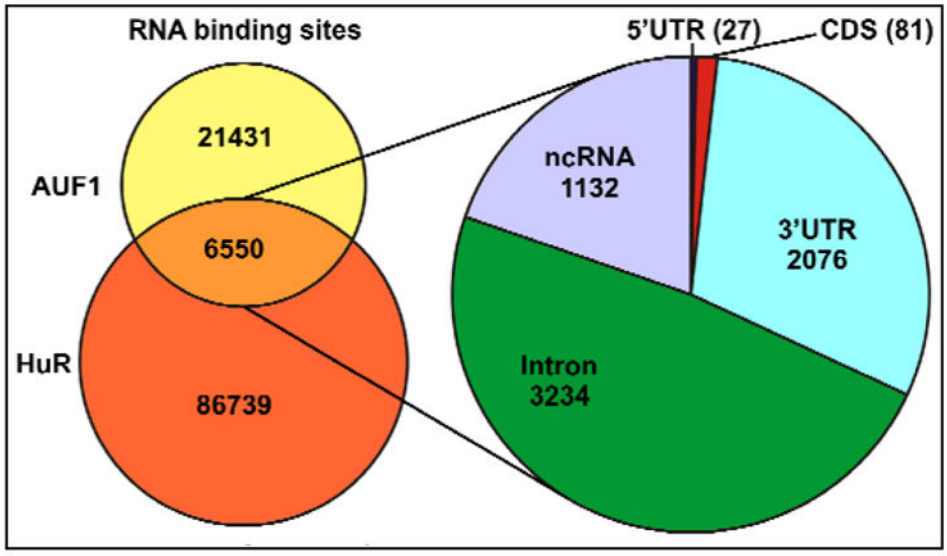


**Fig. 5.**

Distribution and overlap of RNA binding sites recognized by AUF1 and/or HuR resolved by PAR-CLIP studies. The pie chart at right summarizes the locations of RNA targets recognized by both *trans*-factors. Figure adapted from Ref. [36]