Role of RNA Binding Protein HuR in antagonizing the microRNA-mediated Repression

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ABBREVIATIONS

Ab   antibody

ARD   adenine/ uridine rich region D

ARE   adenine/uridine rich element

CAT1   cationic amino acid transporter 1

CRM1   chromosomal region maintenance protein 1

dsRBD   double-stranded RNA binding domain

HNS   HuR nucleocytoplasmic shuttling

IRES   internal ribosomal entry site

miRNA   microRNA

miRISC   miRNA-induced silencing complex

miRNP   micro-ribonucleoprotein

nt   nucleotides

NES   nuclear export signal

NLS   nuclear localization signal

RBD   RNA binding domain

RBP   RNA binding protein

RISC   RNA-induced silencing complex

RL   Renilla luciferase

RNAi   RNA interference

siRNA   short interfering RNA

Trn   transportin

UTR   untranslated region

Less frequently used abbreviations are defined upon their first use in the text.

1 SUMMARY

MicroRNAs (miRNAs) are ~21-nt-long non-coding RNAs regulating gene expression in eukaryotes. In metazoa, miRNAs control gene expression by base-pairing to target mRNAs, bringing about their translational repression and/or deadenylation resulting in mRNA degradation. MiRNA-mediated translational repression is a reversible process in mammalian cells. It was previously demonstrated that target mRNAs containing AU-rich regulatory elements (AREs) in the 3'UTR, can be relieved from miRNA repression in human hepatoma Huh7 or HeLa cells in response to different forms of cellular stress. The derepression required binding of the ELAV family protein HuR to the mRNA 3'UTR (Bhattacharyya et al., Cell 125, 111-1124, 2006). However, whether stress-induced factors other than HuR participate in the process and the actual mechanism of HuR action remain unknown.

In the present study, we have addressed these questions, using different cell-based and *in vitro* assays. Using mutants of HuR accumulating in the cytoplasm in the absence of stress and tumor cell lines constitutively accumulating endogenous HuR in the cytoplasm, we were able to uncouple the HuR effect on miRNA repression from stress. We also found that Ago2 and HuR do not interact with each other and that their binding to target mRNA appears to be largely mutually exclusive. Using an *in vitro* system with purified miRISC and recombinant HuR and its mutants, we demonstrate that HuR, by oligomerizing along RNA, leads to displacement of miRISC from RNA, even when miRISC is positioned at a distance from the primary HuR-binding site. Further, we show that HuR association with AREs can inhibit RISC-mediated endonucleolytic cleavage of target RNAs both *in vivo* and *in vitro*, and also miRNA-mediated deadenylation of RNA in the Krebs-2 ascites extract.

2 INTRODUCTION

The life of an mRNA transcript, from its genesis in the nucleus to its decay in the cytoplasm, is comprised of a highly ordered sequence of events, regulated at multiple levels primarily by its dynamic interactions with numerous RNA binding proteins (RBPs). However, it is the other form of mRNA expression regulatory network, mediated by microRNAs, that has revolutionized our understanding of gene expression over past 12 years.

2.1 miRNAs – a general overview

2.1.1 Discovery

MiRNAs are a large class of non-coding genomically encoded short (~21-24 nt long) RNAs that regulate gene expression in many eukaryotes. The first miRNA, lin-4 was discovered in *Caenorhabditis elegans* simultaneously in the laboratories of Victor Ambros and Gary Ruvkun in an attempt to characterize genes that control the timing of larval development (Lee et al, 1993; Wightman et al, 1993). However, the focus on this class of RNA was renewed only seven years later, after identification of the second miRNA, let-7, also by the forward genetic screen in C. elegans (Reinhart et al, 2000), and its highly conserved orthologues in diverse animal species including humans (Pasquinelli et al, 2000). Soon thereafter, bioinformatic predictions together with cloning and subsequent sequencing approaches revealed many additional miRNAs to be present in worms, flies and mammals (Bentwich et al, 2005; Lagos-Quintana et al, 2001; Lau et al, 2001; Lee & Ambros, 2001).

2.1.2 Biogenesis – a multistep process

Biogenesis of miRNAs in mammals consists of several sequential steps (reviewed by Kim, 2005; Kim et al, 2009b) (Fig. A). In general, miRNAs are transcribed by RNA polymerase II as long primary transcripts (pri-miRNAs) (Lee et al, 2004). The majority of miRNAs is transcribed from independent genes whereas some are present in introns of protein-coding genes (Rodriguez et al, 2004). Resembling the protein-coding transcripts, the pri-

miRNAs transcribed from independent genes contain 5'-cap structures, 3'-polyA tails and may also contain introns (Cai et al, 2004). Pri-miRNAs contain about 60-80 nt stem-loop structures. Their arms harbour the future mature miRNAs.
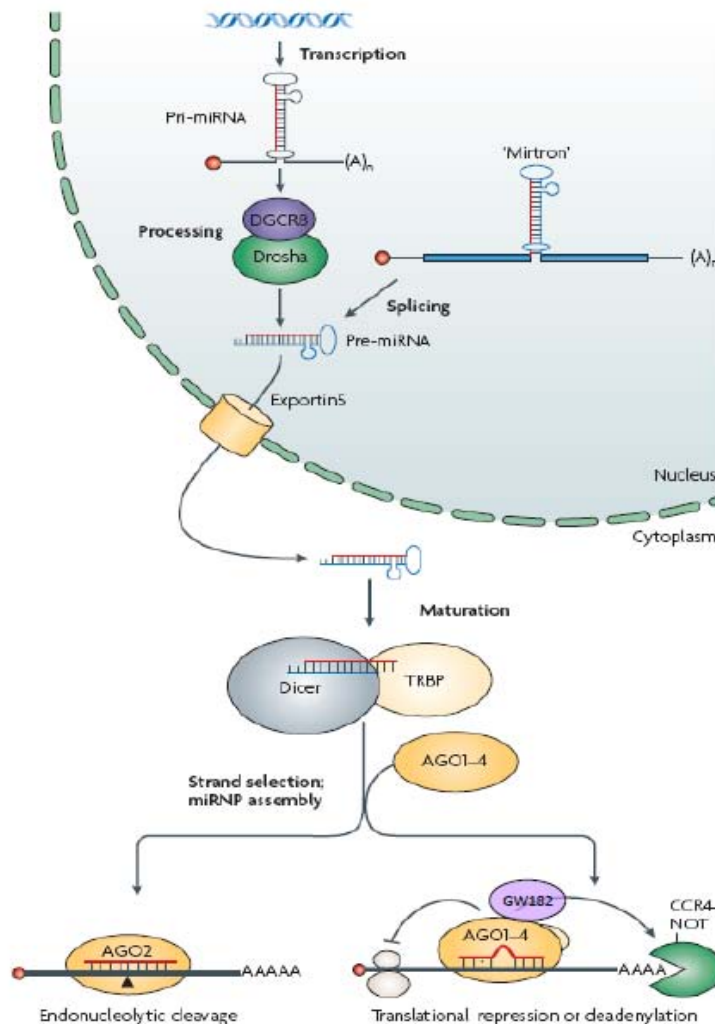
Figure A Canonical pathway of mammalian miRNA biogenesis and function (reproduced from Filipowicz et al, 2008).

In the first step of pri-miRNA processing, the microprocessor complex that includes the RNase III endonuclease Drosha (Lee et al, 2003) and its binding partner DGCR8 (DiGeorge syndrome critical region 8), liberates the hairpin precursor miRNA (pre-miRNA) from pri-miRNAs in the nucleus (Denli et al, 2004; Han et al, 2004; Han et al, 2006). In the subsequent step, the pre-miRNAs are transported from the nucleus into the cytoplasm by Exportin-5, in

a Ran-GTP-dependent manner (Bohnsack et al, 2004; Lund et al, 2004; Yi et al, 2003).

Following export to the cytoplasm, pre-miRNAs are further processed by another RNase III endonuclease, Dicer. Removal of the terminal loop generates a ~22-23 nt miRNA duplex with 2 nt single-stranded 3' overhangs at both ends (Bernstein et al, 2001; Hutvagner et al, 2001). During the process, the mammalian Dicer works in association with dsRBD protein partner, TRBP (TAR RNA binding protein) (Chendrimada et al, 2005; Haase et al, 2005). However, recent three independent studies revealed also a Dicer-independent processing of at least one miRNA, miR-451 (Cheloufi et al, 2010; Cifuentes et al, 2010; Yang et al, 2010).

Following dicing, one strand of the miRNA/miRNA* duplex, representing mature miRNA, is selectively incorporated into a muticomponent complex referred to as miRNA-induced silencing complex (miRISC or miRNP).

2.1.3 Target recognition and function

In metazoa, miRNAs regulate gene expression by base-pairing to target mRNAs and inhibit protein synthesis by repressing translation and/or bringing about deadenylation which leads to mRNA degradation. Most investigated miRNAs base-pair imperfectly with sequences in mRNA 3'-untranslated regions (3'UTRs), with perfect complementarity to positions 2 to 8 of the miRNA, referred to as a seed sequence, being the most important for association with mRNA (Brennecke et al, 2005; reviewed in Bartel, 2009). However, some miRNAs base-pair to mRNA with perfect or nearly perfect complementarity and induce endonycleolytic cleavage of mRNA by the RNA interference (RNAi) mechanism, similar to that mediated by short interfering RNAs (siRNAs).

In mammals, hundreds of different miRNAs are expressed (according to the latest miRBase Version 17, the number of human miRNAs is 1733) and they are predicted to control the activity of ~50% of all genes. Thus, it is not surprising that miRNAs regulate most of the investigated developmental and cellular processes and their altered expression is observed in many human

pathologies, including cancer (reviewed by Bartel, 2009; Bushati & Cohen, 2007; Farazi et al, 2011 and Flynt & Lai, 2008).

2.2 Silencing by RNAi – an overview

Almost coincident with the elucidation of miRNA pathway, another form of gene silencing by small non-coding RNAs, known as RNAi was discovered. Broadly, RNAi can be defined as double-stranded RNA (dsRNA) triggered gene silencing phenomenon in which, dsRNA is processed to siRNAs, that direct the cleavage of complementary RNA transcripts by the RNA-induced silencing complex (RISC) (Fig. B).
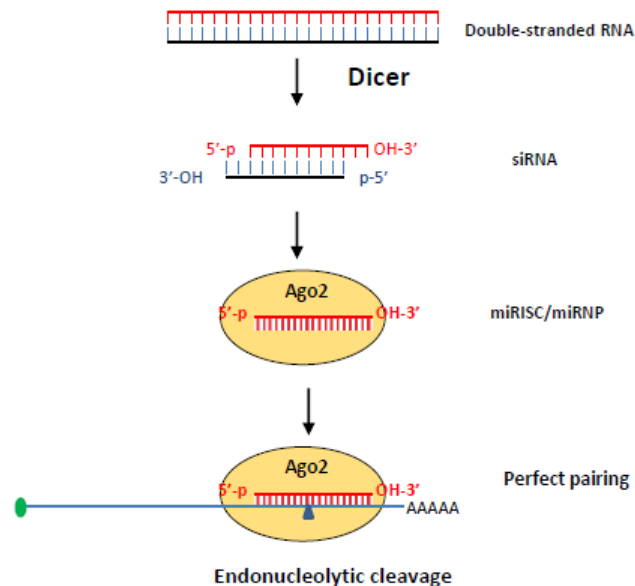
Figure B Silencing induced by RNAi.

It was five years after the discovery of first miRNA, when the studies conducted by Fire, Mello and co-workers (Fire et al, 1998) observed this phenomenon of gene silencing in *C. elegans*, triggered by long dsRNA. Soon thereafter, the work with the biochemical recapitulation of dsRNA-induced silencing i*n vitro* by *Drosophila melanogaster* embryo lysates (Tuschl et al, 1999) and *Drosophila* S2 cells (Hammond et al, 2000), collectively demonstrated that the dsRNA is converted into 21-23 nt duplex siRNAs. It was soon realized that both siRNA- and miRNA-guided silencing pathways

converge to form similar effector complex by sharing a common set of proteins in biogenesis and effector functions. Like pre-miRNAs, dsRNA is processed by Dicer (Bernstein et al, 2001; Billy et al, 2001; Ketting et al, 2001) to generate siRNAs which associate with effector proteins, to form RNA-induced silencing complexes (siRISCs), and guide it to specific transcripts in a sequence-specific manner, bringing about the silencing (Elbashir et al, 2001b; Hammond et al, 2000; Zamore et al, 2000). However, while human cells contain only one Dicer protein to generate small miRNA or siRNA duplexes, in Drosophila two distinct dicer proteins are designated to function separately in two pathways. DCR1 processes pre-miRNAs and DCR2 generates siRNAs (Lee et al, 2004).

2.3 The effector phase of RNA silencing

2.3.1 RISC – RNA induced silencing complex

In order to identify the core component of RISC, both genetic (Tabara et al, 1999) and biochemical studies (Hammond et al, 2001) were performed and it was demonstrated that members of the Argonaute family of proteins form the central and defining component of all RNA-silencing effector complexes.

As mentioned earlier, the mode of miRISC mechanism depends on the degree of complementarity between a miRNA and its target. So it is not surprising to observe that siRNAs can act as miRNAs if made to base pair imperfectly to target sites (Zeng et al, 2003) and, reversely, miRNAs can act as siRNAs if made to base pair perfectly (Doench et al, 2003). Indeed, there is an example of HoxB8 mRNA which shows an extensive complementarity to miR-196 in mammals and it was experimentally demonstrated that miRISC indeed cleaves the HoxB8 message during mouse development (Yekta et al, 2004).

2.3.2 RISC assembly

Since RISC must anneal to the target RNA by base pairing through its RNA component, the two strands of either siRNA or miRNA duplex have to be separated and whereas one strand (guide strand or mature miRNA strand) in

association with the Argonaute protein forms a functional RISC, the other strand (siRNA passenger strand or miRNA* strand) is discarded. Selection of a single strand from a small RNA duplex is not random; instead, one of the two strands is preferred to serve as the guide. Such asymmetric strand selection (often referred to as the 'asymmetry rule') depends on the relative thermodynamic stability of the first 1–4 bases at each end of the small RNA duplex; the strand whose 5' end is less stable serves as the guide whereas the other strand is discarded (Khvorova et al, 2003; Schwarz et al, 2003).

2.3.3 Argonautes

The Argonaute protein family consists of Ago and Piwi subfamilies. The siRNAs and miRNAs bind to the members of Ago subfamily. In mammals, each of the four Ago subfamily proteins (Ago1-4) can form silencing complex while only Ago2 posesses a target cleavage (slicer) activity leading to RNAi (Liu et al, 2004; Meister et al, 2004). In flies, AGO2 mediates RNAi via siRNAs, whereas AGO1 acts in miRNA-mediated gene silencing (Forstemann et al, 2007; Okamura et al, 2009), although recently both of them have been shown to retain target cleavage and translational repression activies (Iwasaki et al, 2009).

Argonaute proteins contain four structural domains as shown below:
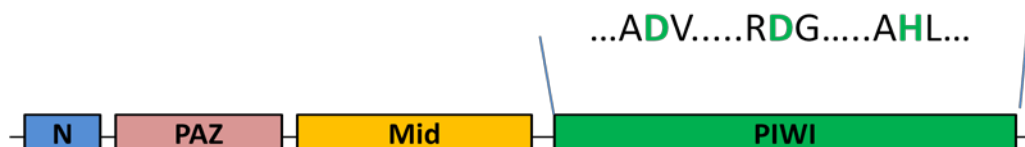
Figure C Domain organization of Ago proteins. The catalytic residues are indicated in green.

The PAZ (piwi-argonaute-zwille) domain is shared with Dicer enzymes whereas PIWI domain is unique to the Argonaute superfamily. The third functionally crucial domain is positioned between the PAZ and PIWI domains, thus referred to as MID domain. Previous crystallographic studies of bacterial and archaeal Argonaute proteins (Parker et al, 2004; Parker et al, 2005; Song et al, 2004; Yuan et al, 2005) have significantly contributed to our understandings of various aspects of Argonaute function. Even more insight

has been provided by structures of *Thermus thermophilus* Argonaute protein in complex with a guide nucleic acid (DNA in this case) with or without a base-paired target RNA (Wang et al, 2008a; Wang et al, 2009; Wang et al, 2008b). The 3' end of the small RNA is anchored to the PAZ domain whereas the MID domain contains a highly basic pocket specifically binding to the characteristic 5' phosphate of the terminal nucleotide of the small RNA. The PIWI domain shows similarity to RNase H fold and harbours three residues, usually Asp-Asp-His (D597, D669, H807 in the case of human Ago2), which form the catalytic triad (Liu et al, 2004; Rivas et al, 2005; Song et al, 2004). Thus, the cleavage-competent Ago2 carries out the guide strand-dependent endonucleolytic cleavage of a base-paired target through its PIWI domain. Interestingly, human Ago3 is catalytically inactive, even though the catalytic triad DDH is conserved (Rivas et al, 2005), indicating that additional factors such as posttranslational modification or interaction with specific proteins may modify the activity of Ago proteins.

2.3.4 Target RNA cleavage by RISC

The minimal RISC that cleaves target RNA complementary to the guide RNA is a ~160 kDa complex containing small single-stranded RNA and Ago2 protein (Martinez et al, 2002; Schwarz et al, 2002). RISC is a Mg^{2+}-dependent endonuclease (Martinez & Tuschl, 2004; Schwarz et al, 2004) and its catalytic center is located within the Piwi domain of Ago2 (Liu et al, 2004; Rivas et al, 2005). RISC cleaves the target RNA once, in the middle of region complementary to the guide RNA (Elbashir et al, 2001a), and the cleavage reaction does not require ATP (Haley & Zamore, 2004; Martinez & Tuschl, 2004; Nykanen et al, 2001; Rivas et al, 2005). The guide RNA is presumed to form an A-form helix with the target RNA for the cleavage to occur (Chiu & Rana, 2003; Haley & Zamore, 2004). In an attempt to determine how RISC finds its target RNA it has been shown that the RISC is unable to unfold structured RNA and, during the course of target recognition, RISC transiently contacts single-stranded RNA nonspecifically and promotes the siRNA-target RNA annealing (Ameres et al, 2007). The 'scissile phosphate' is located opposite the 10th phosphodiester bond of the guide strand, counting from its

5' end (Elbashir et al, 2001b). Cleavage by RISC leaves 3' hydroxyl and 5' monophosphate termini (Martinez & Tuschl, 2004; Schwarz et al, 2004). The mRNA cleavage products are subsequently degraded; the 5' mRNA fragment is degraded from its 3' end by the exosome, whereas the 3' fragment is degraded from its 5' end by an exoribonuclease XRN1 (Orban & Izaurralde, 2005). RISC is a multiple-turnover enzyme – the siRNA guides RISC to its RNA target, the target is cleaved and the siRNA remains intact within the RISC (Haley & Zamore, 2004; Hutvagner & Zamore, 2002; Martinez & Tuschl, 2004).

2.4 Effects of miRNAs on translation

2.4.1 Repression at post-initiation stage

Initial studies performed in *C. elegans* found that miRNA lin-4 inhibits the expression of targets lin-14 and lin-28 without a reduction in mRNA abundance or a shift in polysome. These findings indicated that the repression occurred at the level of translation but after translation had been initiated (Olsen & Ambros, 1999; Seggerson et al, 2002). Later studies in the mammalian cell cultures also supported the idea of the miRNA-mediated inhibition at the post-initiation step (Maroney et al, 2006; Nottrott et al, 2006; Petersen et al, 2006). As additional evidence for the model, Petersen et al (2006) showed that cap-independent translation, driven by HCV and CrPV IRES, which does not require most of the initiation factors for translation, was also repressed by miRNA. In these studies, the target RNAs were observed to be present in the actively translating polysome with a significant reduction in the corresponding protein level. To rationalize these observations, Nottrott et al (2006) proposed the co-translational degradation of the nascent polypeptide chain whereas Petersen et al (2006) suggested that miRNAs cause ribosomes to dissociate prematurely from mRNA (ribosome drop-off).

2.4.2 Repression at the initiation stage

In contrast to these studies, others reported conflicting results indicating that miRNAs inhibit translation at the initiation step. The first study to establish this mechanism used both the tethering approach and reporters targeted by the

endogenous let-7 miRNA (Pillai et al, 2005). Polyribosomal profiling of let-7-targeted mRNAs demonstrated a shift of the targeted mRNA into the upper gradient fractions when the reporter mRNA contained target sites for let-7, consistent with inhibition of translation initiation. Furthermore, cap-independent translation was not affected by miRNA action. This was determined by using cap analogs as well as bicistronic constructs containing an IRES or tethered eIF4E or eIF4G initiation factors. Work from the Preiss laboratory was published soon thereafter, and derived similar conclusions using an artificial miRNA (CXCR4) that targeted a transfected reporter mRNA (Humphreys et al, 2005). Additionally, a recent study argued that both lin-4 and let-7 miRNAs in *C.elegans* target the initiation step of translation rather than the post-initiation step (Ding & Grosshans, 2009).

Further evidences in support of the initiation to be the target of the miRNA-mediated repression were obtained from in vitro reconstituted systems that recapitulated miRNA-mediated repression. The first cell-free system to be functional was reported by Novina laboratory using a rabbit reticulocyte lysate (Wang et al, 2006). Subsequently, several other groups demonstrated similar effects in extracts from *Drosophila* embryos (Thermann & Hentze, 2007; Zdanowicz et al, 2009), Krebs II-ascites cells (Mathonnet et al, 2007) and HEK 293 cells (Wakiyama et al, 2007). Overall, all these studies provided evidences for the miRNA-mediated inhibition of cap-dependent translation initiation.

2.4.3 Translational activation

Challenging the well established inhibitory role of miRNAs in translation, recent studies from Steitz laboratory suggested that miRNAs may act to enhance translation under certain cellular conditions (Vasudevan et al, 2007; Vasudevan et al, 2008). Specifically, under serum-starved condition, miR369-3 was shown to enhance the translation of TNFα mRNA in mammalian cells. This miRNA-mediated enhancement of translation required the interaction of FXR1 protein with Ago2. Translational enhancement was not limited to miR369-3, as let-7 and CXCR4 miRNAs also enhanced translation of target mRNAs under serum-starvation conditions. Overall, they presented a model whereby miRNAs act as translational repressors in proliferating cells, but as

activators when cells quiesce. Extending this observation, they also demonstrated up-regulation of the target Myt1 kinase mRNA by miR16 and of miRNA reporters in immature *Xenopus laevis* oocytes (Mortensen et al, 2011).

2.5 Target destabilization by miRNAs

Despite the original discovery that miRNAs repress translation of target genes without changing the corresponding mRNA levels, many subsequent studies have challenged this notion. Microarray analyses showed that ectopic expression of a miRNA reduced the levels of target mRNAs carrying regions complementary to the seed (Lim et al, 2005). In an independent study, endogenous targets of two C. elegans miRNAs (let-7 and lin-4) were shown to be degraded during the larval stage, when the relevant miRNAs were produced (Bagga et al, 2005), contrary to earlier reports (Olsen & Ambros, 1999; Wightman et al, 1993). In a recent study, investigators found that for both ectopic and endogenous miRNA regulatory interactions, lowered mRNA levels account for most (~84%) of the decreased protein production (Guo et al, 2010).

In contrast to siRNA-mediated mRNA endonucleolytic cleavage, miRNA-mediated mRNA decay was demonstrated to operate via a more traditional deadenylation-dependent degradation pathway. The first evidence that deadenylation is triggered by miRNP interactions, suggesting that mRNAs are generally destabilized by the action of the miRNP complex, originated from studies in zebrafish (Giraldez et al, 2006). Concurrently, miRNA-dependent deadenylation and subsequent decay of target mRNA was shown to occur in the mammalian cells (Wu et al, 2006). Subsequently, numerous studies with reporter constructs in various biological models demonstrated reduction in mRNA abundance that correlated with the silencing of gene expression. These connections between miRNAs and RNA degradation, as supported by biochemical and genetic studies, described GW182 proteins to be the mediator of the phenomenon. GW182 proteins (Drosophila has only one GW182 protein and humans contain three paralogues, TNRC6A, B and C) were found to be the crucial components of miRISC which act downstream

of Ago proteins. GW182 proteins recruit the poly(A)−binding protein (PABP) and the deadenylase complex CCR4:NOT1 to the target mRNA resulting in its deadenylation (Behm-Ansmant et al, 2006; Eulalio et al, 2009; Fabian et al, 2009; reviewed by Fabian et al, 2010; Huntzinger & Izaurralde, 2011). These reports unequivocally demonstrated that mRNA decay is an important component of the miRNA-mediated gene silencing.

2.6 Role of P-bodies in miRNA silencing

Ago and GW182 proteins are often found to be enriched in P-bodies (Processing bodies). P-bodies are discrete cytoplasmic structures that accumulate translationally silent mRNAs. They are implicated in translational repression and mRNA degradation (reviewed in Eulalio et al, 2007; Parker & Sheth, 2007). P-bodies are devoid of ribosomes and most translation initiation factors, consistent with the idea that they store translationally inactive mRNAs (Teixeira et al, 2005). P-bodies were found to be enriched in RNA degradative enzymes (Dcp1/2, Xrn1, Lsm1-7, and others) as well as to contain miRISC proteins, and miRNAs and their targets (Bhattacharyya et al, 2006; Eystathioy et al, 2002; Ingelfinger et al, 2002; Liu et al, 2005a; Liu et al, 2005b; Pillai et al, 2005; Sen & Blau, 2005; van Dijk et al, 2002). Moreover, depletion of the miRISC-associated proteins such as GW182 or RCK was shown to disaggregate P-bodies leading to the impaired gene silencing (Chu & Rana, 2006; Jakymiw et al, 2005; Liu et al, 2005a). Collectively, these reports suggest a role of P-bodies in the miRNA-mediated repression.

2.7 HuR – regulatory RBP with many functions

Control of mRNA turnover and translatability by the RNA binding proteins (RBPs) is an intensively studied research area in the field of posttranscriptional gene regulation. For example, many RBPs interact with *cis* acting AU-rich elements (AREs) present in the 3'UTR of mRNAs in a regulated manner to bring about the effect on mRNA translation and stability. Approximately 3,000 human genes contain AREs, representing 8% of human

genome (Khabar et al, 2005). The majority of known ARE binding proteins promote mRNA degradation (e.g., TTP, AUF1, BRF1) and/or inhibition of translation (Barreau et al, 2005; Chen & Shyu, 1995; Zhang et al, 2002). On the contrary, the ELAV/Hu family of RBPs acts to stabilize ARE-containing transcripts.

2.7.1 Functional studies on HuR

The ELAV protein family is comprised of a number of conserved RBPs named after its first member, ELAV (embryonic lethal abnormal visual), identified in Drosophila (Robinow et al, 1988). In mammals and in Xenopus, the ELAV/Hu family consists of three neuron-specific members [HuB (Hel-N1), HuC and HuD] and one ubiquitously expressed member (HuR). Mammalian HuR was first cloned by Furneaux laboratory (Ma et al, 1996). In mammalian cells, HuR has been shown to bind and stabilize a large number of ARE-containing transcripts such as cyclin A, cyclin B1 (Wang et al, 2000a), p21 (Wang et al, 2000b), p53 (Zou et al, 2006), tumor necrosis factor alpha (TNF-α) (Dean et al, 2001), interleukin-3 (IL-3) (Ming et al, 2001), vascular endothelial growth factor (VEGF) (Levy et al, 1998), β-actin (Dormoy-Raclet et al, 2007), urokinase plasminogen activator (uPA) and its receptor (uPAR) (Tran et al, 2003). In many cases, HuR promoted the translation of the target RNAs [e.g., glucose transporter 1 (GLUT1) (Gantt et al, 2006), cationic amino acid transporter 1 (CAT1) (Yaman et al, 2002), prothymosin alpha (ProTα) (Lal et al, 2005), p53 (Mazan-Mamczarz et al, 2003), cytochrome c (Kawai et al, 2006), hypoxia-inducible factor 1 alpha (HIF1α) (Galban et al, 2008)]. Thereby, by acting upon these various mRNAs, HuR has been implicated in several key biological functions including cell growth, differentiation, apoptosis, signal transduction, hematopoiesis, nutrient transport and metabolism. Although HuR is predominantly a nuclear protein, its mRNA-stabilizing function and ability to modulate translation is highly linked to its transport to the cytoplasm. Indeed, overexpression and cytoplasmic localization of HuR, leading to stabilization of several cancer relevant genes, have been the hallmark of many cancers.

However the molecular mechanism underlying the HuR function is not very well understood. Initial report demonstrated direct binding of Hu proteins

via RRM3 to polyA tail of the target mRNA (Ma et al, 1997) whereas subsequent study suggested that HuRs act by protecting the body of the transcript from degradation (Peng et al, 1998). In general, it is believed that HuR stabilizes mRNAs primarily by competing with other decay promoting RBPs (Lal et al, 2004; reviewed by Pascale & Govoni, 2011).

In order to understand the *in vivo* function of HuR, the investigators generated transgenic mice and showed that the misregulation of HuR, due to expression of a HuR transgene, prevents the production of fully functional gametes in mice providing evidences for its involvement in spermatogenesis (Levadoux-Martin et al, 2003). Recently, one study reported that global deletion of murine HuR in knock-out mouse induced atrophy of hematopoietic organs, extensive loss of intestinal villi, obstructive enterocolitis, and lethality within 10 days after birth. Upon HuR deletion, progenitor cells in the bone marrow, thymus, and intestine underwent apoptosis, whereas hematopoetic quiescent stem cells and differentiated cells were unaffected. Binding of HuR to Mdm2 mRNA, a critical negative regulator of p53 was demonstrated to be the reason behind the phenotype (Ghosh et al, 2009). In another study, the investigators showed that HuR-null embryos exhibited a stage retardation phenotype and failed to survive beyond midgestation. By means of conditional knock-out, they restricted HuR's deletion to either embryonic or endothelial compartments to demonstrate that embryonic lethality is consequent to defects in extraembryonic placenta. HuR-null embryos rescued from these placental defects proceeded to subsequent developmental stages but displayed defects in skeletal ossification, fusions in limb elements, and asplenia. By further analysis, they identified transcription and growth factor mRNAs controlled by HuR, primarily at the posttranscriptional level responsible for guiding morphogenesis, specification, and patterning in skeletal development (Katsanou et al, 2009).

2.7.2 Structure of HuR

In general, RBPs are modular in composition consisting of one or more RNA binding domains (RBDs) and at least one additional auxiliary domain. In eukaryotes, the most frequently occurring RBD is the ~80-90 amino-acid long RNA recognition motif (RRM) containing two consensus ribonucleoprotein

(RNP) motifs separated by 25-35 amino acids that interact directly with the RNA. Each of the RNP motifs, octameric RNP1 and hexameric RNP2, contains conserverd aromatic residues that are engaged in the interactions with RNA. The consensus structure of RRM consists of four-stranded antiparallel β-sheets and two α-helices (β1-α1-β2-β3-α2-β4) and the location of the RNP motifs in the first and third β-strands of the RRM is highly conserved (Birney et al, 1993; Burd & Dreyfuss, 1994). It is usually the variable regions between the RNP motifs and RRMs that impart the sequence specificity to the RBP. HuR, like all other ELAV proteins, has a characteristic structure of three RRMs, with RRM1 and RRM2 positioned next to each other and followed by a hinge region and a terminal RRM3 (Fig. D) (Good, 1995). Although the RRMs share a high degree of sequence homology among the ELAV family, the N-terminus and the linker region reflect greater diversity (Sakai et al, 1999).

Figure D Schematic diagram of HuR structure. The positions of different domains are indicated by amino acid numbers.

2.7.3 Nucleocytoplasmic shuttling and regulation through modification

The hinge region of the protein contains the HuR nucleocytoplasmic shuttling domain (HNS, spanning residues 205-237) (Fan & Steitz, 1998) that regulates the cytoplasmic abundance of HuR by interacting with components of nucleocytoplasmic transport machinery. The investigation by the Steitz group demonstrated that HuR uses two alternative export pathways. In the CRM1-dependent indirect pathway, HuR moves out of the nucleus in association with ligands pp32 and APRIL (acidic protein rich in leucine), which contains nuclear export signal (NES) recognized by the export receptor CRM1(Gallouzi et al, 2001; Gallouzi & Steitz, 2001). The other pathway involves direct interaction with Transportin 2 (Trn2) (Gallouzi & Steitz, 2001). However, later Trn2 was found to be the nuclear import receptor for HuR (Guttinger et al, 2004; van der Giessen & Gallouzi, 2007) and, in an independent study, from

the Steitz laboratory also showed that both, Trn1 and Trn2 can function as redundant import receptors for HuR (Rebane et al, 2004). Importin-α was also shown to be involved in a nuclear import of HuR (Wang et al, 2004).

As mentioned earlier, movement of HuR from the nucleus to the cytoplasm is linked with its ability to promote mRNA stability and influence translation. In normal non-transformed cells, this cytoplasmic translocation is observed in response to various kinds of external stimuli; most of them induce stress i.e., UV irradiation, heat shock, starvation, hypoxia, ER stress, or global transcription block (Abdelmohsen et al, 2008; Atasoy et al, 1998; Fan & Steitz, 1998; Levy et al, 1998; Wang et al, 2000b; Westmark et al, 2005; Yaman et al, 2002; Zou et al, 2006). Several posttranslational modifications of HuR mediated by several effectors of the signaling cascades activated by these stimuli or otherwise have been found to influence HuR subcellular localization.

The first reported modification of HuR, a methylation at residue R217 by CARM1 (coactivator-associated arginine methyltransferase 1) was implicated in its translocation to the cytoplasm in response to lipopolysaccharide (Li et al, 2002). Recently, studies from the Gorospe and Eberhardt laboratories correlated HuR phosphorylation within the hinge region to the nucleo-cytoplasmic localization of HuR. Phosphorylation at S202 by Cdk1/Cdc2 leads to retention of HuR in the nucleus through sequestration by 14-3-3 protein during the G_2/M phase of cell cycle (Kim et al, 2008a). A phosphomimetic HuR modification at S242 (S242D) also renders the protein nuclear (Kim et al, 2008b). On the contrary, PKC-α and PKC-δ induce phosphorylation of HuR at S221 in response to ATP analog or angiotensin II respectively, was implicated in its translocation to the cytoplasm and consequent stabilization of cyclooxygenase-2 (COX-2), cyclin D1 and cyclin A mRNAs (Doller et al, 2008; Doller et al, 2007; Doller et al, 2010). Another example of phosphorylation-mediated cytoplasmic accumulation of HuR results from the p38/MAPK-mediated phosphorylation of HuR on Thr118, present in RRM2 outside of the hinge region, which leads to the enhanced binding to p21 mRNA (Lafarga et al, 2009).

The AMP-activated protein kinase (AMPK) was shown to indirectly affect the nuclear retention of HuR by phosphorylating and enhancing the acetylation of importin α1, thus favouring the nuclear import of HuR. Stress

conditions that inhibit AMPK activity can repress the import pathway allowing cytoplasmic accumulation of HuR (Wang et al, 2004). During apoptosis and myogenesis, an intriguing mechanism has been documented for the cytoplasmic accumulation of HuR. In response to lethal stress or myogenic stimuli, the caspase-3-mediated cleavage of HuR at Asp226 was found to generate two cleavage products: HuR-CP1 (24KDa) and HuR-CP2 (8KDa) (Beauchamp et al, 2010; Mazroui et al, 2008). HuR-CP1, by interfering with the TRN2-mediated import of HuR, helps non-cleaved HuR to accumulate in the cytoplasm (Beauchamp et al, 2010; von Roretz et al, 2011).

2.7.4 Role of HuR in the relief of miRNA-mediated repression

As HuR generally has a positive effect on stability and translatability of mRNA, Bhattacharyya et al (2006) hypothesized that HuR may also interfere with the inhibitory action of miRNAs under certain conditions. Indeed they demonstrated that HuR relieves the miR-122-mediated repression of CAT-1 mRNA. This study demonstrated that, in human hepatoma cells, CAT-1 mRNA is translationally repressed and localized to P-bodies in miR-122-dependent manner. In response to the cellular stress, HuR translocated from the nucleus to the cytoplasm and, by binding to the CAT-1 mRNA 3'UTR, brought about the release of the mRNA from P-bodies and its mobilization into actively translating polysomes, resulting in the relief from the miRNA-directed inhibition (Fig. E).
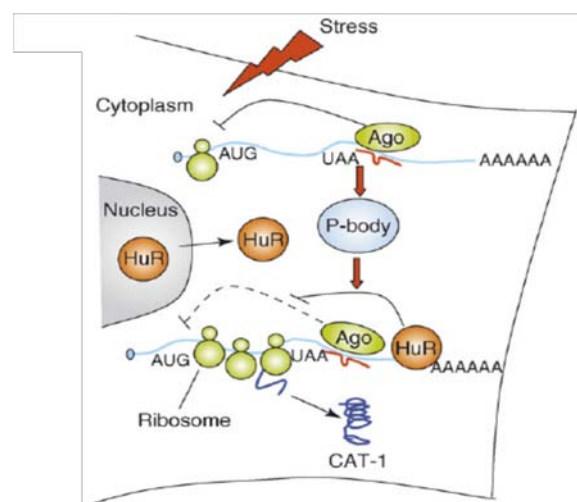
Figure E HuR-mediated relief of CAT-1 mRNA repression by miR-122 in human hepatoma Huh7 cells subjected to different stress conditions. In response to stress,

the HuR protein translocates from the nucleus to the cytoplasm, binds to the 3' UTR of CAT-1 mRNA and helps in its exit from P-bodies and re-entry into translating polysomes (reproduced from Pillai et al, 2007).

This study provided the first example for a cross-talk between miRNPs and RBPs interacting with the 3'UTR. Subsequently there have been several other reports demonstrating that the miRNA repression of individual mRNAs can be strongly modulated or even reversed in response to factors as diverse as cellular stress, developmental cues, and neuronal stimulation (Ashraf et al, 2006; Glorian et al, 2011; Huang et al, 2007; Jafarifar et al, 2011; Kedde et al, 2007; Kedde et al, 2010; Kim et al, 2009a; Nolde et al, 2007; Schratt et al, 2006; Siegel et al, 2009; reviewed by Filipowicz et al, 2008; Kedde & Agami, 2008; Leung & Sharp, 2010; Schratt, 2009). Many of these reports document a crucial role for RBPs in modulating miRNA function. Since 3'UTRs of mammalian mRNAs can be as long as 10 or more kilobases and can associate with many different miRNAs and RBPs, these findings indicated a potentially very complex interplay between the two classes of regulators interacting with the 3'UTR (reviewed by Filipowicz et al, 2008; Kedde & Agami, 2008).

3 BACKGROUND AND OBJECTIVES

The previous study of Bhattacharyya et al from our lab demonstrated that translocation of HuR from the nucleus to the cytoplasm, occurring in response to cellular stress, leads to the relief of miRNA-mediated repression of CAT-1(cationic amino acid transporter 1) mRNA. In human hepatoma Huh7 cells, the expression of CAT-1 mRNA remained translationally repressed by miR-122 under normal non-stress condition. The expression of endogenous CAT-1 was found to increase rapidly, within one hour, in response to different types of stress [i.e., amino acid starvation, endoplasmic reticulum (ER) or oxidative stress], applied to Huh7 cells. The upregulation occurred without discernible effects on mRNA abundance and was independent of Pol II transcription, since neither actinomycin D nor α-amanitin, inhibitors of pol II, had any influence. In contrast, the stimulation was inhibited by cycloheximide, an inhibitor of translational elongation. Taken together, these data indicated that the induction of CAT-1 expression was the result of translational mobilization of the pre-existing mRNA pool.
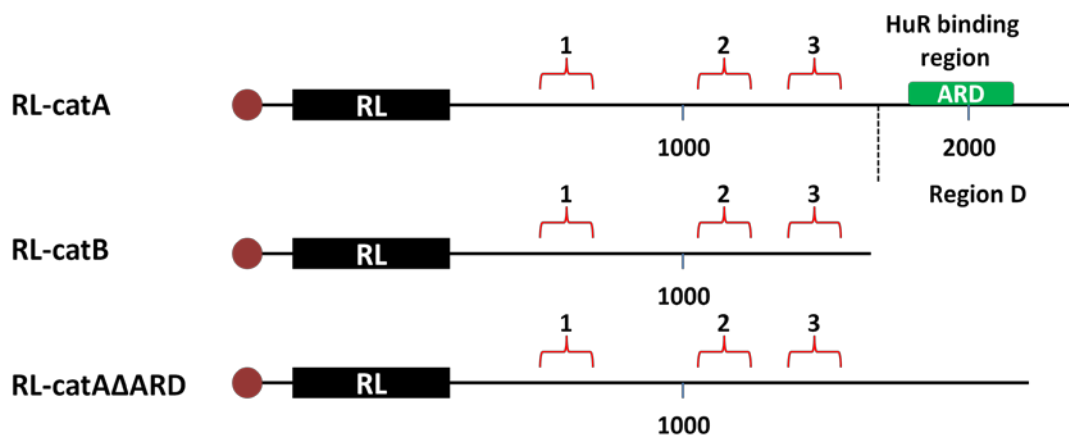
Figure F Schematic diagram of reporters bearing different parts of CAT-1 3'UTR fused to the Renilla luciferase (RL) coding region. The red brackets represent the potential positions of miR-122 binding sites (1-3). The AU-rich fragment at the central part of region D (ARD), characterized as HuR binding site, is denoted by the green box.

In an attempt to dissect the requirements for this stress-induced activation, Renilla luciferase (RL) reporters bearing different segments of the CAT-1

mRNA 3'UTR reporters were used (Fig. F). The induction of luciferase activity was only observed with reporters (RL-catA) bearing both the miR-122 sites and the 250-nt long HuR binding region (ARD), and deletion of the ARD (reporters RL-catB and RL-catAΔARD) completely eliminated the stimulatory effect of stress on translation. The stress-mediated upregulation was disrupted by depletion of HuR with small interfering RNAs (siRNAs). Immunofluoresence and in situ hybridization experiments demonstrated that in nonstressed Huh7 cells, a considerable fraction of CAT-1 mRNA was localized to P bodies. When Huh7 cells were subjected to stress, the CAT-1 mRNA was found to relocalize from P bodies to the soluble fraction of the cytosol. Experiments with RNAi depletion of HuR showed that this relocalization was dependent on HuR and this redistribution was associated with the increase in the fraction polysome-bound CAT-1 mRNA, which is diagnostic of enhanced mRNA translation. Importantly, reporter RNAs bearing sites specifically targeted by let-7 miRNA also showed regulation similar to that of CAT-1 mRNA and reporters bearing its sequences.

Although the role of HuR in the reversal of miRNA repression of CAT-1 and selected reporter mRNAs was well documented in this study, the mechanism of this HuR effect had not been established. Likewise, it was not known whether factors other than HuR, possibly induced in cells subjected to stress, participate in the process; or how HuR antagonize the effector function of miRISC. The present study was aimed to address these questions, using a variety of cell-based and in vitro systems.

4 RESULTS

4.1 PART I - Uncoupling of miRNA-mediated repression from stress

In this study, we first asked if the HuR-mediated relief of miRNA repression can be uncoupled from stress. To address this question, we used three different cellular conditions, under which HuR accumulates in the cytoplasm in the absence of stress to analyze the repression of different miRNA targets.

4.1.1 Effect of cytoplasmic accumulation of HuR mutants
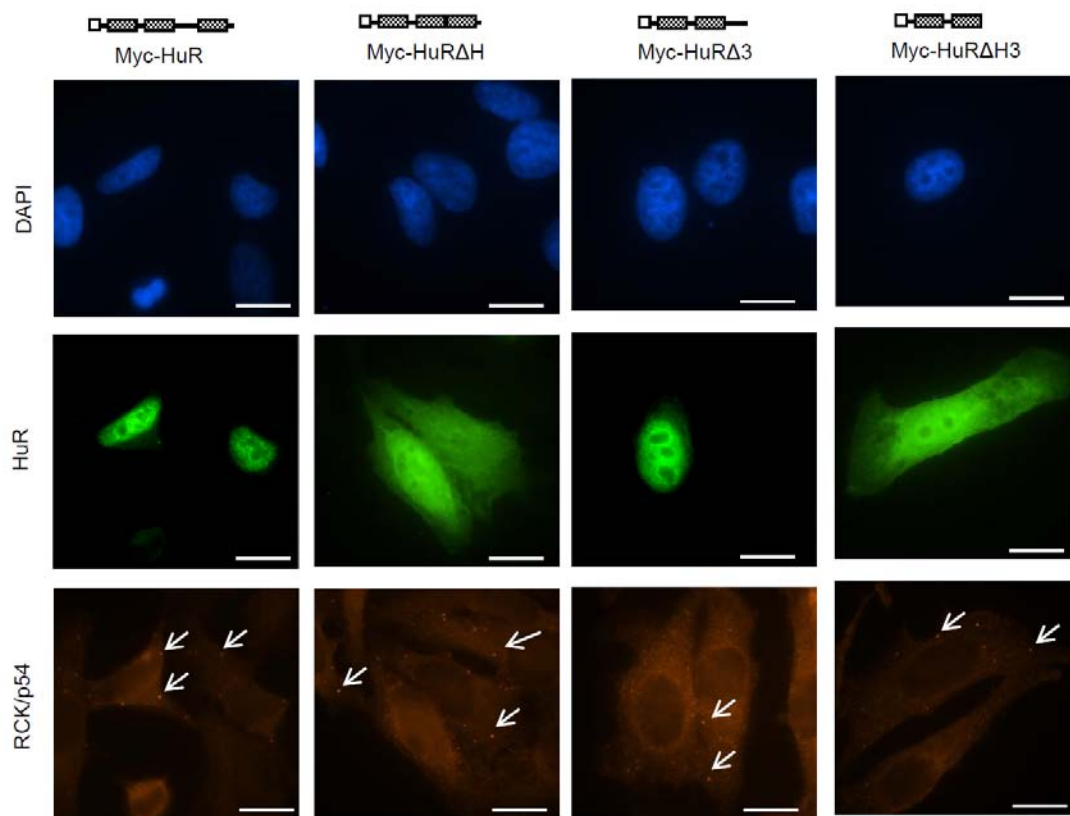
Figure 1 Subcellular localization of Myc-tagged HuR and its deletion mutants in cells (HeLa). The schemes of the proteins are shown above the panels. The HuR proteins were detected by indirect immunoflurescence (IF) using α-Myc antibody (Ab) (shown in green). DAPI stained the nucleus (in blue) and P bodies are visualized by IF using Ab against marker protein RCK/p54 (shown in red, some are marked with white arrow). Bar, 10 μm.

HuR protein consists of three RRM domains and a hinge region separating RRM2 and RRM3. The hinge region was shown to be essential for nuclear localization of HuR (Fan et al, 1998; Chen et al, 2002). In accordance with these previous reports we also observed that the mutant HuR proteins with the hinge region deleted (HuRΔH, devoid of the hinge region only, and HuRΔH3, missing both the hinge region and RRM3) accumulate in the cytoplasm in non-stressed cells while a full-length HuR and a deletion mutant devoid of RRM3 but retaining the hinge region (HuRΔ3) remain predominantly nuclear. Importantly, overexpression of these mutant HuR proteins had no effect on the status of P-bodies, structures implicated in the miRNA repression (Fig. 1).

Figure 2 Repression of let-7 reporters is alleviated by the cytoplasm-accumulating HuR mutant, HuRΔH, in HEK293 cells. (**A**) Schematic diagram of RL reporters bearing let-7 sites (small boxes) and the HuR-binding site (the CAT-1 ARD; large box). (**B**) HEK293 cells were transfected with plasmids expressing indicated RL reporters and different forms of HuR. Activity of RL was normalized for activity of FL expressed from co-

transfected plasmid. The normalized values are related to activities of RL-Con which are set to 1. Representative data from two independent experiments (means ± SD), each including three transfections, is shown. (**C**) A representative western blot analyzing the expression levels of Myc-HuR and its mutants is shown; β-tubulin serves as loading control.

In our first attempt to test whether cytoplasmic presence of HuR can bring about the derepression, Myc-epitope-tagged HuRΔH and HuRΔH3 (both accumulate in the cytoplasm in the absence of stress), and, as controls, full-length HuR and HuRΔ3 (both accumulate in the nucleus; Fig. 1) were transiently expressed in HEK293 cells and their effect on activity of different Renilla luciferase (RL) reporters regulated by let-7 miRNA was measured. Reporter RL-3xB contained three imperfect complementary let-7 sites but no HuR binding site, while reporter RL-3xB-ARD also contained HuR-binding AU-rich region (ARD) derived from 3'-UTR of CAT-1 mRNA; control reporters were devoid of let-7 sites (Fig. 2A). Expression of Myc-HuRΔH, but not Myc-HuR or Myc-HuRΔ3, relieved silencing of the ARD-containing RL reporter regulated by let-7 RNA (RL-3xB-ARD) in non-stressed HEK293 cells. Interestingly, the Myc-HuRΔH3, inspite of being cytoplasmic, did not alleviate the RL-3xB-ARD repression. None of the mutant proteins had an effect on activity of control reporters RL-3xB and RL-ARD (Fig. 2B).
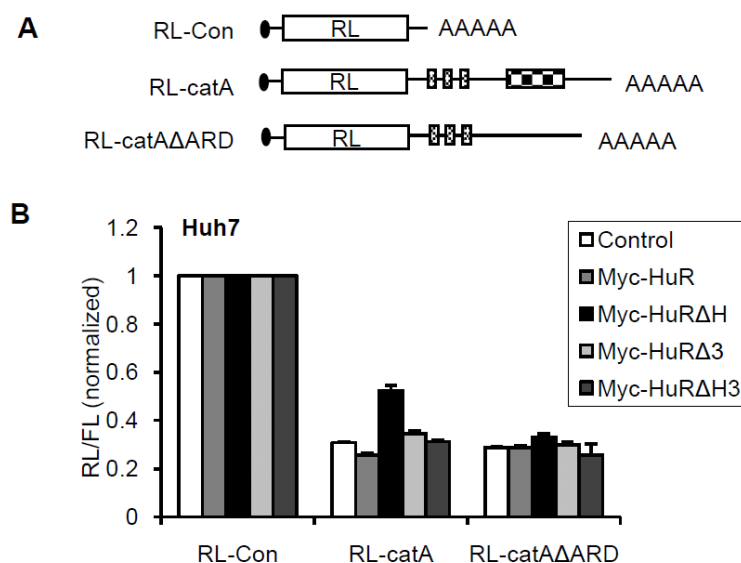
Figure 3 Repression of miR-122 reporters is alleviated by the cytoplasm-accumulating HuR mutant, HuRΔH, in Huh7 cells. (**A**) RL-cat reporters used for luciferase assays.

The rectangles in the 3'UTR represent miR-122 sites (small boxes) or HuR-binding ARD element (single large box). RL-catAΔARD is devoid of the 250-nt-long ARD and RL-Con represents control reporter devoid of miR-122 and ARD sites. (**B**) Activity of RL-cat reporters in Huh7 cells expressing HuR or its mutants. Activity of RL was normalized for activity of co-expressed firefly luciferase (FL). The values, representing means (±SD) from two experiments, each including three transfections, are related to activities of RL-Con which are set to 1.

In similar experiments performed in Huh7 cells, the effect of expression of HuR mutants was measured on activity of RL reporters bearing different regions of the CAT-1 mRNA 3'-UTR. The RL-catA reporter contains a full-length CAT-1 3'-UTR, including miR-122 sites and the HuR-inding AU-rich region (ARD). RL-catAΔARD reporter contains miR-122 sites but no ARD (Fig. 3A). In Huh7 cells also we found that expression of Myc-HuRΔH but not Myc-HuR, Myc-HuRΔ3 or Myc-HuRΔH3 increased activity of RL-catA in non-stressed Huh7 cells but had no significant effect on activity of RL-catAΔARD, reporter bearing no ARD (Fig 3B). Collectively, the cytoplasmic HuRΔH-mediated derepression of miRNA reporters in both cell lines provided us with the first evidence that stress is not required to alleviate the miRNA repression by HuR. In addition, the neutral effect of cytoplasmic Myc-HuRΔH3 on derepression suggested that both the hinge region and RRM3 are likely to be important for HuR to be functional in the derepression.
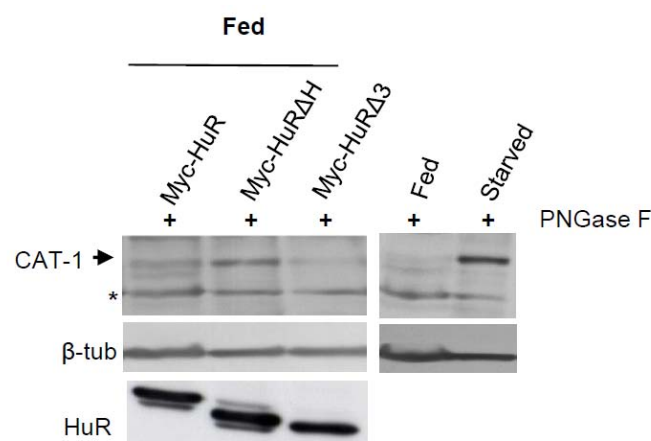
Figure 4 Expression of CAT-1 protein in Huh7 cells over-expressing different HuR proteins. Expression levels of Myc-HuR and its mutants are shown below the CAT-1 and β-tubulin Westerns. The arrow marks the CAT-1 protein. Asterisk denotes a cross-

reacting protein band. Samples were deglycosylated by treatment with Peptide N-Glycosidase F (PNGase F) before loading as indicated. Panel on the right represents a control comparing non-starved (Fed) Huh7 cells with cells starved for 2 h for amino acids.

To address the uncoupling issue in the light of physiological context, we analyzed the level of endogenous CAT-1 protein upon expression of functionally active cytoplasmic mutant Myc-HuRΔH, in Huh7 cells. As shown in Fig. 4, expression of Myc-HuRΔH, but not Myc-HuR or Myc-HuRΔ3 (as controls), resulted in the increase of the CAT-1 protein level, similar to that seen in Huh7 cells subjected to the amino acid starvation stress. HuR proteins were expressed at similar level. In control experiments, we also ascertained, by determining the phosphorylation status of the translation initiation factor eIF2-α (Fig. 5A) and observing stress granule formation (Fig. 5B) that overexpression of neither of the proteins resulted in induction of stress in transfected cells. This eliminated the possibility that derepressive effect of Myc-HuRΔH was observed because of induction of stress in the cells upon its transfection.
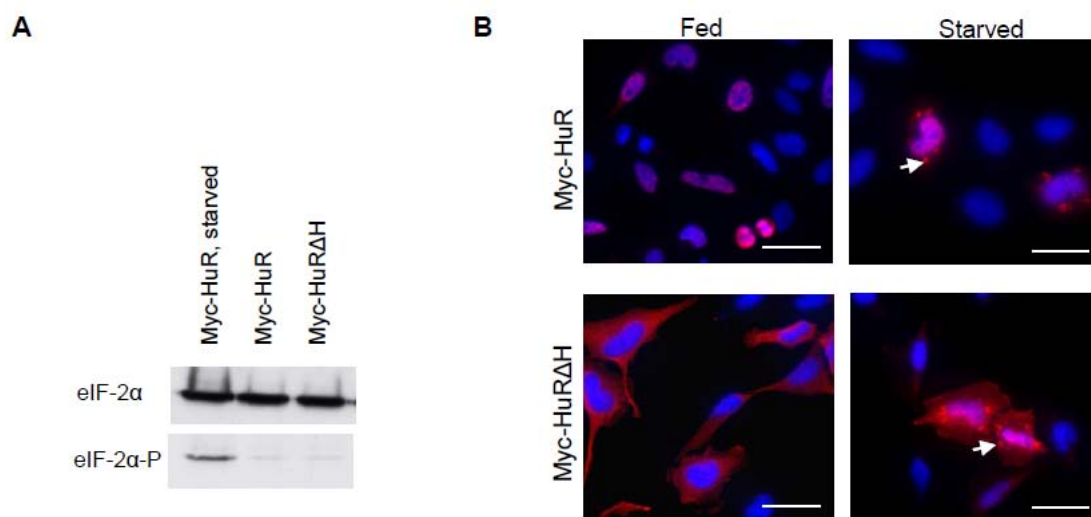
Figure 5 Expression of mutant HuRΔH does not induce cellular stress. (**A**) Huh7 cells were transfected with Myc-tagged HuR or its mutant HuRΔH and whole cell extracts were analyzed by western blotting using Abs recognizing eIF2α or its phosphorylated form. Extract prepared from Huh7 cells transfected with Myc-HuR and starved for amino acids was used as a positive control. (**B**) Expression of indicated HuR proteins in

transfected cells do not induce stress granule formation (left column) but the expressing cells accumulate stress granules in response to amino acid starvation stress (right column). HeLa cells were transfected with indicated plasmids expressing either Myc-tagged HuR or its mutant HuRΔH and cellular localization of the proteins in control and stressed cells determined using α-Myc Ab. Nuclei were stained with DAPI. Stress granules accumulating in stressed cells are marked by arrows. The scale bar is 10 μm.

Taken together, these data suggest that stress-independent cytoplasmic accumulation of HuR can attenuate the miRNA repression of mRNAs bearing HuR binding sites in.

4.1.2 Cytoplasmic accumulation of HuR in a cancer cell line attenuates the miRNA-mediated repression
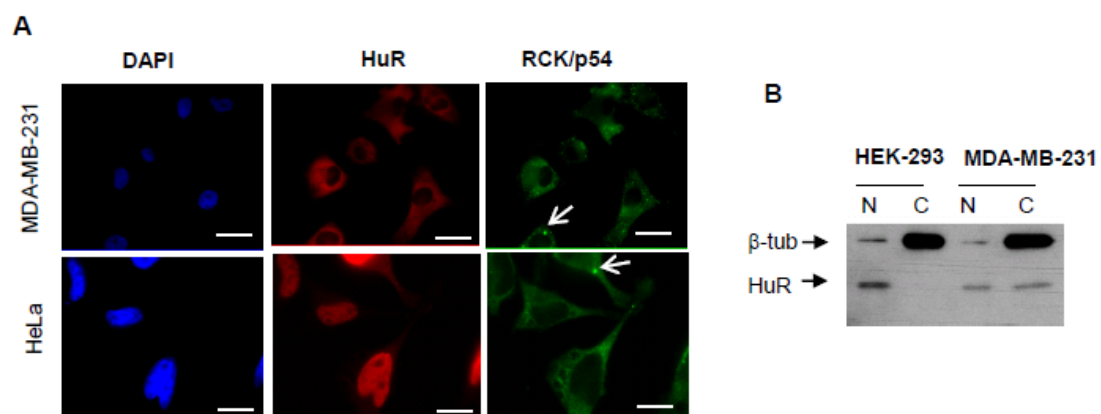
Figure 6 Cytoplasmic localization of HuR in breast cancer MDA-MB-231 cells. (**A**) Cellular localization of HuR (red) in MDA-MB-231 and HeLa cells assayed by IF using α-HuR Ab. Nuclear staining with DAPI is in blue and P-body staining with α-RCK/p54 is in green (marked by white arrows). Scale bar represents 10 μm. (**B**) Subcellular distribution of HuR in HEK293 and MDA-MB-231 cells. Cell extracts were fractionated to cytosolic (C) and nuclear (N) fractions and used for western blotting with α-HuR and α-tubulin Abs.

In breast cancer cell line MDA-MB-231, HuR accumulates in the cytoplasm even in the absence of stress (Fig. 6A and 6B; Tran et al, 2003) whereas in non-stressed HeLa (Fig. 6A) and HEK293 (Fig. 6B), HuR is predominantly localized in the nucleus. As evident from Fig. 6A, the integrity of the P-body structure also remained intact in MDA-MB-231 cells. In order to prove the

stress-independent effect of HuR in mitigating miRNA-mediated repression, we decided to compare the activity of different RL-reporters (Fig. 7A) in MDA-MB-231 and HEK293 cell lines. In non-stressed HEK293 cells, in which HuR was primarily localized in the nucleus all three reporters bearing let-7 sites were repressed 2.5- to 3.5-fold (Fig. 7B, upper panel). The RL-3xB reporter was also strongly repressed (2.8-fold) by let-7 miRNA in MDA-MB-231 cells. However, in MBA-MD-23 cells, the repression of let-7 reporters containing HuR-binding AREs, RL-3xB-ARD and RL-3xB-p53a was largely attenuated (1.4-fold repression; Fig. 7B, lower panel). Consistent with the HuR protein being responsible for the relief of repression in MDA-MB-231 cells, the siRNA-mediated knockdown of HuR resulted in a marked increase in RL-3xB-ARD repression in these cells (Fig. 7C).
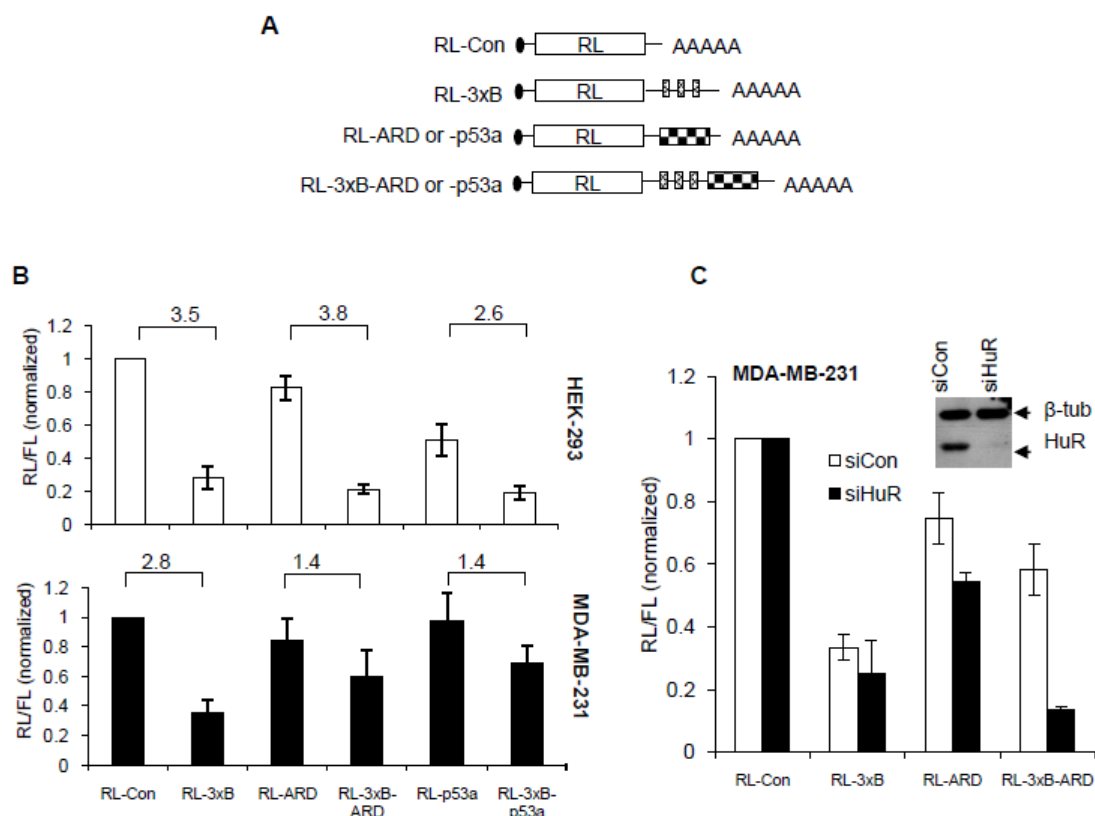
Figure 7 HuR-dependent relief of let-7-mediated repression of reporters in MDA-MB-231 cells. (**A**) Scheme of RL reporters bearing let-7 sites (small boxes) and the HuR-binding region (large box) from the p53 3'UTR or the CAT-1 ARD. (**B**) Activity of RL reporters described in A in HEK293 (upper panel) or MDA-MB-231 (lower panel) cells. The values are related to activities of RL-Con which are set to 1. Fold repression for each pair of reporters is indicated in each panel. The values are from three

independent transfections (means ± SD). (**C**) Alleviation of RL-3xB-ARD repression in MDA-MB-231 cells is HuR-dependent. MDA-MB-231 cells were treated with either control (siCon) or anti-HuR (siHuR) siRNA, and effectiveness of HuR depletion is shown in the inset. Data obtained from three independent transfections (means ± SD).

The activity of RL-cat reporters bearing the miR-122 sites was also tested in MDA-MB-231 cells. Transfection into these cells of the mimic of miR-122 but not that of let-7 strongly repressed RL reporters devoid of the ARD element (RL-catAΔARD and RL-catB; Fig. 8A) but had only a minimal effect on the activity of RL-catA, which contained both miR-122 and ARD sites (Fig. 8B). Collectively, these data suggest that the let-7 and miR-122 induced repression of the targets is attenuated in MDA-MB-231 cells by the stress-independent cytoplasmic presence of HuR.
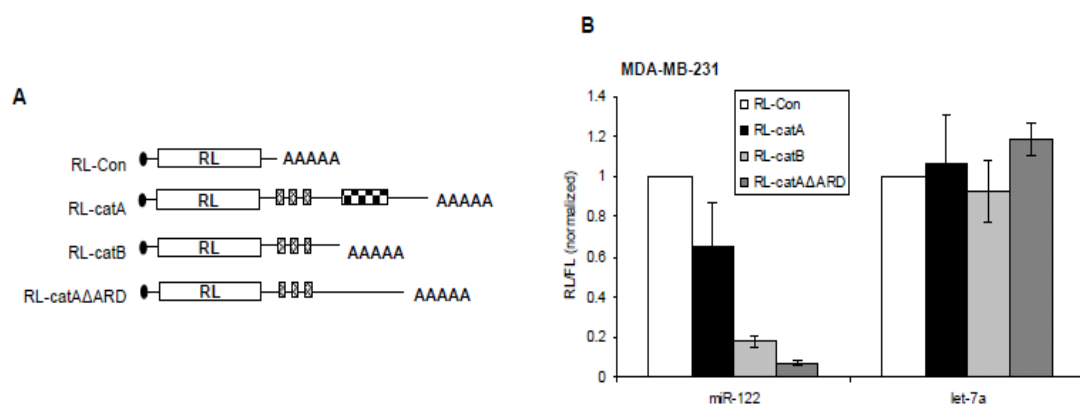
Figure 8 Relief of the miR-122-targeted RL-cat reporters in MDA-MB-231 cells. (**A**) RL-cat reporters used for luciferase assays. Small boxes in the 3'UTR represent miR-122 sites and a single large box denotes the HuR-binding ARD element. (**B**) Reporters containing miR-122 sites but no HuR-binding element ARD are strongly repressed by miR-122 transfected into MDA-MB-231 cells but reporter containing both miR-122 and HuR sites, RL-catA, is largely immune to miR-122 repression. Indicated reporters and either miR-122 mimic or let-7 mimic (used as control) were transfected to MDA-MB-231 cells and activity of RL and FL was measured. The values (means ± SD), originating from two experiments, each including three transfections, are related to activities of RL-Con which are set to 1.

4.1.3 Treatment of cells with an inhibitor of HuR prevents its activity in the relief of miRNA repression

To obtain further evidence of whether alleviation of miRNA repression is specifically mediated by HuR, we used the HuR inhibitor MS-444, which interferes with HuR activity and also prevents its cytoplasmic localization resulting in its nuclear accumulation (Meisner et al 2007). We found that in Huh7 cells subjected to amino acid starvation stress the addition of MS-444 prevented increase in CAT-1 protein level (Fig. 9A). Likewise, treatment with MS-444 prevented starvation-induced derepression of RL-catA reporter in a dose dependent manner, having no effect on the activity of reporter bearing no ARD site (RL-catΔARD) (Fig. 9B).
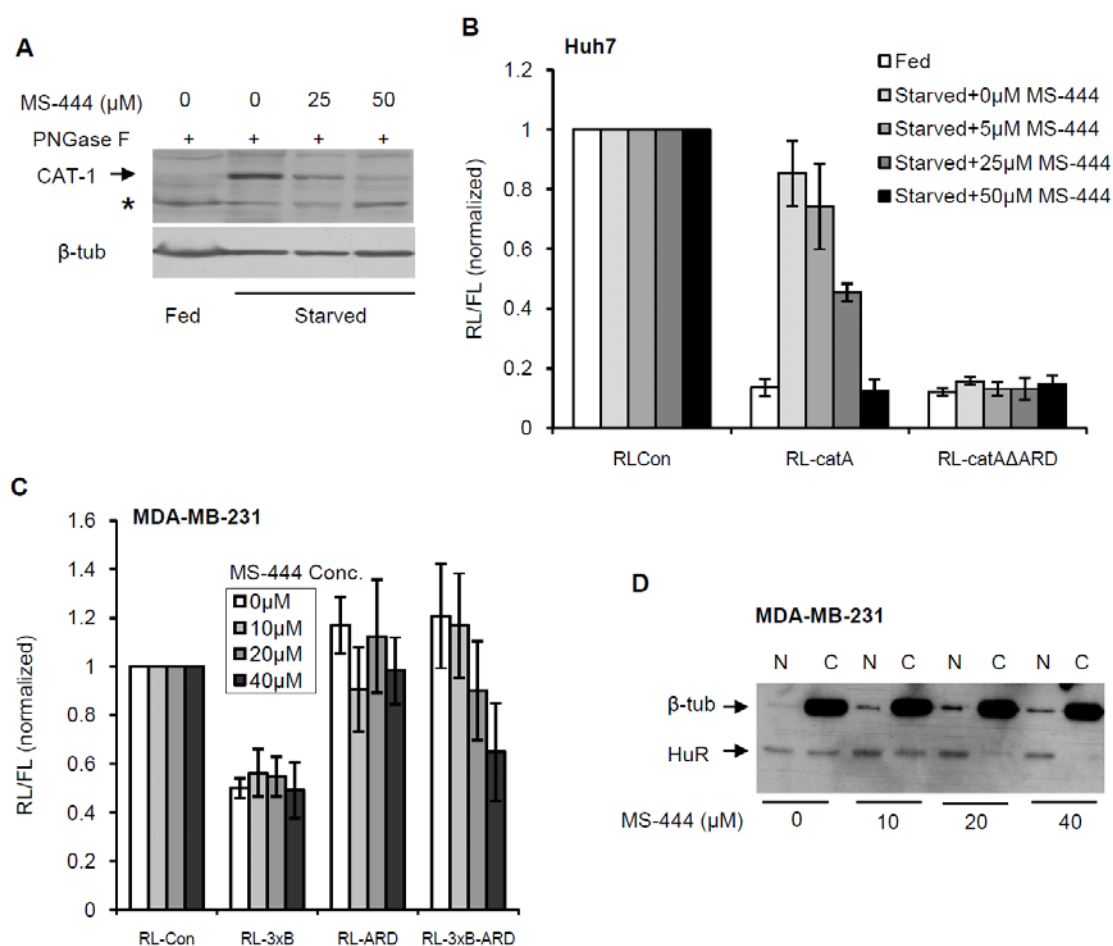
Figure 9 Inhibition of HuR by MS-444 prevents alleviation of miRNA repression. (**A**) Effect of increasing concentrations of the HuR inhibitor MS-444 on CAT-1 protein

expression in Huh7 cells starved for amino acids. (**B**) MS-444 prevents stress induced alleviation of miRNA repression of reporter in Huh7 cells. Huh7 cells expressing indicated RL reporters were subjected to amino acid starvation for 2 h and treated with indicated concentrations of MS-444. The values (means ± SD), originating from three transfections, are related to activities of RL-Con which are set to 1. (**C**) Effect of MS-444 on activity of let-7 RL reporters in MDA-MB-231 cells. Data obtained from three independent transfections (means ± SD). (**D**) Effect of MS-444 on HuR localization in MDA-MB-231 cells. Extracts from cells treated with different concentration of MS-444 were fractionated to cytosolic (C) and nuclear (N) fractions and analyzed by western blotting.

In MDA-MB-231 cells, addition of MS-444 also decreased the activity of the HuR-responsive let-7 reporter RL-3xB-ARD but had no influence on the expression of three control mRNAs (Fig. 9C). Importantly, treatment with MS-444 was associated with a dose-dependent shift of HuR from the cytoplasm to the nucleus (Fig. 9D), correlating with its effect on HuR-mediated derepression of target reporter. This is in line with the requirement for the cytoplasmic localization of HuR for its suppressive effect on miRNA repression.

Collectively, the data presented so far demonstrate that accumulation of HuR in the cell cytoplasm is sufficient for mitigating miRNA-mediated repression of targets bearing HuR binding sites in the 3'-UTR. Furthermore, the results with HuR mutants and the carcinoma MDA-MB-231 line also provide evidence that stress-independent accumulation of HuR in the cytoplasm can alleviate the miRNA-mediated repression. Taken together, these data indicate that HuR-induced derepression can be uncoupled from stress.

4.2 PART II Characterization of the HuR-miRISC interplay on target RNA

4.2.1 HuR does not inhibit miRISC by interacting with its component proteins

Having obtained evidence for HuR being the major factor involved in antagonizing the miRNA repression, we envisaged several potential mechanisms how HuR could attenuate the miRNA repression. HuR could interact with miRISC components and inhibit their effector function in miRNA repression. Alternatively, binding of HuR to mRNA might lead to the disassembly of miRISC or its displacement from target mRNA. To obtain insight into the mechanism of HuR-mediated suppressive activity, we first tested whether HuR interacts with the miRISC components such as Ago or GW182 proteins and thus possibly inhibits the miRISC function.

4.2.1.1 Endogenous HuR does not interact with miRISC proteins
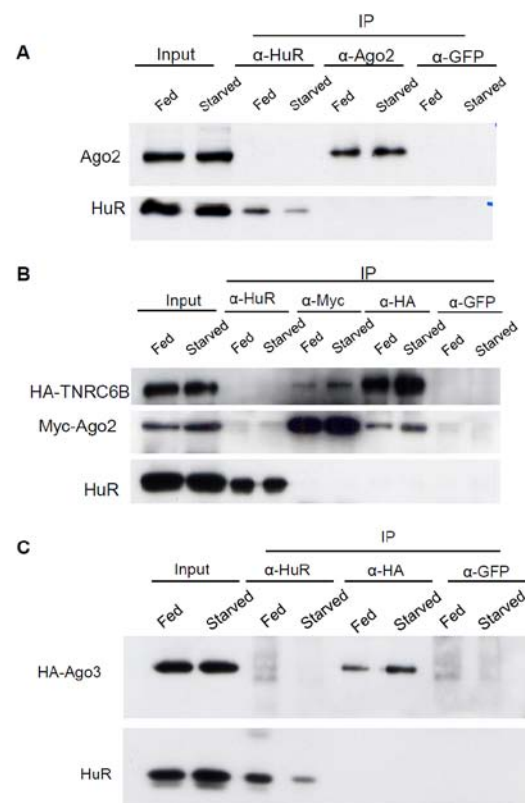
Figure 10 Immunoprecipitation (IP) experiments reveal no interaction between HuR and miRISC components (**A, B**) HuR does not interact with either Ago2 (**A, B**), Ago3

(**C**) or TNRC6B (**B**). Extracts prepared from HeLa cells, either control (Fed) or starved (Starved) for amino acids for 2 h, were used for IP reactions with Abs as indicated. α-GFP was used for control IP reactions. Material immunoprecipitated with indicated Abs was used for western blotting.

We performed immunoprecipitation (IP) experiments with HuR and Ago proteins from cell extracts of either stressed or non-stressed cells. HuR did not co-immunoprecipitate endogenous Ago2 and Ago2 did not co-immunoprecipitate HuR (Fig. 10A) in either cell condition. The IP experiments with ectopically expressed epitope-tagged proteins similarly showed no evidence of endogenous HuR interaction with Ago3 (Fig. 10C), and Ago2 or a GW182 protein TNRC6B (Fig. 10B). As expected, the IP experiments revealed interaction between Ago2 and TNRC6B (Fig. 10B) and immunoprecipitation with control GFP antibody was unable to pull down any of the investigated proteins (Fig. 10).

4.2.1.2 Cytoplasmic mutant HuRΔH does not interact with Ago2

To find out if the cytoplasm-accumulative HuR mutant (HuRΔH), which is active in mediating derepression, behaves in a similar manner, we also performed IP experiments with ectopically expressed HuRΔH and Ago2 proteins, using extracts from non-stressed cells. Like in case of a full length HuR, the HuRΔH mutant also did not show any association with Ago2 (Fig. 11).
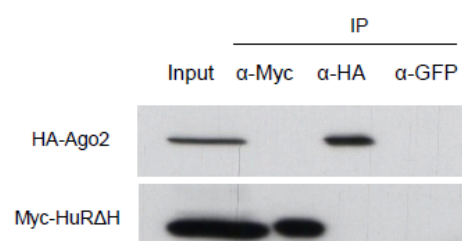
Figure 11 Immunoprecipitation (IP) experiments reveal no interaction between cytoplasmic HuR and miRISC components. HEK293 cells were transfected with HA-Ago2 and Myc-HuRΔH. Extracts prepared after 48 h of transfection were used for IP reactions with Abs as indicated. α-GFP Ab was used for control IP reactions.

Taken together, these results point out the fact that HuR does not inhibit the miRISC activity by directly interacting with its components. The absence of appreciable interaction of HuR with miRISC components even in the absence of RNase digestion (Figure 10A, B, C and 11) suggested that association of HuR and Ago complexes with mRNA may be mutually exclusive.

4.2.2 Interaction of HuR and miRNPs with target mRNA is mutually exclusive

Next, we measured association of HuR and Ago proteins with target mRNAs by isolating RNA from HuR and Ago2 IPs prepared from cells subjected to different treatments.
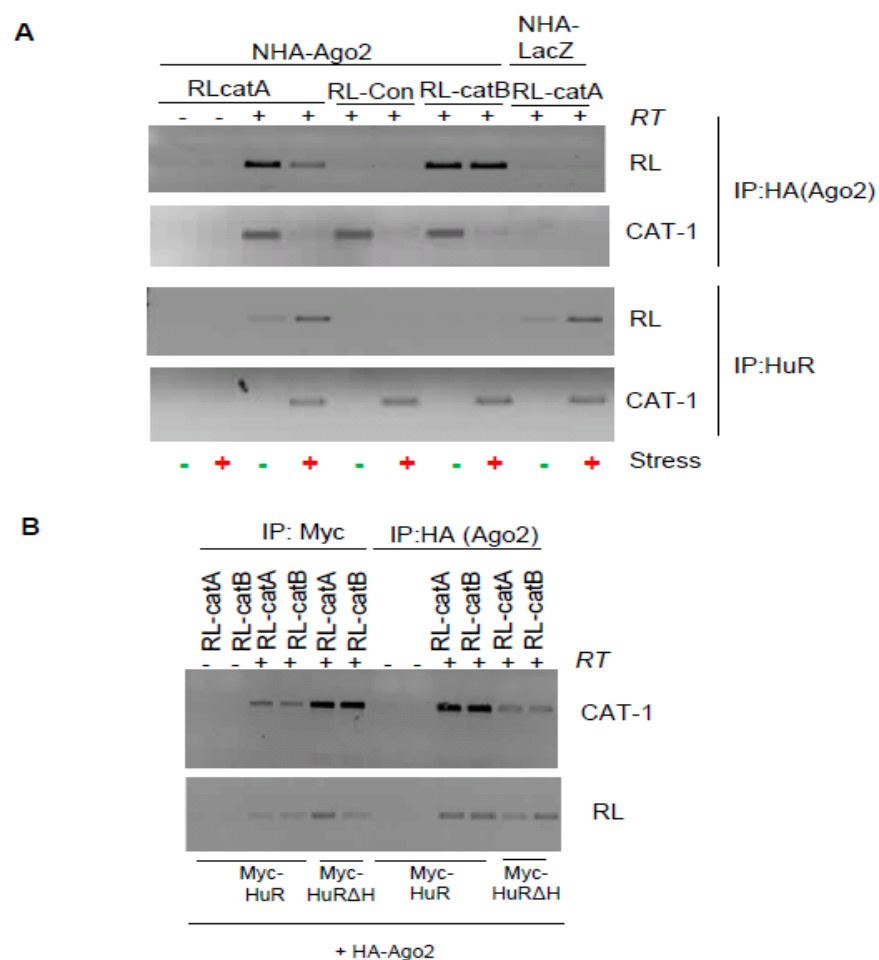
Figure 12 Immunoprecipitation (IP) experiments indicate that association of HuR and Ago2 with target mRNA is mutually exclusive. (**A**) Huh7 cells were transfected with

plasmids expressing NHA-Ago2 or NHA-LacZ and indicated RL reporters (RL-catA, RL-catB or RL-Con). Material immunoprecipitated with α-HA or α-HuR Abs from extracts prepared from either control cells (Fed) or cells starved (Starved) for amino acids was used for RNA extraction. Following reverse transcription (RT), RNA was PCR-amplified to detect reporter mRNAs and endogenous CAT-1 mRNA. (**B**) HuRΔH, which accumulates in the cytoplasm, but not other HuR forms, decrease association of CAT-1 and reporter mRNAs with Ago2 in non-stressed Huh7 cells. Huh7 cells were co-transfected with indicated RL-cat reporters and plasmids expressing HA-Ago2 and Myc-HuR or Myc-HuRΔH. Cell extracts were subjected to IP reactions with α-HA or α-Myc Abs. RNA was isolated from IP material and RT-PCR-amplified using primers specific for CAT-1 or RL mRNAs.

We first analyzed the association of endogenous HuR and HA-tagged Ago2 with either endogenous CAT-1 mRNA or different RL-cat reporters expressed in Huh7 cells, either control or stressed by amino acid starvation. Analysis of α-Ago and α-HuR precipitates by semi-quantitative RT-PCR revealed reciprocal enrichment of CAT-1 and RL-catA mRNAs (these mRNAs bear both miR-122 and ARD elements and are thus subjects of the regulation) with either Ago2 or HuR as a function of stress (Figure 12A). They were enriched in Ago2 IPs in non-stressed cells but in HuR IPs in stressed cells. Control RL-reporters did not follow this pattern. RL-Con was not pulled down by either protein in any condition, and RL-catB with miR-122 sites but no ARD was pulled-down by Ago2 but not HuR; this result was independent of stress (Figure 12A).

In a further experiment, we measured the association of CAT-1 mRNA and RL-cat reporters with Ago2 as a function of full lenght HuR and its active cytoplasmic mutant HuRΔH, which were co-expressed (as Myc fusions) with HA-Ago2 in non-stressed Huh7 cells. In α-Myc precipitates, CAT-1 mRNA was markedly enriched in the IP originating from cells expressing the HuRΔH mutant, which accumulates in the cytoplasm and has potential to derepress CAT-1 mRNA (see Fig. 4); IPs from extracts expressing HuR, which accumulates in the nucleus, contained lower levels of CAT-1 mRNA (Figure 12B). In contrast, analysis of α-Ago-2 precipitates revealed the reciprocal pattern, with the lowest level of CAT-1 mRNA present in IP from cells

expressing HuRΔH. Analysis of the levels of RL-catA reporter, which is subject to the regulation, revealed a pattern similar to that of endogenous CAT-1 mRNA, i.e., in extracts originating from the Myc-HuRΔH transfections, the RL-catA mRNA was enriched in α-Myc IPs but was depleted in α-Ago2 IPs. Distribution of RL-catB followed the opposite pattern (Fig. 11B).

Taken together, the results presented above support the notion that HuR functions by displacing miRISC from mRNA, rather than by interfering with the inhibitory function of the miRISC remaining associated with the RNA target.

4.2.3 HuR does not antagonize repression induced by the Ago tethering to mRNA

The results of IP experiments described above indicated that interactions of miRISC and HuR with target mRNAs are mutually exclusive, suggesting that HuR displaces miRISC from its target upon binding to mRNA. To gain more insight into a possible mechanism, we investigated whether HuR interferes with the repression induced by direct tethering of Ago2 to target mRNA. We and others showed previously that tethering of Ago2, expressed as a fusion with the N-peptide that recognizes BoxB elements in the mRNA 3'-UTR (Fig. 13), represses mRNA by a miRISC-like mechanism but without involving miRNA or its base-pairing to mRNA (Pillai et al, 2004).

Figure 13 Tethering of Ago2 to reporter RNA. The tethering reporters contain five 19-nt BoxB hairpins which are recognized by the N-peptide. Ago2 fused to N-peptide interacts with the tethering reporter (left panel) and that devoid of the N-peptide does not (right panel).

We inserted HuR-binding ARD element into the RL-5BoxB tethering reporter downstream of the BoxB sites (Fig. 14A). Both in the fed (stressed) and starved (non-stressed) conditions, the RL activity of RL-5BoxB was inhibited by NHA-Ago2 but not HA-Ago2 or NHALacZ control proteins. Interestingly, we found that the presence of HuR-binding ARD element in the tethering reporter RL-5BoxB-ARD had no effect on reporter activity, either in stressed or non-stressed cells (Fig. 14B, left), which is in marked contrast to reporters responding to the let-7-mediated repression (Fig. 14B, right). This indicated that HuR binding to the reporter RNA cannot antagonize repression by Ago-tethering but can mitigate the repression by miRNAs suggesting that HuR alleviates repression in reactions depending on base-pairing of small RNAs to mRNA.
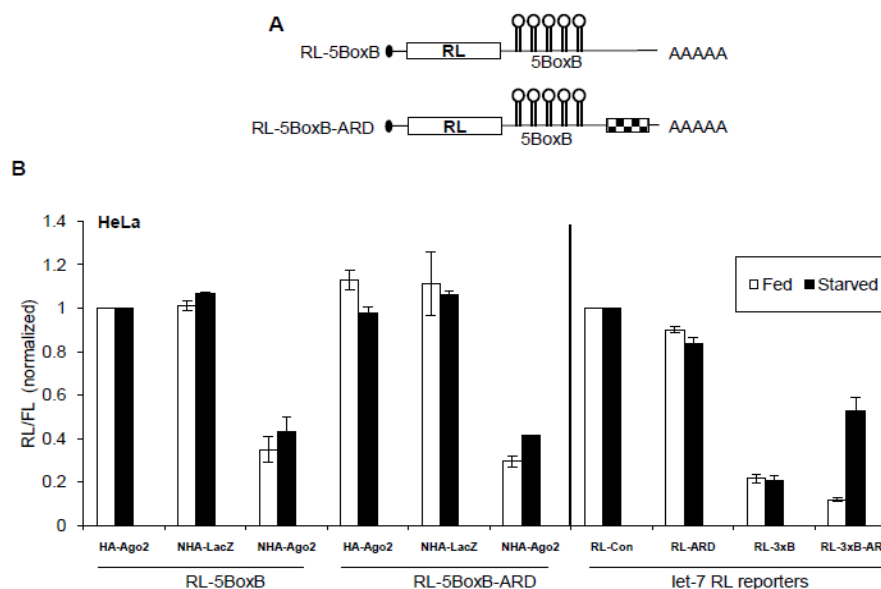
Figure 14 HuR does not inhibit silencing by the direct tethering of Ago2 to mRNA. (**A**) Schemes of reporters used for tethering. The HuR-binding ARD element is marked with a large box. (**B**) Repression induced by Ago2 tethering is not alleviated by the amino acids starvation stress. HeLa cells were transfected with indicated reporters and plasmids expressing NHA-Ago2 and either HA-Ago2 or NHA-LacZ as controls. The let-7 RL reporters were used as further controls. The values, representing means (±SD) from three independent transfections, are related to activities of RL-Con or to RL-5BoxB transfected with HA-Ago2, which are set to 1.

4.3 PART III - HuR interferes with the RNAi-induced gene silencing in mammalian cells

Having found that binding of HuR prevents miRNP interaction with the target RNA and the base-pairing to be required for HuR-mediated alleviation of repression, we hypothesized that HuR bound mRNAs might be also refractory to the RNAi-mediated gene silencing.

4.3.1 Antagonizing of the let-7-mediated RNAi
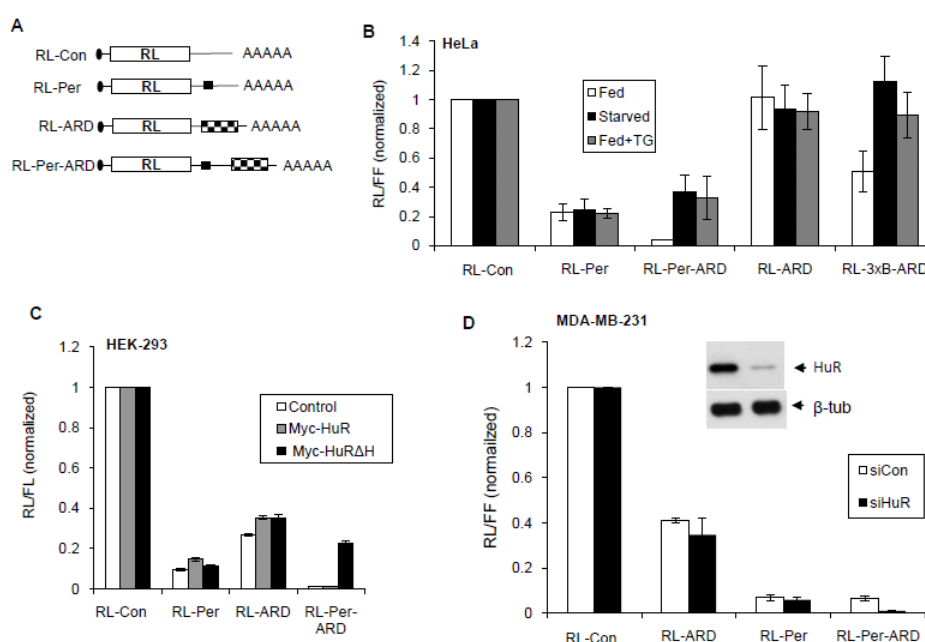
Figure 15 HuR inhibits let-7-induced RNAi in different cells. (**A**) Schemes of reporters used for RNAi assays. The HuR-binding ARD element is marked with a large box and the perfectly complementary let-7 site with a small black rectangle. (**B**) Stress induced alleviation of RNAi reporters bearing sites perfectly complementary to let-7 miRNA. HeLa cells were transfected with indicated reporters and RL activity was measured after 2 h of treatment with thapsigargin (TG), or 2 h starvation for amino acids. Activity of RL-Con, used for normalization, was set to 1. Data represent means (±SD) from three independent transfections. (**C**) Expression of the cytoplasm-accumulating Myc-HuRΔH in non-stressed HEK293 cells relieves repression of the let-7 RL-Per-ARD reporter. One representative experiment out of three experiments, each including three transfections is shown. Values represent means (±SD). (**D**) Alleviation of RL-Per-ARD repression in MDA-MB-231 cells is HuR-dependent. MDA-MB-231 cells were treated with either control (siCon) or anti-HuR (siHuR) siRNA, and effectiveness of

HuR depletion is shown in the inset. Data represent values from three independent transfections (means ± SD).

We first investigated whether HuR can interfere with repression of RL reporters bearing a single site perfectly complementary to let-7 miRNA. Such reporters are repressed by an RNAi mechanism involving endonucleolytic cleavage of target mRNA. RL-Per contains one perfect let-7 site while RL-per-ARD also contains an ARD originating from CAT-1 mRNA (Fig. 15A). We found that expression of RL-Per-ARD but none of the control reporters was markedly increased in cells starved of amino acids or subjected to endoplasmic reticulum stress by addition of thapsigargin (TG) (Fig. 15B). Moreover, expression of HuRΔH, which accumulates in the cytoplasm, but not full-length HuR, specifically attenuated repression of RL-Per-ARD in HEK-293 cells (Fig. 15C). Consistent with our previous finding that the HuR protein is responsible for the relief of miRNA-induced repression in MDA-MB-231 cells (see Figs. 7 and 8), the siRNA-mediated knockdown of HuR also prevented the relief of RL-Per-ARD repression in these cells (Fig. 15D).
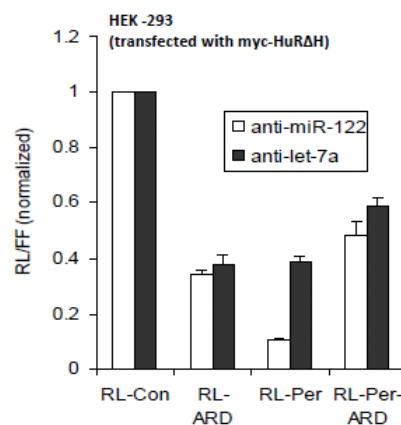
Figure 16 Blocking of the let-7 miRNA with an anti-sense 2'-*O*-methyl oligonucleotide in HEK293 cells expressing the cytoplasmic HuR mutant, HuRΔH, results in activity of the RL-Per-ARD reporter which is similar to that seen in response to the HuRΔH-mediated rescue. HEK293 cells expressing Myc-HuRΔH were transfected with indicated RL reporters and anti-sense 2'-*O*-methyl oligonucleotides blocking either miR-122 (as a control) or let-7. Values were normalized to activities of RL-Con which were set to 1. Data represent means (±SD) from three independent transfections.

To eliminate the possibility that HuR is opposing the effect of ARD but not let-7 mediated RNAi, we blocked the let-7 activity by 2'-O-methyl oligonucleotide in the HEK293 cells expressing HuRΔH (Fig. 16). In control condition (treatment with 2'-O-methyl oligonucleotide against miR-122), consistent with the previous result (Fig. 15C), the repression of RL-per-ARD was mitigated in the presence of cytoplasmic HuRΔH. Upon blocking of let-7 activity, the RL-per-ARD activity was elevated to a similar level. If HuRΔH was opposing the effect of ARD, one would expect an additional increase of RL-per-ARD activity in the absence of let-7. This result supported the idea that the antagonistic effect of HuR applies specifically to the reporters inhibited by RNAi.

4.3.2 HuR antagonizes activity of exogenous siRNA

We further tested the effect of HuR on activity of an exogenous siRNA, siRL, targeting the coding sequence of the RL reporter. Activity of RL-ARD but not RL-Con, which is devoid of ARD, was strongly rescued from siRL-mediated repression by the HuRΔH mutant, but expression of a full-length HuR had no effect (Fig. 17A). Similarly, repression of RL-ARD by siRL in MDA-MB-231 cells was attenuated (~1.8 fold) when compared to RL-Con (Fig.17B).
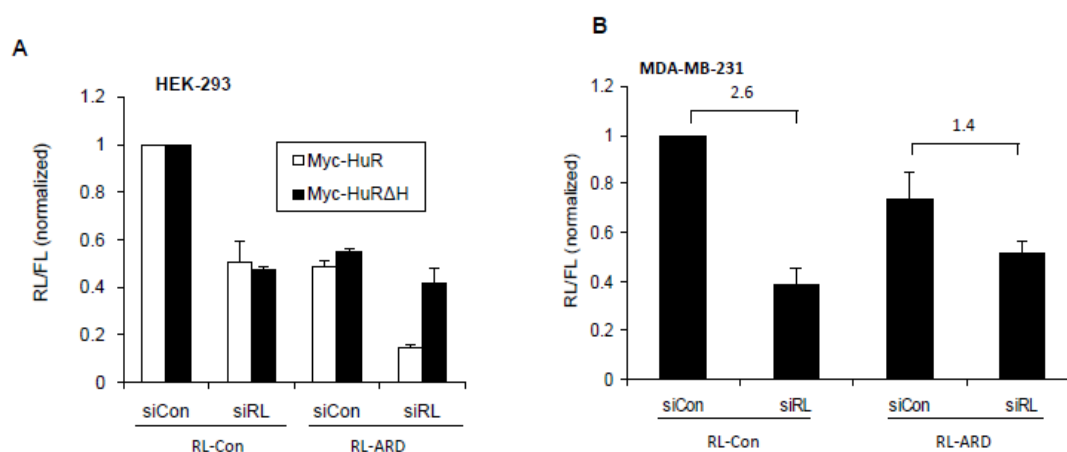
Figure 17 HuR antagonizes repression of RL-ARD reporter induced by siRNA (siRL) targeting the RL coding region (**A**) HEK293 cells were transfected with indicated reporters and plasmids encoding either Myc-HuR or Myc-HuRΔH. Cells were additionally transfected with either siCon or siRL as indicated. Data represent means

(±SD) from three independent transfections. **(B)** In MDA-MB-231 cells, the repression of RL-ARD by the RL-specific siRNA, siRL, is attenuated when compared to RL-Con. Cells were transfected with a control siRNA (siCon) or siRL, along with either RL-Con or RL-ARD plasmids. Values were normalized to the activity of RL-Con in cells transfected with siCon which was set to 1. Fold repression by siRL (as compared to control siCon) for each reporter is indicated. The values (means ± SD) originate from two experiments, each including three independent transfections.

Taken together, the data indicate that like in case of miRISC, repression involving siRISC, which also depends on base-pairing of small RNA to the target mRNA, is attenuated under conditions when HuR (or its mutant) accumulates in the cytoplasm.

4.4 PART IV - Recombinant HuR antagonizes effects of miRISC *in vitro*

Having found that HuR is essential for alleviating both the miRNA- and siRNA-mediated gene silencing and that the HuR effect applies only to situations when the repression involves base-pairing of small RNAs to mRNA, we attempted to dissect the mechanism further, by recapitulating the derepression reaction *in vitro*, using the purified components.

4.4.1 Effect of HuR proteins on endonucleolytic cleavage of RNA by miRISC

As a first approach we established the *in vitro* miRISC-mediated cleavage assay to test whether HuR can interfere with the endonucleolytic cleavage of target RNA by miRISC, occurring by the RNAi mechanism.

4.4.1.1 Purified miRISC specifically cleaves its target RNA

The partially purified miRISC was prepared by co-expressing human FLAG-HA-tagged Ago2 and let-7a pri-miRNA in HEK293T cells, followed by affinity purification of Ago2 on anti-FLAG conjugated antibody (Ab) beads. We rationalized that miRISC purified from cells co-expressing the pri-miRNA would be enriched in let-7-miRNA and be catalytically active in cleaving target RNA bearing perfectly complementary let-7 site. The expression of tagged proteins was verified by Western blotting using the anti-HA Ab (Fig. 18A). The cleavage competency of the purified miRISC was ascertained by incubating it with the 5'-^{32}P-labeled model RNA substrates (Fig. 18B) bearing one perfectly complementary let-7 site (MBSp) or mutated let-7 site (MutMBSp). As shown in Fig. 18C, miRISC prepared by co-expression of human FLAG-tagged Ago2 and let-7a pri-miRNA cleaved the target MBSp whereas that prepared from cells not co-expressing let-7a pri-miRNA was unable to cleave the same target, indicating only let-7-enriched miRISC can carry out the cleavage. The RNA with mutated let-7 complementary site was refractory to cleavage by the purified miRISC (Fig. 18D). Together these two initial experiments confirmed the specificity of purified miRISC.
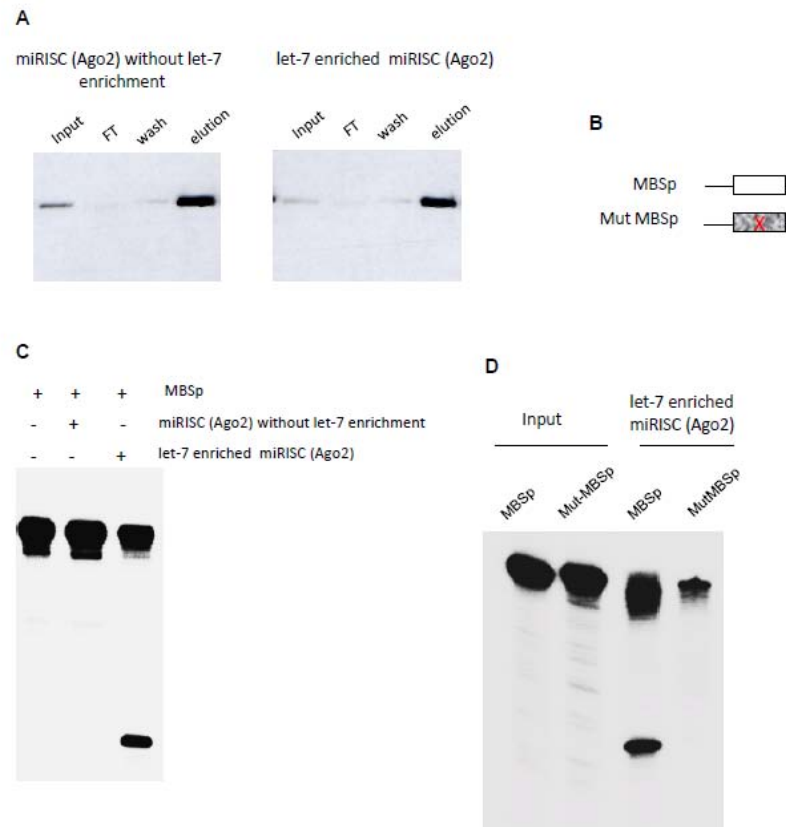
Figure 18 miRISC specifically cleaves the target RNA. (**A**) Western blot showing the similar level of purification for both let-7 enriched and without let-7-enriched miRISC (The latter purified from cells not co-transfected with the pri-let7). FT denotes flowthrough extract after immunoprecipitation. (**B**) Schematic representation of target RNAs. (**C**) MBSp, but not MutMBSp, is specifically cleaved by let-7-enriched miRISC. (**D**) Only let-7 enriched miRISC specifically cleaves MBSp (1nM).

4.4.1.2 Binding of recombinant HuR specifically inhibits endonucleolytic cleavage of RNA

HuR protein was expressed bacterially as a His-tag fusion and purified to near homogeneity (Fig.19A). We first investigated whether inclusion of HuR affects the miRISC-mediated cleavage of 5'-^{32}P-labeled model RNA substrates containing perfectly complementary let-7 site (MBSp) and HuR-binding site (HBS; a high affinity ARE originating from IL-1β mRNA; Meisner et al, 2004), separated by either 20-nt (HBS_20_MBSp) or 50-nt (HBS_50_MBSp)(Fig. 19B). As control, substrates with mutated HBS (HBS Mut_20_MBSp) or without HBS (ΔHBS_20_MBSp) were prepared (Fig.19B). The latter two

substrates did not bind to HuR whereas HBS-harbouring target, HBS_20_MBSp was specifically bound to HuR as verified by native gel electrophoretic mobility shift assay (EMSA) (Fig. 19C).
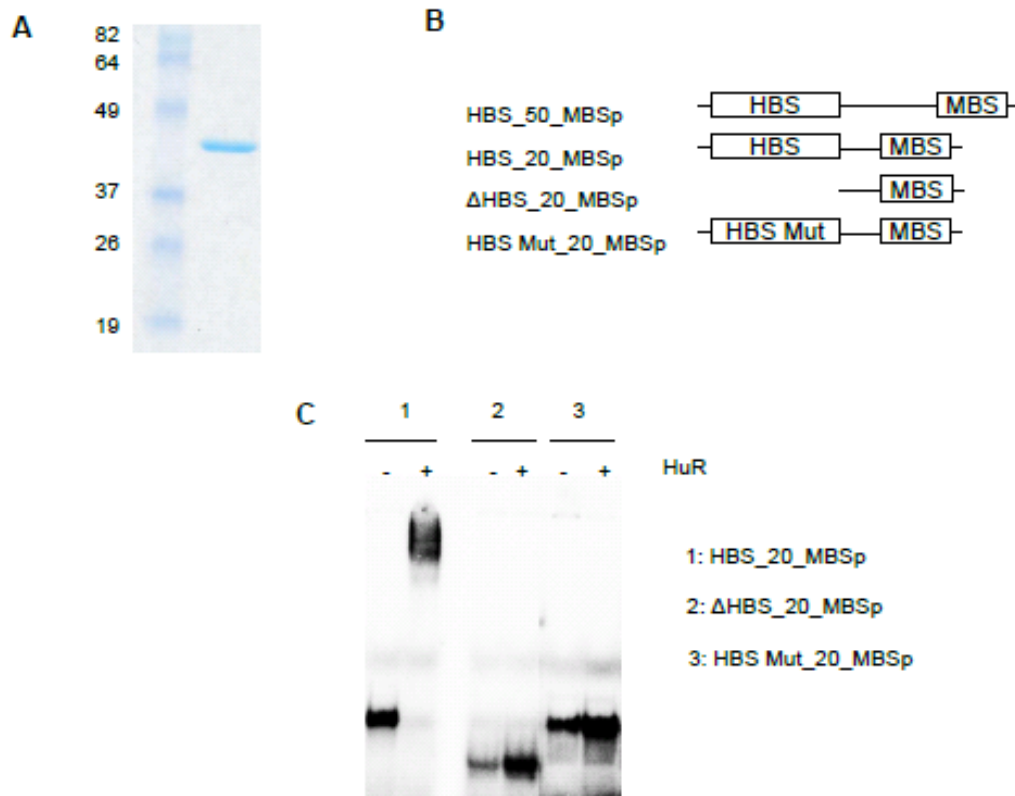
Figure 19 Specific binding of recombinant HuR to model substrates. (**A**) Coomassie-Blue-stained 12% SDS-polyacrylamide gel of recombinant full-length HuR. (**B**) Schemes of target RNAs used for the cleavage assay. HBS, <u>H</u>uR <u>B</u>inding <u>S</u>ite; MBS, <u>m</u>iRNA <u>B</u>inding <u>S</u>ite; ΔHBS and HBS Mut, HBS deleted or mutated. p denotes perfect let -7 complementarity of the MBS site. (**C**) EMSA showing target specificity of HuR binding. 1 nM of RNA was incubated with or without 200 nM HuR.

During the analysis of the effect of HuR on the miRISC-mediated cleavage of target RNAs, we added HuR and miRISC simultaneously to the reaction. As shown in Fig. 20A, addition of HuR resulted in a concentration dependent repression of cleavage of substrates containing HBS and MBS elements. Since similar effect was seen with RNAs bearing the elements either 20-nt (HBS_20_MBSp) or 50-nt (HBS_50_MBSp) apart (Fig. 20A and 20C), it is unlikely that binding of HuR to HBS caused a direct steric hindrance

for miRISC association. HuR had no effect on cleavage of RNAs bearing mutated HBS or having no HBS (Fig. 20B and 20C) as they did not bind HuR.
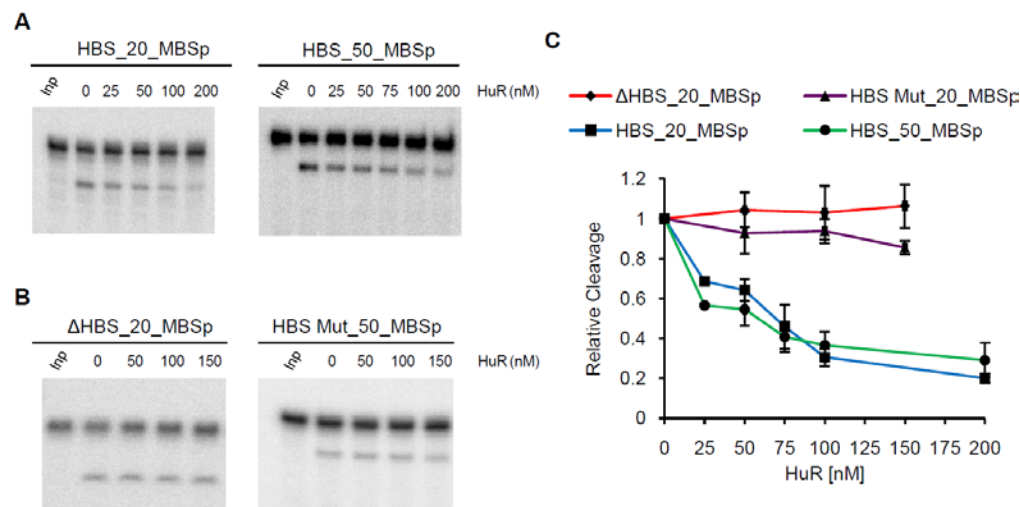
Figure 20 Purified recombinant HuR specifically inhibits RNA cleavage mediated by the let-7 miRISC. (**A** and **B**) Representative in vitro cleavage reactions performed with different 5'-^{32}P-labeled target RNAs and increasing amounts of HuR. (**C**) Phosphorimaging quantification of cleavage reactions similar to those shown in panels A and B. Values (means ± SD; n ≥ 3) were normalized to the reactions in the absence of HuR (the procedure of quantification is decribed in 'Materials and methods').

4.4.2 HuR oligomerization mutants are defective in attenuating miRISC cleavage

The *in cellulo* experiments revealed that the cytoplasm accumulating HuR mutant having no hinge region but having the RRM3 domain (HuRΔH) effectively mediated the derpression while the predominantly nuclear mutant lacking RRM3 (HuRΔ3) could not exert the effect. However, mutant HuR lacking both RRM3 and a hinge region (HuRΔH3) was unable to alleviate miRNA-mediated repression, despite its sufficient cytoplasmic abundance, suggesting that both of these two domains contribute to the derepressive function of HuR. HuR, and also proteins related to it such as, for example, HuB, HuD, and *Drosophila* ELAV, are known to oligomerize along RNA substrates (Devaux *et al*, 2006; Fialcowitz-White *et al*, 2007; Gao & Keene, 1996; Kasashima *et al*, 2002; Soller & White, 2005; Toba & White, 2008).

Furthermore, for HuR and Drosophila ELAV, the hinge separating RRM2 and RRM3, and the RRM3 were identified as domains contributing to formation of cooperative oligomeric HuR-ARE complexes (Fialcowitz-White et al, 2007; Toba & White, 2008). Aiming to separate the effect of different mutants (Fig.21A) on miRNA-directed inhibition from the context of their localization in cells and to gain insight whether HuR oligomerization plays a role in the interference with miRISC, we used the *in vitro* cleavage assay.

Bacterially expressed His-tagged mutant HuR proteins were purified to near homogeneity (Fig. 21B). A full-length HuR and its three different purified mutants, HuRΔH, HuRΔ3, HuRΔH3 were tested using EMSA for their potential to form oligomeric complexes on HSB_50_MBSp target RNA (Fig. 21C). Consistent with previous findings (Fialcowitz-White et al, 2007), a full-length HuR, and HuRΔH and HuRΔ3 mutants formed complexes gradually increasing in size in a concentration-dependent manner. In contrast, the potential of HuRΔH3 to oligomerize was clearly diminished (Fig. 21C). The two mutants able to oligomerize, HuRΔH and HuRΔ3, inhibited miRISC-mediated cleavage of HSB_50_MBSp RNA, although at a concentration higher than that of full-length HuR. In contrast, the cleavage of target RNA remained unaffected in the presence of the mutant HuRΔH3, which is defective in oligomerization (Fig. 21D and 21E).

To test further whether HuR oligomerization is important for interference with miRISC-mediated target cleavage, we pre-hybridized the HSB_50_MBSp RNA with a 50-nt-long oligodeoxynucleotide complementary to the spacer separating HBS and MBSp sequences in target RNA. The resulting duplex migrated with a slower mobility than the HSB_50_MBSp RNA and also showed a largely reduced potential to form multimeric complexes with HuR, as analyzed by EMSA (Fig. 21F; note that the IL-1β HBS element is 33-nt long and probably able to accommodate two HuR molecules). Importantly, hybridization to the complementary oligonucleotide fully eliminated the inhibitory effect of HuR on RNA cleavage by miRISC (Fig. 21G), thus providing additional evidence that oligomerization of HuR on target RNA is important for the protein getting into proximity of miRISC and suppression of miRISC activity.
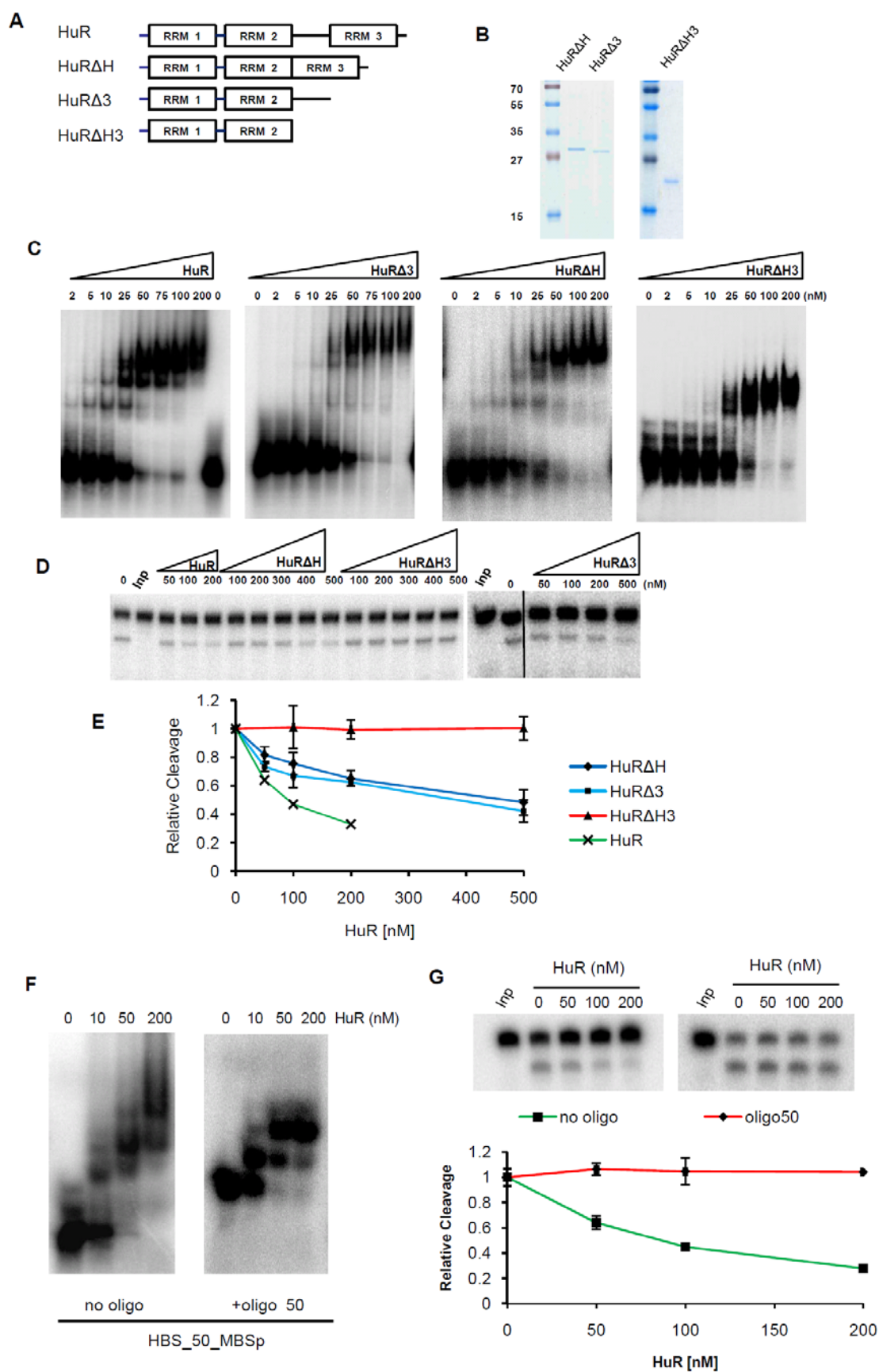
Figure 21 Alleviation of the miRISC cleavage in vitro involves oligomerization of HuR along target RNA. (**A**) Schemes of HuR and its deletion mutants. (**B**) Coomassie-Blue-

stained 12% SDS-polyacrylamide gel of different recombinant mutant HuR. (**C**) Oligomerization potential of HuR and its mutants as determined by EMSA, using ^{32}P-labeled HBS_50_MBSp RNA as a substrate. Identity and concentration (nM) of proteins is indicated at the top of the gels. (**D**) Representative in vitro cleavage reactions of HBS_50_MBSp RNA in the presence of increasing amounts of indicated HuR mutants. (**E**) Quantification of the effect of different HuR mutants on HBS_50_MBSp RNA cleavage. Values represent means ± SD≥ n₃ in case of the mutant proteins and for full length HuR, the value from the presented experiment in B plotted in the graph. (**F**) Prehybridization of 50-nt oligonucleotide complementary to the spacer region of HBS_50_MBSp RNA interferes with HuR oligomerization along RNA. (**G**) Prehybridization of 50-nt oligonucleotide also interferes with the alleviating effect of HuR on miRISC cleavage. (Upper panels) Effect of increasing amounts of HuR on cleavage of HBS_50_MBSp RNA or its duplex. (Lower panel) Quantification (means ± SD; n=3) of experiments similar to those shown in upper panels.

4.4.3. Activity of HuR and its mutants in alleviation of miRNA-mediated deadenylation

It was previously described that an *in vitro* extract derived from Krebs-2 ascites cells can faithfully recapitulate RNA deadenylation induced by miRNA let-7 (Fabian et al, 2009; Mathonnet et al, 2007) and this miRNA-mediated deadenylation was shown to be m^{7}GpppG-cap- and translation-independent event. To obtain an additional evidence for activity of different HuR proteins interfering with the miRISC function, we used this established system in collaboration with Sonenberg lab. We tested whether addition of recombinant HuR or its mutants interferes with the miRNA-induced deadenylation of RNA bearing let-7 miRNA sites and the HuR binding region ARD, derived from the CAT-1 3'UTR (6xB-ARD-3'UTR). We found that addition of HuR or its mutants HuRΔH and HuRΔ3, which relieved repression in cleavage assay strongly inhibited deadenylation of 6xB-ARD-3'UTR RNA in vitro. In contrast, addition of the HuR mutant HuRΔH3 that is inactive in derepression had no effect (Fig. 20B, upper row). In control experiments, we verified that addition of any of the four forms of HuR had no effect on deadenylation of RNA without ARD (6xB-

3'UTR; middle row). As expected, addition of anti-let-7 blocked deadenylation of 6xB-ARD-3'UTR, and RNA bearing mutated let-7 sites (6xBMut-ARD-3'UTR) did not undergo deadenylation (Fig. 22B, lower row). In conclusion, HuR can interfere with the inhibitory function of let-7 in the *in vitro* system recapitulating miRNA-mediated deadenylation and like in the case of miRISC-mediated cleavage assay, presence of either the hinge region or the RRM3 domain was required for HuR to oppose inhibition by let-7 indicating that though each of them are not essential for HuR function, together they confer HuR with the derepressive property which correlate nicely with the oligomerization potential of different HuR proteins.
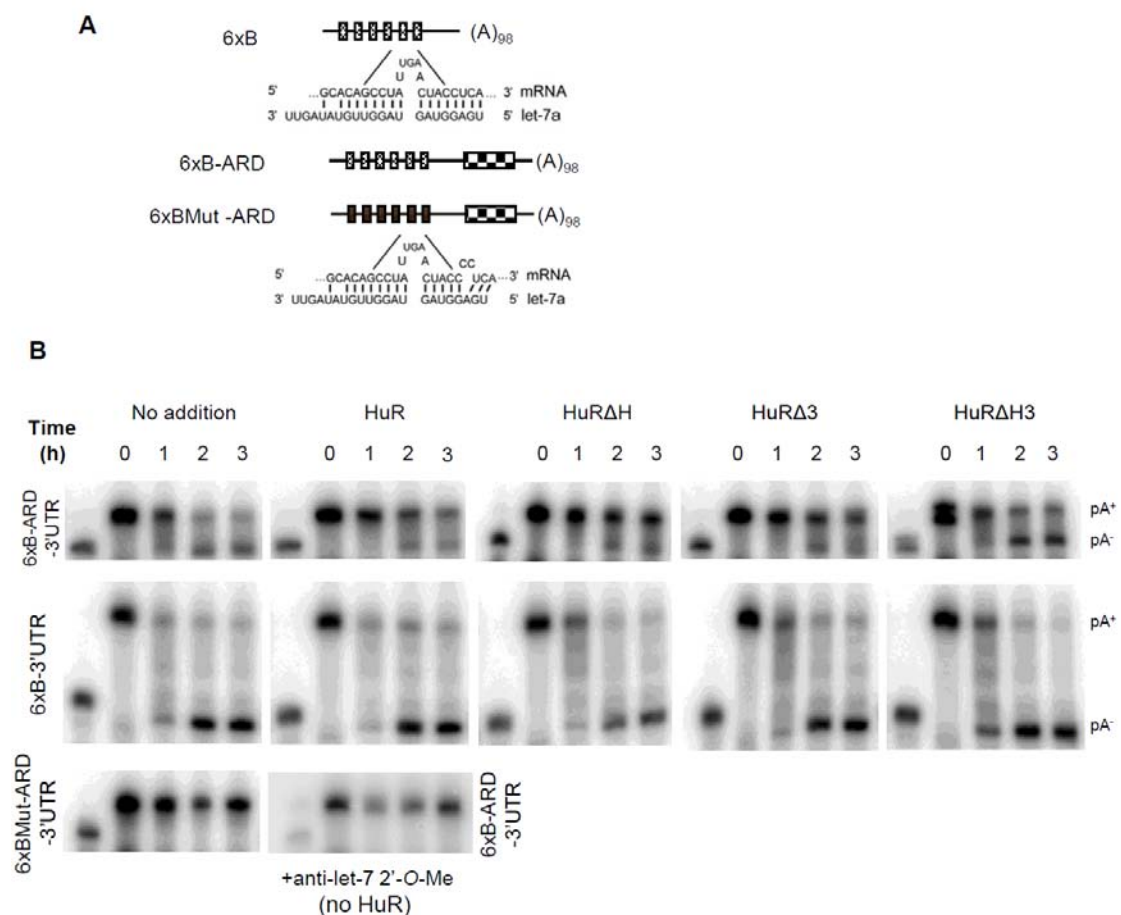
Figure 22 Effect of HuR and its mutants on deadenylation of reporter RNAs. (**A**) Schematic representation of reporter mRNAs. Sequences of the let-7-binding sites (RL-6xB) and mutated seed sites (RL-6xBMut) are shown below the drawings. (**B**) Effect of HuR and its mutants on deadenylation of 6xB-ARD-3'UTR (upper row) and 6xB-3'UTR (middle row) RNAs in Krebs-2 ascites extract. RNAs were incubated in the presence or

absence of 250 nM of HuR or its mutants for indicated time. Positions of pA^+ and pA^- RNAs are marked on the right. (Lower row) Control assays performed in the absence of HuR with 6xBMut-ARD-3'UTR and 6xB-ARD-3'UTR RNAs, the latter assay containing anti-let-7 2'-O-methyl oligonucleotide.

4.4.4 HuR can displace miRISC from the target RNA

4.4.4.1 miRISC is displaced from target RNA bearing the site perfectly complementary to miRNA

In previously described experiments, which involved the use of the cleavage assay, miRISC and HuR were always mixed together prior to the addition of target mRNA. In the experiments presented in this section, we investigated whether addition of HuR can inhibit miRISC or displace it from the target even when miRISC has been pre-bound to RNA.
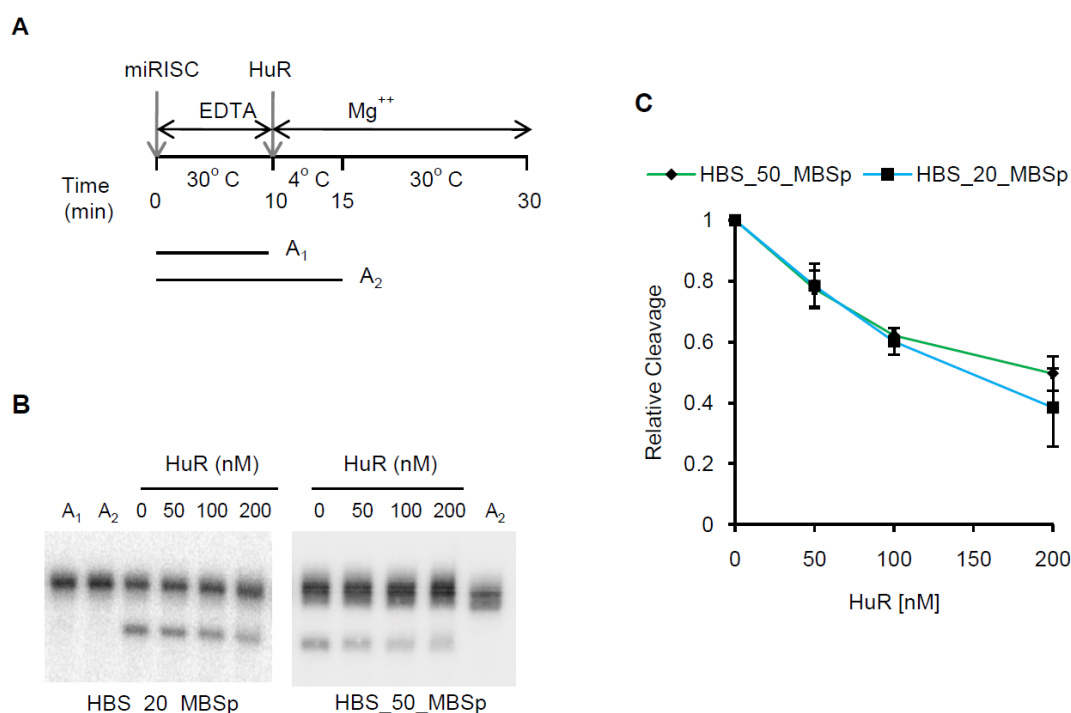
Figure 23 HuR can inhibit cleavage by miRISC pre-bound to RNA bearing perfectly complementary let-7 site. (**A**) Overview of the experimental set up for cleavage assay with miRISC pre-bound to target RNA. RNA was prebound to miRISC in the presence of EDTA to ensure blocking of the cleavage (timepoint A_1), followed by incubation on

ice for 5 min (timepoint A_2) after simultaneous addition of HuR and Mg^{2+}. The cleavage reaction was then incubated for 15 min at 30°C. (**B**) Representative in vitro cleavage reactions of HBS_20_MBSp and HBS_50_MBSp RNAs, as a function of increasing concentration of HuR. Reactions followed the experimental set up presented in **A**. The RNA remains uncleaved when incubated with miRISC in the presence of EDTA at 30 °C (lane A_1) or upon addition of Mg^{2+} when the incubation is performed on ice (A_2). (**C**) Quantification of reactions similar to the one shown in **B** (mean ± SD; n=3).

Pre-incubation of miRISC with either HBS_20_MBSp or HBS_50_MBSp was performed in the presence of EDTA to inhibit Mg^{2+}-dependent cleavage of RNAs containing perfectly complementary let-7 sites (Ameres et al, 2007; Schwarz et al, 2004). Following 10 min incubation at 30ºC in the presence of EDTA, Mg^{2+} and HuR were added and incubation continued first at 4ºC (to inhibit miRISC cleavage but to allow HuR binding) and then at 30ºC (scheme of the experiment is shown in Fig. 23A; association of miRISC with the target RNA and effect of HuR on target cleavage assay in the presence of EDTA were verified as discussed in detail in Appendices 1 and 2). As shown in Figs. 23B and C, addition of HuR led to the concentration-dependent inhibition of target cleavage, suggesting that HuR can lead to the dissociation of miRISC which has been pre-bound to RNA.

To determine directly whether HuR is indeed able to displace miRISC pre-bound to the target, we measured association of the HBS_50_MBSp RNA with miRISC following the HuR addition. Besides the miRISC containing a wild-type (wt) Ago2, we also used miRISCs in which let-7 is loaded onto FLAG-HA-Ago1 or the FLAG-HA-tagged Ago2 mutants Ago2D669A and Ago2H634A which, like Ago1, are catalytically inactive (Liu *et al*, 2004) (Fig. 24A). Pre-binding of the wt-Ago2 miRISC was carried out in the presence of EDTA (see above) but pre-binding of the three catalytically-incompetent miRISCs was performed in the presence of Mg^{2+}. Following incubation with HuR, amount of the HBS_50_MBSp RNA remaining associated with the immobilized miRISC was quantified by quantitative real-time PCR (qRT-PCR) (Fig. 24B). We found that addition of HuR decreased by 2- to 3-fold the association of HBS_50_MBSp RNA with all tested miRISC variants,

consistent with the HuR-mediated dislocation of miRISC from the RNA target
(Fig. 24C). We have verified that miRISC associated with RNA bearing the
let-7 site but not with the one having the let-7 site deleted (data not shown).
Taken together, these results indicated that dissociation of miRISC from the
target mRNA in response to HuR binding is likely to mediate its derepressive
function.
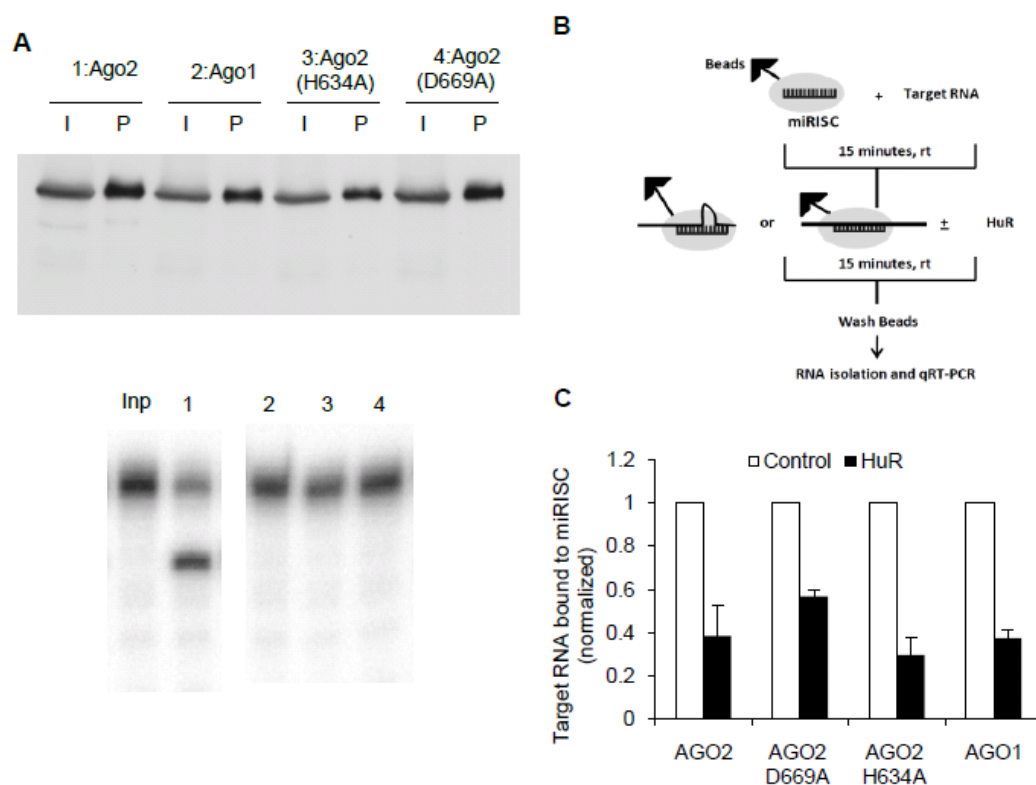
Figure 24 HuR can inhibit displace miRISC from target RNAs bearing perfectly
complementary let-7 site. (**A**) Characterization of purified miRISC complexes
containing Ago1, Ago2, or indicated Ago2 mutants. Upper panel, Western blot
performed with anti-HA Ab, showing purification of miRISCs containing Ago1, Ago2,
or indicated Ago2 mutants. "I" denotes input cell extracts and "P" denotes miRISCs
purified on anti-FLAG M2 Affinity beads. Lower panel, miRISC cleavage assay
demonstrating that only miRISC containing wt Ago2 is catalytically active (numbers
correspond to the indicated Ago protein as in the upper panel). "Inp", input $5'-^{32}$P-
labeled RNA substrate bearing a site perfectly complementary to let-7 miRNA. (**B**)
Schematic overview of experiments to determine the effect of HuR on association of

miRISC with target RNA. The miRISC-containing beads were incubated with target RNA for 15 min at 23°C followed by additional 15 min incubation at 23°C in the absence (control) or presence of 150 nM HuR. The RNA remaining bound to beads was subjected to qRT-PCR (for details see Materials and methods). (**C**) Quantification, by qRT-PCR, of the effect of HuR on association of indicated different forms (Ago2, Ago1, Ago2D669A, and Ago2H634A) of miRISC with HBS_50_MBSp RNA. Values for RNA remaining bound with different miRISC forms upon addition of 150 nM HuR are normalized to values measured in the absence of HuR which are set to 1 (means ± SD; n ≥ 3)

4.4.4.2 HuR effectively displaces miRISC from target RNAs bearing bulged miRNA site

We also tested the effect of HuR addition on association of the Ago2-miRISC with targets containing the let-7 site which is not complementary to let-7 RNA in its central region ("bulged" let-7 site, identical to that used in RL-3xB reporters and 6xB-3'UTR fragments, responding to let-7 miRNA, Fig. 22A). Two analyzed RNA targets, HBS_50_MBSb and MBSb_50_HBS, differ in the relative orientation of HBS and MBSb sites in RNA (Fig. 25A).
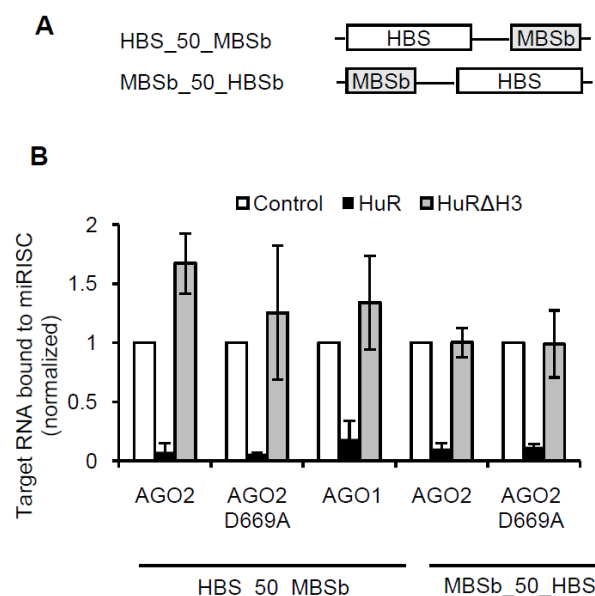
Figure 25 HuR can displace miRISC from target RNAs bearing bulged let-7 site. (**A**) Schemes of target RNAs bearing bulged let-7 site positioned either downstream (HBS_50_MBSb) or upstream (MBSb_50_HBS) of the HuR binding site. b denotes bulged let-7 MBS site (**B**) Quantification, by qRT-PCR, of the effect of HuR or HuRΔH3

mutant on association of indicated forms (Ago2, Ago1, and Ago2D669A) of miRISC with RNA targets. Values for RNA remaining bound with different miRISC forms upon addition of 150 nM HuR or HuRΔH3 are normalized to values measured in the absence of HuR (means ± SD; n ≥ 3).

We found that addition of HuR decreased association of targets with both wt (Ago2 and Ago1) and mutant (Ago2D669A) miRISCs by approximately 10-fold. This effect was independent of the orientation of MBSb and HBS sites, and, most significantly, was not observed when non-oligomerizing HuR mutant, HuRΔH3, was used instead of the wt protein (Fig. 25B). We note that efficiency of miRISC displacement from HBS_50_MBSb was much higher from that seen with HBS_50_MBSp, containing the perfectly complementary let-7 site.

Taken together, the presented results indicate that HuR can displace the miRISC from substrates containing both perfectly complementary and "bulged" miRNA sites and that a potential of HuR to oligomerize along RNA is important for this effect.

5 DISCUSSION

We showed previously that CAT-1 mRNA and also reporter mRNAs bearing AREs in their 3'UTR can be relieved of miRNA-mediated repression in mammalian cells subjected to various stress conditions. The derepression required the binding of the ELAV family protein HuR to the 3'-UTR AREs (Bhattacharyya *et al*, 2006). This raised questions about the mechanism of HuR action and that of other possibly stress-induced factors that may participate in the process. In this present study, we show that relief of miRNA-mediated repression involving HuR can be uncoupled from stress and that HuR appears to be sufficient to execute derepression upon binding to the mRNA ARE. Using an in vitro system with recombinant miRISC and HuR and its mutants, we show that HuR, by its property to oligomerize along RNA, leads to the displacement of miRISC from target RNA. Further, we show that HuR association with AREs can inhibit RISC-mediated endonucleolytic cleavage of target RNAs both *in vivo* and *in vitro*, and also miRNA-mediated deadenylation of RNA in the Krebs-2 ascites extract.

Several approaches demonstrated that the HuR effect can be uncoupled from stress: (1) an HuR mutant accumulating in the cytoplasm in the absence of stress led to the alleviation of miRNA-mediated repression of both endogenous CAT-1 mRNA and ectopically expressed reporters upon its expression in cells. (2) In breast cancer MDA-MB-231 cells, in which HuR accumulates in the cytoplasm in the absence of stress, miRNA repression of ARE-containing mRNAs was strongly compromised in a process dependent on HuR. (3) MS-444, a specific inhibitor of HuR, eliminated much of the ARE-dependent suppressive effect of HuR on miRNA repression when added to MDA-MB-231 cells, and this effect of MS-444 was similar to that of the siRNA-mediated knock-down of HuR (Figs. 7C and 9C). (4) Recombinant HuR interfered with the inhibitory function of let-7 miRNA in the *in vitro* system recapitulating miRNA-mediated deadenylation (Fig. 22). (5) Finally, other *in vitro* experiments with purified components unequivocally showed that HuR can exert its derepressive effect independent of stress signals (Figs. 20, 23,

24, and 25). The experiments listed above also provided strong evidence that HuR alone, either when present in the cytoplasm or added to the reaction *in vitro*, is sufficient to interfere with the RISC-induced repression. However, our results do not exclude the possibility that further factors can assist HuR in its activity in a cellular context.

Interestingly, we found that binding of HuR to the mRNA 3'UTR can also reduce repression occurring via the RNAi mechanism, when induced by RISC targeting either a perfectly complementary let-7 site in the 3'UTR (Figs. 15 and 16) or the site in the coding region responding to the exogenously supplied siRNA (Fig. 17). As in the case of reporters containing bulged miRNA sites, the effect on RNAi required the cytoplasmic presence of HuR, as indicated by its response to cellular stress (Fig. 15B) or to the expression of the HuR mutant that accumulates in the cytoplasm (Fig. 15C) or to the cytoplasmic abundance of HuR protein in MDA-MB-231 cells (Fig. 15D); the effect also depended on the presence of an HuR-binding ARE in the 3'UTR. The effect of HuR on RNA targets bearing a perfectly complementary miRNA site was also observed in the *in vitro* system involving the use of recombinant components. In marked contrast to the alleviating effect of HuR on RNAi-induced repression, HuR did not interfere with inhibition induced by direct tethering of Ago2 to mRNA in transfected cells (Fig. 14B).

The findings that HuR alleviates repression in reactions depending on base-pairing of small RNAs to mRNA but not when Ago2 is directly tethered to mRNA suggested that HuR-induced interference is unlikely to target steps in the miRNA/RNAi pathway that are downstream of the miRISC binding. HuR more likely compromises miRISC association with mRNA, leading to displacement of miRISC from the target. The latter possibility is also supported by the results of Ago and HuR IP experiments, which indicated that association of HuR and miRISC with the investigated target mRNAs is largely mutually exclusive (Fig. 12). Moreover, we found no evidence that Ago or GW182 proteins interact with HuR (Figs. 10 and 11). We note, however, that some authors have reported that HuR, either endogenous (Kim et al, 2009a)

or overexpressed (Hock et al, 2007), interacts in an RNA-dependent manner with Ago2. In contrast, others did not detect HuR-Ago2 interaction even in the absence of RNase treatment (Jing et al, 2005) or did not find HuR among proteins identified by mass spectrometry as associating with tagged Ago1, Ago2, or three human GW182 proteins, but found it in complexes with Ago3 and Ago4 (Landthaler et al, 2008).

The notion that binding of HuR causes displacement of miRISC from RNA bearing both HuR and miRNA sites was confirmed by *in vitro* experiments performed with purified bacterially-expressed HuR and let-7-enriched recombinant miRISC. The experiment using the *in vitro* cleavage assay revealed that addition of HuR even after miRISC has been pre-bound to RNA, results in a concentration-dependent dislodging of miRISC from the target RNA (Fig. 23). The immunoprecipiation experiments showed that this effect occurred irrespective of whether miRISC was base-paired with target RNA by perfect or imperfect complementarity (Figs. 24 and 25), although the HuR effect was much more pronounced for substrates associating with miRISC through an imperfect, central bulge-forming interaction. This is likely due to the perfect complementarity exerting stronger association with target RNA compared to that mediated by imperfect base-pairing.

The observation that addition of HuR could interfere with miRISC activity irrespective of whether the miRISC site was located 20- or 50-nt away from the HuR-binding region suggested that the HuR effect is unlikely to be caused by direct sterical occlusion of the miRISC site by the protein. Since HuR and related proteins such as HuD and *Drosophila* ELAV are known to oligomerize along RNA substrates (Devaux et al, 2006; Fialcowitz-White et al, 2007; Gao & Keene, 1996; Kasashima et al, 2002; Soller & White, 2005; Toba & White, 2008), we investigated whether this property of HuR plays a role in the suppressive effect of HuR on miRISC activity. Two types of experiments provided strong support for the multimerization of HuR being important: (1) only full-length HuR and its mutants HuRΔH and HuRΔ3, all able to multimerize, inhibited the miRISC-mediated cleavage of RNA. In contrast, an

HuR mutant (HuRΔH3) that is devoid of a hinge and RRM3 and that binds target RNA but is impaired in multimerization did not interfere with miRISC activity (Figs. 21C, 21D, and 21E). (2) In addition, pre-hybridization of target RNA with a 50-mer oligodeoxynucleotide complementary to the spacer separating HBS and MBS sequences, which effectively blocks HuR oligomerization, wholly eliminated its inhibitory effect on RNA cleavage by miRISC (Figs. 21F and 21G). However, a long RNA-DNA helix formed with a 50-mer oligonucleotide puts HBS and MBS sites of the target RNA approximately 50Å apart what likely prevents any contact between HuR and miRISC. Hence, inactivity of HuR to derepress miRNA-mediated cleavage of the hybrid, may be partially also explained by inability of HuR to contact (even transiently) the miRISC. HuR has recently been reported to have a RNA 3'-terminal adenosyl transferase activity, residing in RRM3 (Meisner et al, 2009). However, the positive effect of HuR RRM3 deletion mutants in preventing cleavage and deadenylation of let-7 targeted RNAs suggest this enzymatic activity is unlikely to contribute to the derepressive property of HuR.

Regulation of miRNA repression by RBPs interacting with the 3'UTR is probably a widespread phenomenon (reviewed by Fabian *et al.* 2010). Apart from the study of Bhattacharyya et al, till date, several other cases of miRNA-RBP interplay on the target RNA have been reported. Dead end (Dnd1), an RBP expressed in germ cells of zebrafish and mammals, prevents miRNA repression by binding to sequence elements in the 3'UTR that overlap with miRNA sites, thus effectively blocking miRNA access to target mRNA (Kedde et al, 2007). Similarly, hnRNP protein L, which translocates from the nucleus to the cytoplasm during hypoxia, competes with miRNA for binding to the same CA-rich elements present in the 3'UTR of VEGFA mRNA (Jafarifar et al, 2011). In contrast, Pumilio 1 (PUM1), a ubiquitously-expressed RBP, was found to facilitate miRNA repression. Binding of PUM1 to the p27 mRNA 3'UTR induces a local change in RNA structure that favors association with specific miRNAs (Kedde et al, 2010). Furthermore, a comparative study of mRNAs interacting with Pumilio (PUF) proteins showed a considerable

enrichment of PUF-binding sites in the vicinity of predicted miRNA recognition sequences in human mRNAs (Galgano et al, 2008), suggesting that PUF-miRNA cross-talk is a common event.

In addition to the examples discussed above, further cases of a cross-talk between RBPs and miRNAs have been recently reported (Huang et al, 2007; Nolde et al, 2007), although their mechanisms are unknown. Moreover, in colon cancer cell lines, the insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) was found to bind the coding region of the β-transducin repeat-containing protein 1 (βTrCP1) mRNA, preventing its degradation by alleviating the association of miR-183-RISC with its target site, also residing in the coding region (Elcheva et al, 2009). Additionally, IGF2BP1 was shown to target the 3'UTR of microphthalmia-induced transcription factor (MITF) in melanoma cells to prevent miR-340-induced repression of MITF (Goswami et al, 2010).
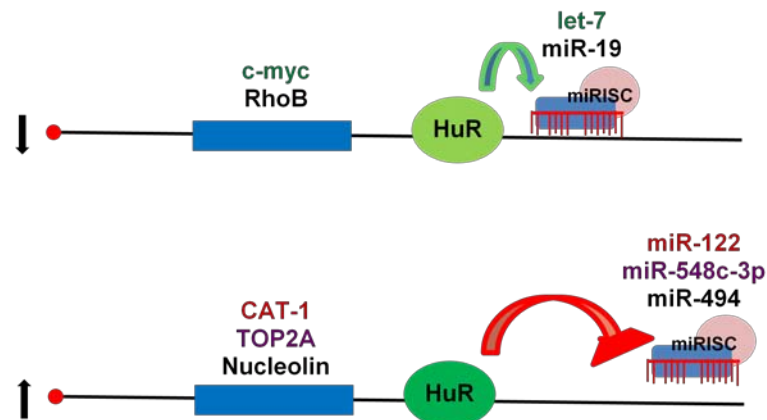
Figure G HuR-mediated regulation of miRNA repression. The miRNA is drawn linearly for simplicity. (Upper panel) HuR acts as a positive modulator of the miRNA function resulting in the inhibition of mRNAs. (Lower panel) HuR acts as a negative modulator of the miRNA function resulting in the upregulation of mRNAs. The miRNA and its corresponding target are shown in the same colours.

Interestingly, in all of the mentioned examples of RBP-mediated modulation of miRNA function, the RBP binding site on the target was positioned in close proximity of the miRNA binding site (~10-20 nt apart) except the case of CAT-1 derepression, which occurred even when AREs were several hundreds of bases apart from the miRNA sites (~263 nt). Recently, two studies involving HuR have demonstrated that the protein imparts a positive effect on miRNA repression rather than alleviating it (Fig. G, upper panel). In both cases, the miRISC access to the target RNA was shown to be dependent on the presence of HuR. This enhancing effect of HuR on c-Myc repression by let-7 (Kim *et al*, 2009a) and Ras homolog B (RhoB) repression by miR-19 (Glorian et al, 2011) was shown to occur in non-stressed HeLa and HaCaT cells, respectively. In later study, the UV exposure resulted in the loss of the interdependent binding between HuR and miR-19 to the RhoB mRNA, leading to the relief of mRNA repression. Interestingly, in both these studies, like in the majority of cases, HuR binding site on the target RNA was present close to the miRNA binding site. This is not surprising since recent transcriptome-wide analysis of HuR targets found that miRNA sites are often enriched near the HuR sites in mRNAs (Lebedeva et al, 2011; Mukherjee et al, 2011) or even sometimes overlapping with them (Lebedeva et al, 2011; Uren et al, 2011). The positive effect of HuR on the miRISC repression was explained by a possibility of HuR modifying local RNA structure and increasing the accessibility of miRNA-binding sites, as was shown for PUM and p27 mRNA (Kedde *et al*, 2010). Intersetingly, two new studies in HeLa cells from the Gorospe laboratory have demonstarted opposite results, showing that HuR antagonizes miR-548c-3p-mediated repression of topoisomerase IIα (TOP2A) (Srikantan et al, 2011) and miR-494-mediated repression of nucleolin at the level of translation. Interestingly, like in the case of CAT-1, the main interaction site of HuR and co-regulated miRNA sites on the target RNA in both examples were positioned at a considerable distance (Fig. G, lower panel). One of the genome-wide studies on HuR targets demonstrated that depletion of miRNA in non-stressed HeLa cells resulted in significantly less upregulation of the transcripts containing

overlapping (<10 nt apart) miRNA and HuR sites compared to the messages of no-overlapping class (Mukherjee et al, 2011), suggesting that the competition between HuR and miRNA to access the target site could prevent the repression. However, this does not explain the effect observed in the case of CAT-1, TOP2A or nucleolin.
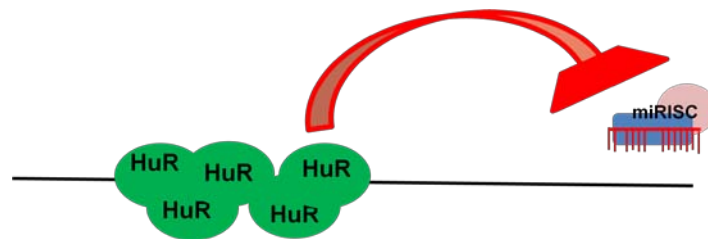
Figure H The property of HuR to oligomerize along the RNA is important for its function to alleviate miRISC-mediated inhibition.

The HuR oligomerization-dependent relief of miRISC repression in the model transcripts, described in this study represents a possible mechanism of miRNA regulation, differing from other examples (Fig. H). However, careful detailed analysis of RNA structure and folding is required to gain more insight about different individual mechanism. One should also consider the fact that the RBP-RNA interactions are highly dynamic and the rates of binding and release of both miRNP and RBP can also influence the outcome of the interaction in a given cellular condition. It will be important to investigate which factors determine the outcome, either negative or positive, of the HuR effect on miRNA repression. Activity of HuR is known to be controlled by various post-translational modifications and proteolytic cleavages, and also by interaction with protein ligands (as mentioned in Introduction and reviewed by Meisner & Filipowicz, 2011). Hence, it is perhaps not be surprising to find that the protein contributes to the miRNA regulation in many different ways.

6 MATERIALS AND METHODS

6.1 Plasmids

Plasmids p-FL, pRL-Con, pRL-3xBulge, pRL-Per, NHA-LacZ, NHA-Ago2, NHA-Ago3, HA-Ago2, and pRL-5BoxB were previously described (Pillai et al, 2005). Construction of plasmids pRL-catA, pRL-catB, pRL-catAΔARD, pRL-ARD, and pRL-3xB-ARD was described in Bhattacharyya *et al*, 2006. To obtain pRL-5BoxB-ARD and pRL-Per-ARD, the ARD region of the 3'UTR of human CAT-1 mRNA (Bhattacharyya et al, 2006) was inserted to pRL-5BoxB and pRL-Per in the *Not*I site positioned downstream of let-7 and 5BoxB sites, respectively. For construction of pRL-p53a and pRL-3xB-p53a, oligonucleotides encoding an AU-rich region "a" of the p53 mRNA 3' UTR (Bhattacharyya et al, 2006) were cloned in pRL-Con and pRL-3xBulge respectively, linearized with *Not*I.

To obtain mammalian expression vectors encoding Myc-tagged full length HuR and its deletion mutants [HuRΔH: hinge region (H) (amino acids 186-242) deleted; HuRΔ3: RRM3 (amino acids 243-326) deleted; and HuRΔH3: both hinge and RRM3 (amino acids 186-326) deleted], appropriate regions were PCR-amplified from plasmids kindly provided by A. B. Shyu (Chen et al, 2002) and were subcloned between *Nhe*I and *Not*I sites of pRL-Con following removal of the RL ORF. For expression of HuR and mutant proteins in *E. coli*, the inserts were cloned in pET42a(+) (Novagen) between *Nde*I and *Xho*I sites for His-tag purification. NHA-TNRC6B was described before (Zipprich et al, 2009).

Plasmids expressing FLAG-HA-Ago1, FLAG-HA-Ago2 and the FLAG-HA tagging vector were obtained from G. Meister (Meister et al, 2004). Myc-Ago2 and Ago2D669A and H634A mutant clones were generously provided by G. Hannon (Liu et al, 2004). Full length cDNAs encoding mutant Ago2 were amplified from Myc-Ago2 plasmids and subcloned into FLAG-HA tagging vector between *Not*I and *Eco*RI sites. Primers for amplification are presented in Table I. pLET7a encoding pri-let-7a was kindly provided by J. Belasco (Wu et al, 2006).

Plasmids used in the synthesis of RNAs tested in deadenylation assays were prepared by inserting the ARD region in the NotI site downstream of 6xB and 6xBMut of RL-6xB and RL-6xBMut (previously described in Mathonnet et al, 2007) to generate RL-6xB-ARD and RL-6xBMut-ARD respectively.

Table I Primers used for construction of plasmids. F and R denote forward and reverse primers, respectively. Restriction sites present in primers are underlined.

Primer No.	Primer Name	Restriction Site	5'→3' Sequence
1	ARD_F	*Not*1	G*GCGGCCGC*GGGAGCTTTATTCTG
2	ARD_R	*Not*1	G*GCGGCCGC*GCTGTCTTGCACGG
3	Ago2_F	*Not*I	CT*GCGGCCGC*ATGTACTCGGGAGCCGGCCC
4	Ago2_R	*Eco*RI	CT*GAATTC*TCAAGCAAAGTACATGGTGCGC
5	myc-HuR_F	*Nhe*I	CGC*GCTAGC*TTGCCACCATGGAGC
6	myc-HuR_R	*Not*I	CGC*GCGGCCGC*AGATCCTAGAGATAC
7	myc-HuRΔ3_R	*Not*I	GCA*GCGGCCGC*GGAGGAGGCGTTTCCTGGC
8	myc-HuRΔH3_R	*Not*I	GCA*GCGGCCGC*TTAGGCTGCAAACTTCACTGTG
9	His-HuR_F	*Nde*I	GATCG*CATATG*TCTAATGGTTATGAAGACC
10	His-HuR_R	*Xho*I	GATCG*CTCGAG*TTTGTGGGACTTGTTGGTTTTG
11	His-HuRΔH_R	*Xho*I	GATCG*CTCGAG*TTTGTGGGACTTGTTGGTTTTG
12	His-HuRΔ3_R	*Xho*I	GATCG*CTCGAG*GGAGGAGGCGTTTCCTGGC
13	His-HuRΔH3_R	*Xho*I	GATCG*CTCGAG*GGCTGCAAACTTCACTGTGATG

6.2 Cell Culture-based assays

6.2.1 Cell culture

Huh7, HEK293, HEK293T, HeLa S3 and MDA-MB-231 cells were grown in monolayers in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 unit/ml penicillin, and 100 µg/ml streptomycin at 37°C in humidified atmosphere containing 5% CO_2. Cells were split in a 1:5 ratio every second day by trypsinization.

6.2.2 Transfection

Cells at high confluency (95%), cultured in DMEM without antibiotics supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's manual. All further assays were carried out 48 h after transfection, with splitting of cells and change of medium at 24 h.

Amounts of transfected plasmids per well of 6-well plates were: 20 ng of indicated RL reporters and 500 ng of FL control reporter for HeLa, Huh7 and MDA-MB231 cells, and 200 ng for HEK293 cells. For expression of epitope-tagged HuR, Ago and TNRC6B proteins, 200 ng of plasmids were used per transfection. For miRNA inhibition, the 2'-*O*-methyl anti-sense oligonucleotides were used at 100 nM concentration. siRNAs were used at 100 nM concentration. When both reporter plasmids and siRNA (or 2'-*O*-methyl oligonucleotide) were used, they were transfected at the same time, 48 hrs before analysis. For sequence information of siRNAs and oligonucleotides, see Table II. AllStars Negative Control siRNA (Qiagen) was used as control siRNA (siCon).

Table II Sequence of 2'-*O*-methyl oligonucleotides and siRNAs

oligonucleotide	Name	Sequence (5'→3')
2'-*O*-methyl	let-7a	UCUUCACUAUACAACCUACUACCUCAACCUU
	miR-122	UCUUCACAAACACCAUUGUCACACUCCAACCUU
siRNA	HuR_sense strand	CGGUUUGCUUCCAAAUGAATT
	HuR_antisense strand	UUCAUUUGGAAGCAAACCGGG
	RL_sense strand	GCGAGAUCCCUCUCGUUAATT
	RL_antisense strand	UUAACGAGAGGGAUCUCGCGG

6.2.3 Stress Induction

For stress induction experiments, cells were grown to 40-50% confluency. For amino acid starvation cells were grown for 2 h in DMEM without amino acids and supplemented with 10% heat-inactivated FCS dialyzed against PBS (Phosphate-buffered saline). For induction of the endoplasmic reticulum (ER) stress, thapsigargin (Calbiochem; dissolved in DMSO, dimethyl sulphoxide) was added at 200 nM final concentration to the existing medium and the cells were grown for 2 h. Stress induction was monitored by measuring the level of eIF2α phosphorylation and relocalization of HuR to cytoplasm, measured by either immunofluorescence (IF) or cell extract fractionation. Cells at early passages were used and special attention was paid to eliminate cell cultures showing signs of stress due to handling or transfection.

6.2.4 Luciferase assay

RL and FL activities were measured using Dual-Luciferase Assay Kit (Promega). Cells were lyzed in 1X Passive Lysis Buffer supplied with the kit and fluorescence was measured using the appropriate substrates. To avoid measurement problems, samples were diluted such that the reading for Renilla (RL) was in the range of 10^6 and the one for Firefly (FL) around 10^4. The luciferase activity was measured in 96 well plates by the LB960 Centro Microplate Luminometer (Berthold Technologies).

6.3 Immunoflurescence analysis

Immunoflurescence (IF) analyses were performed as described in Bhattacharyya et al., 2006. Briefly, cells grown in gelatin-coated glass cover slips (autoclaved in a 0.5% gelatin solution) in 24-well plates, were fixed with PBS containg 4% (v/v) paraformaldehyde for 30 min at room temperature. Then, the cells were permeabilized by incubating with PBS containing 5% goat serum, 1% BSA and 0.1% Triton X-100 for 30 min at room temperature followed by 2-3 h incubation with the appropriate dilution of the primary antibody in PBS containing 5% FCS. After washing three times for 5 min each in PBS, secondary antibodies were applied in 1:500 dilution for 1 h at room temperature in dark. Cover slips were mounted with Vectrashield containing DAPI (Reactolab). For detection of expressed Myc-tagged proteins, mouse 9E10 (Santa Cruz) mAb was used at 1:100 dilutions, and to visualize P-bodies rabbit α-RCK/p54 (DDX6) (Bethyl) was used at 1:500 dilution. α-mouse or α-rabbit Abs with Alexa 488 (green) or Alexa 594 (red) fluorochrome (Invitrogen) were used as secondary antibodies. Images were taken using Zeiss Z1 fluorescence microscope using 60X or 40X objectives.

6.4 Cellular fractionation

Cellular fractionation of MDA-MB-231and HEK293 cells was performed as described previously by Pillai et al, 2005. Briefly cells were lysed by incubating with buffer containing 10 mM tris HCl, pH 7.4, 250 mM sucrose, 25 mM KCl, 5

mM MgCl$_2$, 1 mM DTT, 1x EDTA-free protease inhibitor cocktail (Roche), 40U/ml RNasin and 50 µg/ml digitonin for 15 min on ice with occasional shaking (without vortexing) and were rapidly centrifuged at 1000xg for 5 min at 4°C. The resulting pellet was resuspended in the same buffer but containing no digitonin and washed by centrifugation once at 1,000xg to get the nuclear fraction, which was suspended in 1x Passive lysis buffer supplied with the Dual-Luciferase Assay Kit (Promega). Supernatant was clarified by centrifugation at 15,000xg for 10 min and saved as cytosolic fractions. For analysis, 20 µg of protein from each fraction was applied to SDS-PAGE.

6.5 Western analysis

Western blot analyses of CAT-1 were performed as previously described (Rotmann et al, 2004). Briefly, cells were lysed with buffer (100 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitors (complete; Roche Applied Science) for 1 h at 4 °C. After removal of the cellular debris protein concentration of the lysates was determined using the Bradford reaction followed by treatment with Peptide N-glycosidase F (PGNase F, Roche Applied Science; 2 units/100 µg protein, 30 min at 37 °C). An affinity-purified polyclonal antibody raised against the C-terminus of CAT-1 (Gräf et al., 2001) was used at 1:100 dilution.

For analysis of other proteins, cells were lyzed as decribed under either "cellular fractionation" or "luciferase assay". Mouse α-HuR mAb 3A2 (Santa Cruz), rabbit α-eIF2α (Santa Cruz) and rabbit α-eIF2α-P (Cell Signal Technology) Abs were used at 1:500 dilutions. Mouse anti-β-tubulin mAb (B-5.1.2, Sigma) was used at 1:8,000 dilution. Rat α-HA (3F10, Roche), mouse α-Myc (9E10, Santa Cruz) and α-GFP (Roche) mAbs were used at 1:1,000 dilution. HRP-conjugated secondary α-mouse, α-rabbit (GE Healthcare) and α-rat (MP Biochemicals) Abs were used at 1:10,000 dilutions.

6.6 Analysis by Immunoprecipitation and RT- PCR

For immunoprecipitation (IP) reactions performed with cell extracts, cells were lyzed in IP buffer [25 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% Triton X-100, 40 U/ml RNasin (Promega) and 1x EDTA-free protease inhibitor cocktail (Roche)] for 15 min on ice. Supernatants recovered after centrifugation at 14,000xg were used for IP. They were pre-cleared by incubation with pre-blocked [with 100 µg/ml of bovine serum albumine (BSA) in IP buffer] recombinant Protein G Agarose beads (Invitrogen) for 1 h prior to IP reactions. Mouse α-HuR (3A2, Santa Cruz), α-HA (3F10, Roche), α-Myc (9E10, Santa Cruz) or α-GFP (Roche) mAbs at 1:50 dilution, were used for IP reactions. The α-Ago2 mAb [2A8, kindly provided by Z. Mourelatos (Nelson et al, 2007)] was used in 1:20 dilution. Protein G Agarose beads bound to Abs (one hundredth of the lysate volume), were added to the lysates and IP reaction was carried out for 4 h at 4°C. The beads were centrifuged down, washed three times with IP buffer containing 200 mM KCl and used for either Western blotting or RNA extraction.

For analysis of cellular mRNAs, RNA was extracted from IPs with the RNAeasy kit (Qiagen) and re-suspended in 30 µl of RNase-free water. Following consecutive treatments with RNase-free DNase (Promega) and Proteinase K (Promega), RNA was extracted and reverse transcribed, using gene-specific primers and 50 U of Superscript III RT (Invitrogen). One fifth of the reverse transcription (RT) reaction was then used for 25 cycles of PCR amplification with gene-specific primers. The gene-specific RT primers and reverse PCR primers were: CCGCTCTAGAATTACTGCTCGTTC (RL), and CGGAATTCGCAAGGTGAGCAG (CAT-1). Sequences of the forward PCR primers for amplification of RL and CAT-1 were GGTTAGACGGCCTACC and GGCGGCCGCGGGAGCTTTATTCTG, respectively. For PCR analysis, it was ascertained that number of PCR cycles used (25 for all amplifications) is within a linear range of amplification. RNA from the α-Ago2 IP material isolated from cells not subjected to stress was used at 1:1 or 1:10 dilution for RL and CAT-1 amplification at different number of PCR cycles to ensure the linearity of amplification at 25 cycles.

6.7 RNA synthesis and labeling for cleavage assay and EMSA

6.7.1 Generation of templates for in vitro transcription

Templates for generation of HBS_20_MBSp, ΔHBS_20_MBSp and HBSMut_20_MBSp target RNAs were prepared by annealing synthetic antisense DNA oligonucleotides (Microsynth; for sequences, see Table III) to the T7 promoter sense oligonucleotode (T7s; Table III). Mixture of oligonucleotides, each 50 µM, was heated at 95°C for 3 min in 1x annealing buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA) and then allowed to slowly cool down to room temperature.

For synthesis of HBS_50_MBSp, HBS_50_MBSb, and MBSb_50_HBS target RNAs, double-stranded DNA templates were used. The templates were prepared by annealing equimolar amounts of two oligonucleotides (T7s and 5'-phosphorylated sense; Table III), forming a sense strand with two oligonucleotides (Reverse and 5'-phosphorylated antisense; Table III) forming an antisense (template) strand, followed by ligation by T4 DNA ligase (NEB). The resulting double-stranded products were used as templates for PCR amplification with T7s and reverse oligonucleotides as primers, gel-purified, and used as templates for in vitro transcription.

Table III DNA oligonucleotides used for construction of templates for in vitro transcription

Target RNA	DNA oligomer	Sequence of DNA Oligonucleotides (5'→3')
	T7s	TTAATACGACTCACTATAGGG
HBS_20_MBSp	antisense	AGTGTAACGTTGAGGTAGTAGGTTGTATAGTCAATATCACTCTTTGGAAATAATAAAACAAACAAACAAATAAATAAATAAATACCCTATAGTGAGTCGTATTAA
ΔHBS_20_MBSp	antisense	AGTGTAACGTTGAGGTAGTAGGTTGTATAGTCAGTCTCACTCTTTGGACCTCCCTATAGTGAGTCGTATTAA
HBSMut_20_MBSp	antisense	AGTGTAACGTTGAGGTAGTAGGTTGTATAGTCAGTCTCACTCTTTGGACCTAGTAGATACGCACATACGTATGCATGACATATATACCCTATAGTGAGTCGTATTAA
HBS_50_MBSp	sense	TATTTATTTATTTATTTGTTTGTTTGTTTTATTGCTGTACGAGAGAGTCACTGTGCTGATGTGGACTCAACATCTGATGAGCAACTATACAACCTACTACCTCAACGTTACACT
	antisense	GTTGTATAGTTGCTCATCAGATGTTGAGTCCACATCAGCACAGTGACTCTCTCGTACAGCAATAAAACAAACAAACAAATAAATAAATAAATACCCTATAGTGAGTCGTATTAA

	reverse	AGTGTAACGTTGAGGTAGTAG
HBS_50_MBSb	sense	TATTTATTTATTTATTTGTTTGTTTGTTTTATTGCTGTACGAGAGAGTCACTGTGCTGATGTGGACTCAACATCTGATGAGCAACTGCACAGCCTATTGAACTACCTCAACGTTACACT
	antisense	AATAGGCTGTGCAGTTGCTCATCAGATGTTGAGTCCACATCAGCACAGTGACTCTCTCGTACAGCAATAAAACAAACAAACAAATAAATAAATAAATACCCTATAGTGAGTCGTATTAA
	reverse	AGTGTAACGTTGAGGTAGTTC
MBSb_50_HBS	sense	GGTCCAAAGAGTGACTGCACAGCCTATTGAACTACCTCAGCTGTACGAGAGAGTCACTGTGCTGATGTGGACTCAACATCTGATGAGCATATTTATTTATTTATTTGTTTGTTTGTTTTATTAC
	antisense	TAAATAAATATGCTCATCAGATGTTGAGTCCACATCAGCACAGTGACTCTCTCGTACAGCTGAGGTAGTTCAATAGGCTGTGCAGTCACTCTTTGGACCCTATAGTGAGTCGTATTAA
	reverse	GTAACGTAATAAAACAAACAAACAAATAAA

6.7.2 In vitro transcription

Transcription reactions (50 µl), containing 1x Transcription Buffer (Promega), 500 µM each ATP, CTP, and UTP, 200 µM GTP, 3.5 mM guanosine, 1.25 mM MgCl$_2$, 2.5 mM DTT, 40 U of RNase Out (Invitrogen), and 300 units of T7 RNA polymerase (Epicenter Biotechnologies), were performed at 37°C for 3 h. Inclusion of excess of guanosine, compared to GTP, resulted in its incorporation at the RNA 5' end, thus making the 5'-end dephosphorylation step unnecessary (Krol et al, 2004). Sequences of RNA transcripts are shown in Table IV. The transcripts were analyzed by 10% polyacrylamide/8M urea PAGE, excised, eluted from the gel with 0.3 M sodium acetate (pH 5.2), 0.5 mM EDTA, and 0.1% SDS, precipitated, dissolved in water and stored at -80°C.

RNAs MBSp and MutMBS were purchased from Dharmacon.

Table IV Sequences of target RNAs. HBS is underlined and MBS is shown in italics. Nucleotides modified in MBS of MBSMut are underlined. Nucleotides modified in HBS of HBSmut_20_MBSp are in bold.

Target RNA	Sequence (5'→3')
MBSp	GUAUCAACC*ACUAUACAACCUACUACCUCA*ACGUUCAAU
MutMBSp	GUAUCAACC*GAAAGACAGUAGAUUGUAUAG*ACGUUCAAU

HBS_20_MBSp	GG<u>UAUUUAUUUAUUUAUUUGUUUGUUUGUUUUAUU</u>AUUUCCAAAGAGUG AUAUUG*ACUAUACAACCUACUACCUCA*ACGUUACACU
ΔHBS_20_MBSp	GGAGGUCCAAAGAGUGAGACUG*ACUAUACAACCUACUACCUCA*ACGUUA CACU
HBSMut_20_MBSp	GGUAU**A**UAU**GUC**AU**GC**AU**AC**GUA**U**GU**GC**GU**AU**C**U**AC**U**AGGUCCAAAGAG UGAGACUG*ACUAUACAACCUACUACCUCA*ACGUUACACU
HBS_50_MBSp	GG<u>UAUUUAUUUAUUUAUUUGUUUGUUUGUUUUAUU</u>GCUGUACGAGAGAG UCACUGUGCUGAUGUGGACUCAACAUCUGAUGAGCA*ACUAUACAACCUA CUACCUCA*ACGUUACACU
HBS_50_MBSb	GG<u>UAUUUAUUUAUUUAUUUGUUUGUUUGUUUUAUU</u>GCUGUACGAGAGAG UCACUGUGCUGAUGUGGACUCAACAUCUGAUGAGCA*ACUGCACAGCCUA UUGAACUACCUCA*ACGUUACACU
MBSb_50_HBS	GGUCCAAAGAGUG*ACUGCACAGCCUAUUGAACUACCUCA*GCUGUACGAG AGAGUCACUGUGCUGAUGUGGACUCAACAUCUGAUGAGCA<u>UAUUUAUUU AUUUAUUUGUUUGUUUGUUUUAUU</u>ACGUUAC

6.7.3 5'-end labeling

When indicated, the transcripts were 5'-end-labeled, using T4 polynucleotide kinase (NEB) and [γ-^{32}P] ATP (5,000 Ci/mmol; Hartmann Analytic) according to manufacturer's protocol. Following labeling, transcripts were subjected to column purification (Micro Bio-Spin 30 Columns, RNase-free; Bio-Rad Laboratories), precipitated and stored at -20°C in water before use.

6.8 Annealing of DNA oligonucleotide to labeled RNA

For annealing of HBS_50_MBSp RNA to a 50-mer oligodeoxynucleotide, they were mixed at the 1:1.2 ratio, heated at 95°C for 3 min in 1x annealing buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) followed by slow cooling to room temperature. Sequence of the 50-mer (complementary to the entire spacer) is TGCTCATCAGATGTTGAGTCCACATCAGCACAGTGACTCTCTCGTACAGC

6.9 Purification of miRISC

MiRISC complexes, preferentially loaded with let-7a miRNA, were purified from HEK293T cells (grown in 10 cm dish) doubly-transfected with plasmids (5 µg each) expressing pri-let-7a and indicated FLAG-HA-tagged Ago proteins or their mutants. 48 h after transfection, cells were lyzed in 1 ml buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.5%

Triton X-100. After centrifugation at 14,000xg the supernatant was used for IP purification with 40 µl anti-FLAG M2 Affinity Gel (Sigma). The miRISC complex was eluted with 3x FLAG peptide (Sigma), following the manufacturer's protocol.

6.10 Purification of His$_6$-HuR fusion proteins

6.10.1 Growth of bacterial culture and induction

1 ml of LB broth (containing 25 µg/ml chloramphenicol and 50 µg/ml kanamycin) inoculated with a single colony of BL21-CodonPlus® (DE3)-RIPL cells (Stratagene) harbouring the expression plasmid were grown at 37°C overnight. Next day, fresh 200 ml LB medium (containing 50 µg/µl kanamycin) was inoculated with the overnight grown culture in 1:200 ratios by volume, incubated at 37°C with shaking till the A$_{600}$ rise to 0.3-0.4. The culture was induced with IPTG (0.1 mM) and grown overnight with shaking at 18°C.

6.10.2 Lysis of cells

The culture was pelleted down by centrifuging at 3,000xg for 15 min at 4°C and cells were dissolved in 10 ml of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 300 mM KCl, 2 mM MgCl$_2$, 5 mM β-mercaptoethanol (β-me), 50 mM imidazole, 0.5% Triton X-100, 5% glycerol, 0.5 mg/ml lysozyme, 1x EDTA-free Protease Inhibitor Cocktail (Roche), followed by sonication and centrifugation at 16,000 g for 30 minutes at 4°C.

6.10.3 Protein purification

The supernatant was incubated with Ni-NTA Agarose beads (Qiagen) for 2 h at 4°C. After washing the beads with buffer A (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 2 mM MgCl$_2$, 5 mM β-me, 100 mM imidazole, 0.5% Triton X-100, 2.5% glycerol) and buffer B (as buffer A but containing 250 mM imidazole), each for 15 min at 4°C, protein was eluted with buffer C (as buffer A but containing 500 mM imidazole, 5% glycerol, and 1x EDTA-free Protease Inhibitor Cocktail (Roche). The eluted protein was dialyzed against storage buffer (20 mM Tris-HCl, pH 7.5, 200 mM KCl, 2 mM MgCl$_2$, 1 mM DTT, 0.1%

Triton X-100, 5% glycerol, and EDTA-free Protease Inhibitor Cocktail) in cellulose membrane (MW cut off: 12-14 KD).

Protein concentration was measured using the Bradford assay. Purity of proteins was analyzed in coomassie blue-stained 12% SDS-polyacrylamide gel. The purified protein was kept in small aliquots, frozen in liquid nitrogen and stored at -80°C.

6.11 Target RNA cleavage assay

The reactions (10 µl), containing 1x cleavage buffer (20 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM MgCl$_2$, 0.01% Triton X-100, 5% glycerol, 1 mM DTT), 0.1 µg yeast tRNA, purified miRISC (~0.2 nM), 5'-end labeled target RNA (0.1 nM), and increasing amounts of indicated purified HuR proteins, were incubated for 15 min at 30°C. Reactions were stopped by adding urea stop dye (8 M urea, 50 mM EDTA, 0.04% Bromophenol Blue, 0.04% Xylene Cyanol), followed by heating at 95°C for 2 min. The products were analyzed by 10%-8M urea PAGE. The gel was dried and reaction-products were quantified using a PhosphorImager (Molecular Dynamics).

For analysis of the effect of HuR on cleavage of target RNA containing pre-bound miRISC, the 1x cleavage buffer initially contained 5 mM EDTA in place of MgCl$_2$. At the indicated time point, MgCl$_2$ was added to final concentration of 5 mM to allow cleavage to take place.

6.11.1 Determination of active miRISC concentration
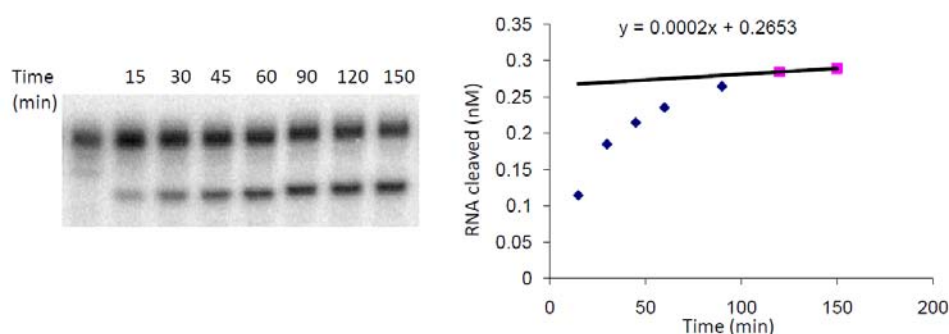
Concentration of active miRISC (Ago2) was determined as described in Ameres et al. The cleavage of excess of target RNA (1nM) was quantified in a

time course (upper panel), the amount of cleaved product (y-axis) was plotted against time (x-axis) (lower panel) and slower steady-state cleavage rate line was extrapolated back to y-axis. The y-intercept at the zero time point denoted the amount of active RISC. In the presented example, it is ~0.27 nM.

6.11.2 Quantification of data

The radioactivity amount in the cleaved (I^C) and uncleaved band (I^U) were measured with ImageQuant v5.2 (Molecular Dynamics). The fraction of the target RNA cleaved was determined from the relative amount of radioactivity present in the respective band when compared to the total amount of radioactivity [$I^C/(I^C+I^U)$]. The fraction cleaved in the absence of HuR was taken as 1 to normalize the fractions cleaved in the presence of HuR.

6.12 RNA deadenylation assay

The preparation of RNAs by the in vitro transcription, labeling and the assay was performed as described by Fabian et al, 2009. Briefly, transcripts 6xB-3'UTR, 6xB-ARD-3'UTR and 6xBMUT-ARD-3'UTR were generated from PCR products derived from corresponding plasmids and T7-3'UTR (GGCGCCTAATACGACTCACTATAGGGGTAAGTACATCAAGAGCTTCG) and Oligo 3R(-) (GGTGACACTATAGAATAGGGCCC) primers. PCR products were digested with ApaI and filled in using the Klenow fragment. Transcription reactions were performed using MAXIscript In Vitro Transcription Kit (Ambion) in 20 µl at 37°C according to the manufacturer's protocol incorporating [α-^{32}P] UTP (800 Ci/mmol, 10 mCi/ml; PerkinElmer. Radiolabeled RNA (0.1 ng) was incubated in the Krebs-2 ascites cell extract in a total volume of 10 µl. Aliquots of the reaction mixture were withdrawn at specific intervals, and the RNA was extracted using TRIzol reagent (Invitrogen) and loaded on a 4% or 4.5% polyacrylamide/urea gel. The gel was dried and analyzed using a PhosphorImager.

6.13 Electrophoretic mobility shift assays (EMSA)

Interactions of HuR and its mutants with target RNAs were analyed by electrophoretic mobility shift assays (EMSA), using non-denaturing gels. The reactions (10 µl) contained 1x cleavage buffer, 1 µg of yeast tRNA, 2 fmol of 5'-^{32}P-labeled RNA, and indicated increasing concentrations of purified proteins. Following 10 min incubation on ice, the reactions were immediately processed for a non-denaturing 6% PAGE performed at 4°C. The gel was dried and the resolved RNA:protein complexes were visualized by phosphorimaging.

6.14 Analysis of miRISC association with target RNA

For measurements of miRISC association with target RNA, the purified miRISC was immobilized on α-FLAG beads and then first incubated with target RNA, followed by addition of HuR. 30 µl α-FLAG M2 Affinity Gel beads (Sigma) were pre-incubated with 4 µl of purified miRISC in 1 ml of binding buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1x EDTA-free Protease Inhibitor Cocktail (Roche)]. The beads were incubated with 50 fmol of target RNA in the binding buffer additionally containing 0.5 mM DTT, 1 µg tRNA and 5 mM EDTA (in case of the Ago2 miRISC) or 2 mM MgCl$_2$ (in a case of other catalytically inactive miRISCs) for 15 min at 23°C followed by additional 15 min incubation at 23°C in the presence or absence of 150 nM of HuR. The beads were spun down by centrifugation at 5,000xg for 1 min and washed twice with the binding buffer.

The RNA bound to beads was extracted with TRIzol reagent (Invitrogen), reverse transcribed using a reverse primer (GCTCATCAGATGTTGAGTCC; complementary to the 3'-end of the spacer region) and 25 U of Superscript III RT (Invitrogen). One fourth of the RT reaction was then used for quantitative real-time PCR (qRT-PCR), using the ABI 7500 Fast Sequence Detection System (Applied Biosystems) and Platinum SYBR Green qPCR Super Mix (Invitrogen). Sequence of the forward PCR primer for HBS_50_MBSp and HBS_50_MBSb is GGTATTTATTTATTTATTTGTTTG and that for MBSb_50_HBS is

GGTCCAAAGAGTGACTGCACAGCC. The reverse primer was the same as that used for RT.

Appendix 1

Target RNA associate with miRSC in the presence of EDTA

Cleavage (normalized)   0.4   1   0.93   0.95
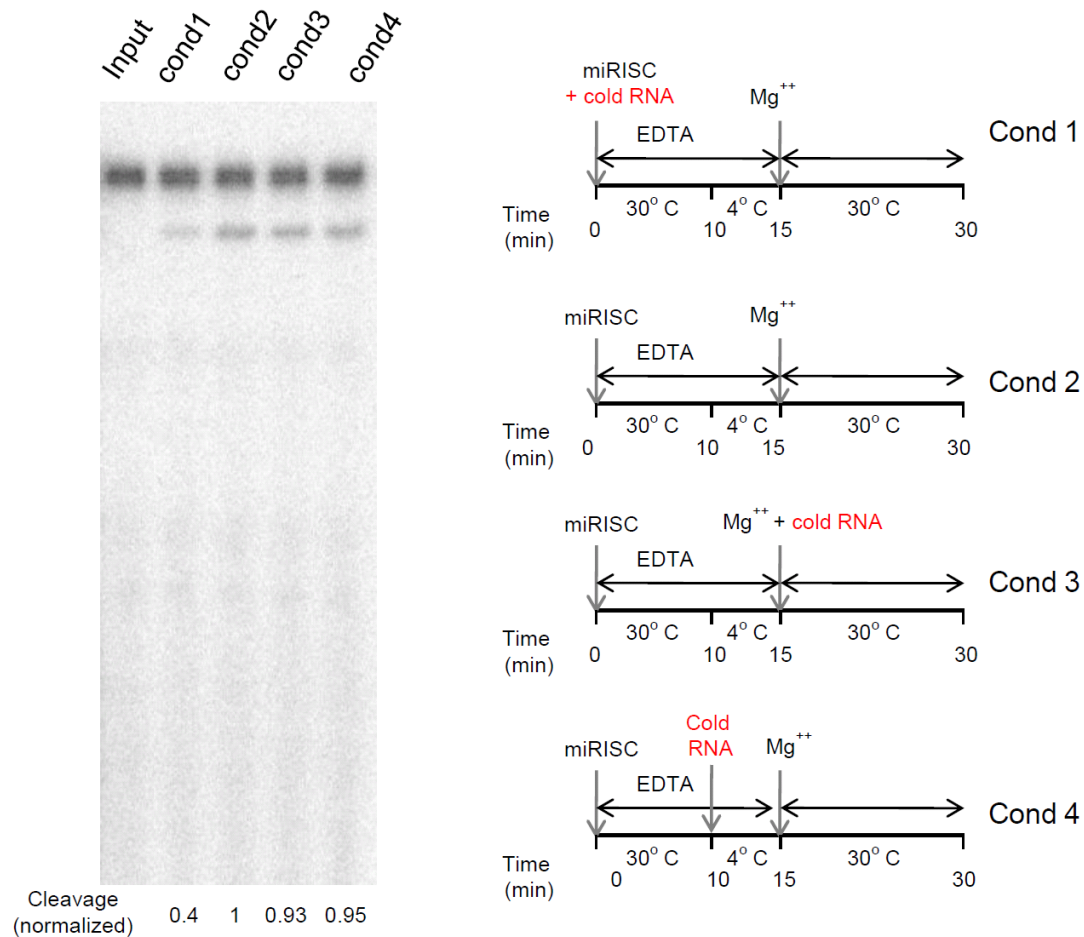
Figure I No effect of pre-incubation with EDTA on miRISC association with target RNA HBS_50_MBSp. The cleavage efficiency was normalized against condition 2, which was set to 1.0.

When performing cleavage assays involving pre-binding of miRISC to target RNA in the presence of EDTA (Fig. 23B), it was necessary to ensure that there is no effective dissociation of miRISC from the target during the process, before addition of HuR. As shown in Figure I, when labeled RNA (0.1 nM) was diluted with cold RNA (0.3 nM) during the pre-binding with miRISC as in condition 1, this led to ~2.5 fold reduction in the cleavage compared to the condition 2 when no cold RNA was added to the reaction. This is consistent

with the cold RNA competing with the radiolabeled RNA for binding to miRISC. On the other hand, when cold RNA was added to the reaction simultaneously with Mg^{++} at the moment when the cleavage is initiated (condition 3) or added to the reaction and incubated on ice for 5 min prior to Mg^{++} addition (condition 4), the cleavage of the target RNA remained unchanged. This indicates that once miRISC is bound to the target complementary site, it remains associated with it throughout the experiment. Hence, the effect we see on the inhibition of cleavage upon addition of HuR is not caused by HuR-independent dissociation of miRISC from the target RNA.

Appendix 2

HuR function is unaffected by preincubation in the presence of EDTA
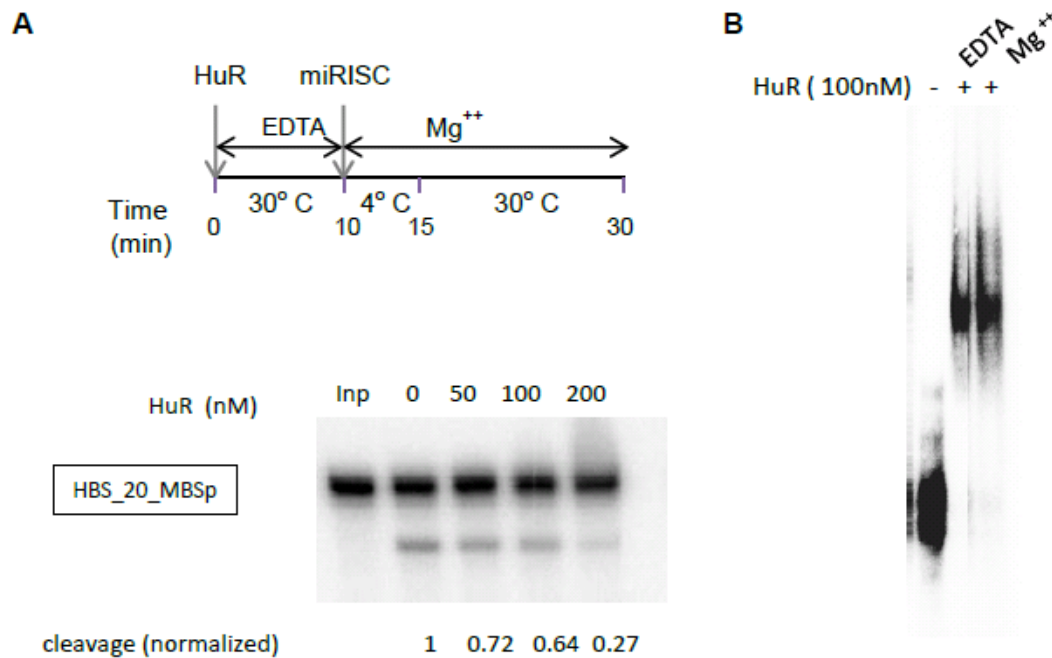
Figure II HuR function remains unaffected by preincubation in the presence of EDTA. (**A**) HBS_20_MBSp was incubated with HuR (as indicated above the gel) in the presence of EDTA followed by addition of miRISC and Mg^{++} to initiate cleavage. (**B**) EMSA showing HuR binding to target RNA either in the presence of 5 mM EDTA or 2 mM Mg^{++} in the buffer.

In order to ensure that the presence of EDTA does not affect the derepressive property of HuR, the *in vitro* cleavage reaction was performed as shown in Figure IIA (upper panel). Consistent with our earlier observation (Figs. 20A and 20C), similar degree of concentration-dependent inhibition of cleavage of target RNA was observed upon addition of HuR (Fig. IIA, lower panel). The binding of HuR to the target RNA in the presence of EDTA also remained unaltered (Fig. II B).

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EDUCATION

2007-date   PhD Studies at the laboratory of Prof. Witold Filipowicz, Friedrich Miescher Institute under the University of Basel, Switzerland

2006-2007   Training (Praktikantin) at the Filipowicz lab, FMI, Basel, Switzerland

2006   Obtained Master of Science degree in Biotechnology, ranked 1st, University of Calcutta, India

2004   Obtained Bachelor of Science degree in Physiology (Honours) and Zoology and Chemistry (general) with distinction, Presidency College, University of Calcutta, India

2000   Qualified Higher Secondary School Examination, ranked 19th in the board, Kolkata, India

1998   Qualified Secondary School Examination with first division, Kolkata, India

SCHOLARSHIPS AND FELLOWSHIPS

Awarded an RNA Society Travel Grant to attend the conference "The Complex Life of mRNA: From Synthesis to Decay" held at EMBL, Heidelberg, Germany on 18 - 20 March 2010.

Awarded Lt. Col. A. N. Bose Scholarship (Foreign Scholarships/Fellowships) from University of Calcutta for travelling to Switzerland.

Qualified NET (National Eligibility Test) for award of Research Fellowship in subject Life Sciences under the CSIR (Council of Scientific and Industrial Research) fellowship scheme by Government of India.

Awarded National Scholarship for performance in both Secondary and Higher Secondary School Examination.

EXPERIENCE

Jul-Sep 2006
Worked under the guidance of Prof. D. J. Chattopadhyay at the University of Calcutta in a project "Role of Leader RNA of Chandipura Virus on the Activity of Casein Kinase II During Transcription-Replication Switch".

Jun-Jul 2005
Worked under the guidance of Prof. Tapas Kundu at Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore in the project entitled "Characterisation of Expression pattern of human Positive Cofactor 4 (PC4) in brain tissues and U87MG (Glioblastoma) cell line".

Nov 2003
Actively participated in the field tour, conducted by the department of Physiology, Presidency College at Tamia and Toula, villages near Panchmari, Madhya Pradesh, India, covering following areas:
      1. Studies on anthropometric measurements of the inhabitants.
      2. Studies on haematological & cardiovascular parameters of the people.
      3. Studies on social and nutritional status of the villagers.

PRESENTATIONS and PUBLICATIONS

FMI annual meetings, 2007-2011 (posters)

EMBO Conference Series on Protein Synthesis and Translational Control
07 - 11 September 2011, EMBL, Heidelberg, Germany (poster)

Conference on The Complex Life of mRNA: From Synthesis to Decay
18 - 20 March 2010, EMBL, Heidelberg, Germany (poster)

A manuscript describing the work of PhD thesis is submitted to Nucleic Acids Research.