Supplementary Data

Supplementary Tables

Supplementary Table 1. 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	Ago2_F	*Not*I	CT*GCGGCCGC*ATGTACTCGGGAGCCGGCCC
2	Ago2_R	*Eco*RI	CT*GAATTC*TCAAGCAAAGTACATGGTGCGC
3	His-HuR_F	*Nde*I	GATCG*CATATG*TCTAATGGTTATGAAGACC
4	His-HuR_R	*Xho*I	GATCG*CTCGAG*TTTGTGGGACTTGTTGGTTTTG
5	His-HuRΔH_R	*Xho*I	GATCG*CTCGAG*TTTGTGGGACTTGTTGGTTTTG
6	His-HuRΔ3_R	*Xho*I	GATCG*CTCGAG*GGAGGAGGCGTTTCCTGGC
7	His-HuRΔH3_R	*Xho*I	GATCG*CTCGAG*GGCTGCAAACTTCACTGTGATG
8	ARD_F	*Not*1	GGCGGCCGCGGGAGCTTTATTCTG
9	ARD_R	*Not*1	GGCGGCCGCGCTGTCTTGCACGG

Supplementary Table 2. 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	AGTGTAACGTTGAGGTAGTAGGTTGTATAGTCAATATCACTCTTTGG AAATAATAAAACAAACAAACAAATAAATAAATAAATACCCTATAGTGA GTCGTATTAA
ΔHBS_20_MBSp	antisense	AGTGTAACGTTGAGGTAGTAGGTTGTATAGTCAGTCTCACTCTTTGG ACCTCCCTATAGTGAGTCGTATTAA
HBSMut_20_MBSp	antisense	AGTGTAACGTTGAGGTAGTAGGTTGTATAGTCAGTCTCACTCTTTGG ACCTAGTAGATACGCACATACGTATGCATGACATATATACCCTATAG TGAGTCGTATTAA
HBS_50_MBSp	sense	TATTTATTTATTTATTTGTTTGTTTGTTTTATTGCTGTACGAGAGAGTC ACTGTGCTGATGTGGACTCAACATCTGATGAGCAACTATACAACCTA CTACCTCAACGTTACACT
	antisense	GTTGTATAGTTGCTCATCAGATGTTGAGTCCACATCAGCACAGTGAC TCTCTCGTACAGCAATAAAACAAACAAACAAATAAATAAATAAATACC CTATAGTGAGTCGTATTAA
	reverse	AGTGTAACGTTGAGGTAGTAG
HBS_50_MBSb	sense	TATTTATTTATTTATTTGTTTGTTTGTTTTATTGCTGTACGAGAGAGTC ACTGTGCTGATGTGGACTCAACATCTGATGAGCAACTGCACAGCCTA TTGAACTACCTCAACGTTACACT
	antisense	AATAGGCTGTGCAGTTGCTCATCAGATGTTGAGTCCACATCAGCACA GTGACTCTCTCGTACAGCAATAAAACAAACAAACAAATAAATAAATAA ATACCCTATAGTGAGTCGTATTAA
	reverse	AGTGTAACGTTGAGGTAGTTC
MBSb_50_HBS	sense	GGTCCAAAGAGTGACTGCACAGCCTATTGAACTACCTCAGCTGTAC GAGAGAGTCACTGTGCTGATGTGGACTCAACATCTGATGAGCATATT TATTTATTTATTTGTTTGTTTGTTTTATTAC
	antisense	TAAATAAATATGCTCATCAGATGTTGAGTCCACATCAGCACAGTGAC TCTCTCGTACAGCTGAGGTAGTTCAATAGGCTGTGCAGTCACTCTTT GGACCCTATAGTGAGTCGTATTAA
	reverse	GTAACGTAATAAAACAAACAAACAAATAAA

Supplementary Table 3. 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	GGUAUUUAUUUAUUUAUUUGUUUGUUUGUUUUAUUAUUUCCAAAGAGUG AUAUUG*ACUAUACAACCUACUACCUCA*ACGUUACACU
ΔHBS_20_MBSp	GGAGGUCCAAAGAGUGAGACUG*ACUAUACAACCUACUACCUCA*ACGUUA CACU
HBSMut_20_MBSp	GGUAU**A**UAU**G**U**C**AU**GC**AU**AC**GU**A**UGU**GC**GU**A**U**C**UA**C**UAGGUCCAAAGAG UGAGACUG*ACUAUACAACCUACUACCUCA*ACGUUACACU
HBS_50_MBSp	GGUAUUUAUUUAUUUAUUUGUUUGUUUGUUUUAUUGCUGUACGAGAGAG UCACUGUGCUGAUGUGGACUCAACAUCUGAUGAGCA*ACUAUACAACCUA CUACCUCA*ACGUUACACU
HBS_50_MBSb	GGUAUUUAUUUAUUUAUUUGUUUGUUUGUUUUAUUGCUGUACGAGAGAG UCACUGUGCUGAUGUGGACUCAACAUCUGAUGAGCA*ACUGCACAGCCUA UUGAACUACCUCA*ACGUUACACU
MBSb_50_HBS	GGUCCAAAGAGUG*ACUGCACAGCCUAUUGAACUACCUCA*GCUGUACGAG AGAGUCACUGUGCUGAUGUGGACUCAACAUCUGAUGAGCAUAUUUAUUU AUUUAUUUGUUUGUUUGUUUUAUUACGUUAC

Supplementary Table 4. Sequences of DNA and LNA oligonucleotides complementary to substrate spacer regions. The modified LNA nucleotides are shown in bold.

oligonucleotide	Sequence (5'→3')
DNA 50	TGCTCATCAGATGTTGAGTCCACATCAGCACAGTGACTCTCTCGTACAGC
LNA 27	C**A**CAT**C**AG**C**A**C**AG**T**GA**C**TCT**C**TC**G**TA**C**
LNA 20	C**A**A**T**AT**C**A**C**T**C**T**T**TG**G**A**A**AT

Supplementary Figure Legends

Figure S1. miRISC specifically cleaves the target RNA bearing sequence perfectly complementary to let-7 miRNA . (**A**) Western blot analysis showing a similar level of purification for both let-7-enriched and non-let-7-enriched Ago2 miRISC (the latter purified from cells not co-transfected with plasmid expressing the pri-let-7 RNA). Input, extract before affinity purification; FT, flow-through fraction not retained on FLAG antibody beads; wash, fraction eluted with a buffer containing no FLAG peptide; elution, fraction eluted with 3xFLAG peptide. (**B**) Schematic representation of target RNAs, MBSp RNA and MutMBSp RNA. (**C** and **D**) MBSp RNA, but not MutMBSp RNA, is specifically cleaved by the let-7-enriched miRISC. Components added to cleavage reactions are indicated at the top. Cleavage product is indicated with arrow.

Figure S2. Representative assay of kinetics of the HBS_20_MBSp RNA cleavage catalyzed by the Ago2 miRISC. Quantification of cleavage reactions is shown in a lower panel.

Figure S3. 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.

Figure S4. Prehybridization of 20-mer LNA oligonucleotide complementary to the spacer interferes with the alleviating effect of HuR on the miRISC-catalyzed cleavage of HBS_20_MBSp. (Upper panels) Effect of increasing concentrations of HuR on cleavage of HBS_20_MBSp RNA alone or its duplex with a complementary LNA oligonucleotide. The schemes of HBS_20_MBSp RNA or its duplex with LNA oligonucleotide are shown above the gels. Inp, input RNA. (Lower panel) PhosphorImaging quantification of the experiment shown in the upper panel.

Figure S5. Immunoprecipitation (IP) experiments performed with HeLa cell extracts reveal no interaction between HuR and miRISC protein components

Ago2 and TNRC6B. (**A**) Extracts prepared from HeLa cells, either control (Fed) or starved (Starved) for amino acids for 2 h, were used for IP reactions with antibodies against endogenous Ago2 and HuR. (**B**) HeLa cells (Fed or Starved) expressing Myc-Ago2 and HA–tagged TNRC6B were used for IP reactions. Material immunoprecipitated with indicated antibodies was used for western blotting with α-HuR, α-HA, α-Myc and α-GFP antibodies. (**C**) HeLa cells were transfected with the HA-Ago3 expression plasmid. 24 h after transfection, the cytoplasmic extract prepared from cells, either control (Fed) or starved (Starved) for amino acids for 2 h, were used for IP reactions with α-HA, α-GFP or α-HuR antibodies. The IP material was analyzed by western blotting for HA-Ago3 and endogenous HuR. α-GFP antibody was used for control IP reactions.

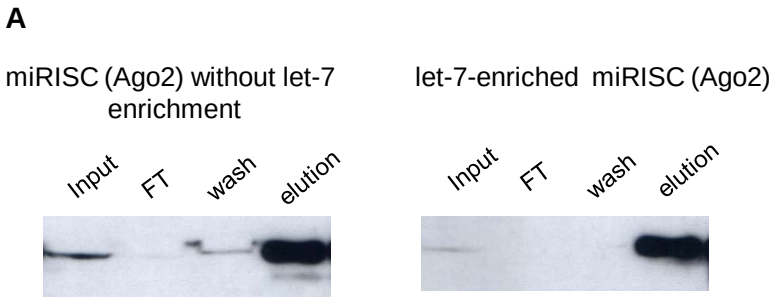
Supplementary Methods

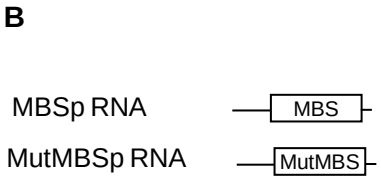
Immunoprecipitaion from cellular extracts and Western analysis

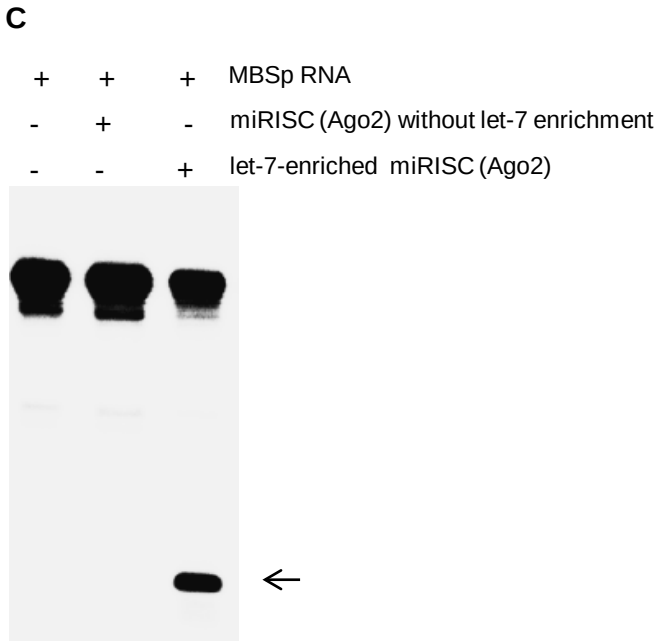
HeLa cells were grown in monolayers in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin at 37°C in humidified atmosphere containing 5% CO_2. Cells at high confluency (95%), cultured in 6-well plates DMEM without antibiotics supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, were transfected with 200 ng of indicated plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's manual. Further assays were carried out 48 h after transfection, with splitting of cells and change of medium at 24 h. For stress induction experiments by amino acid starvation, cells at 50-60% confluency were grown for 2 h in DMEM without amino acids and supplemented with 10% FCS dialyzed against PBS. Cell extracts, from either transfected or non-transfected cells, were prepared by lysing the cells 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 (67)] was used in 1:20 dilution. Protein G Agarose beads bound to antibodies (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 Western blotting, using, a rabbit α-Ago2 antibodies (Abnova; 1:1,000 dilution), mouse α-HuR 3A2 (Santa Cruz), rat α-HA 3F10 (Roche), mouse α-Myc 9E10 (Santa Cruz), and mouse α-GFP (Roche) mAbs (all at 1:1,000 dilution), and HRP-conjugated secondary anti-mouse, anti-rabbit (both from GE Healthcare), and anti-rat (MP Biochemicals) antibodies (all at 1:10,000 dilution).

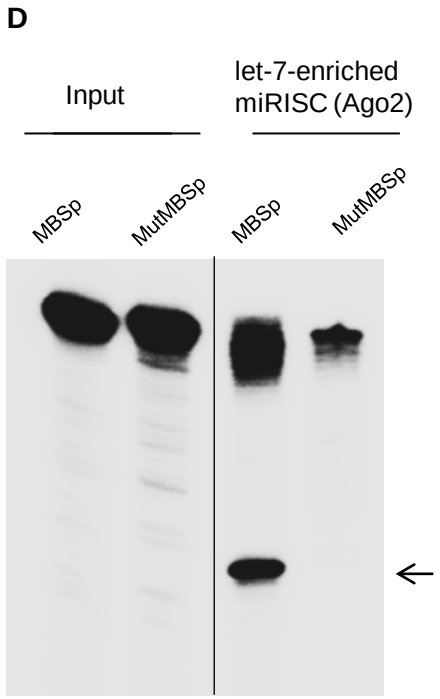
Supplementary Reference

67. Nelson, P.T., De Planell-Saguer, M., Lamprinaki, S., Kiriakidou, M., Zhang, P., O'Doherty, U. and Mourelatos, Z. (2007) A novel monoclonal antibody against human Argonaute proteins reveals unexpected characteristics of miRNAs in human blood cells. *RNA*, **13**, 1787-1792.

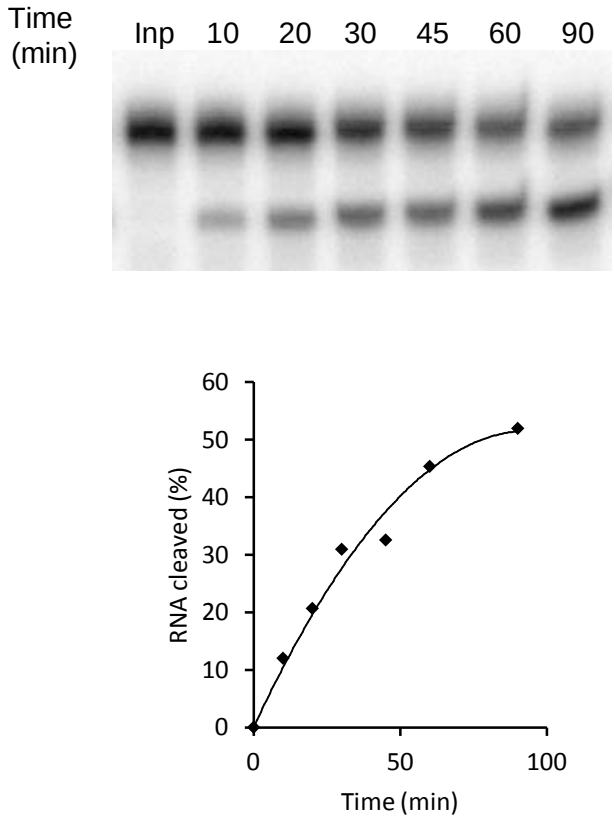




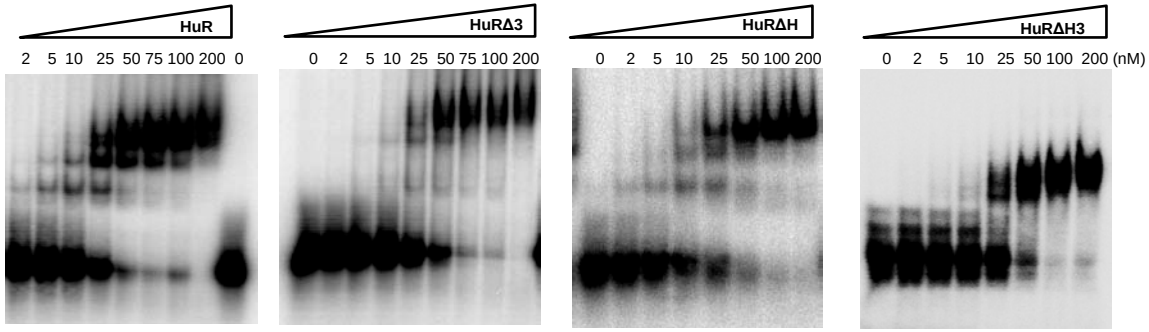




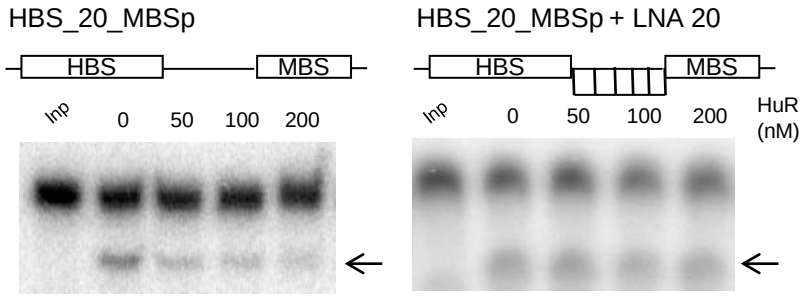
Supplementary Figure S1

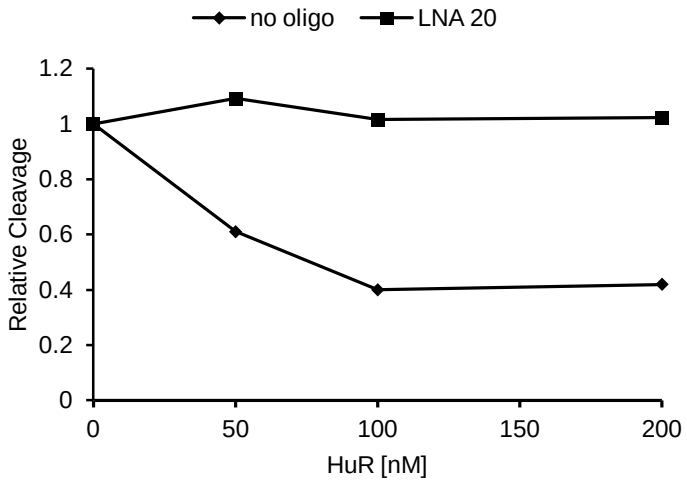


Supplementary Figure S2

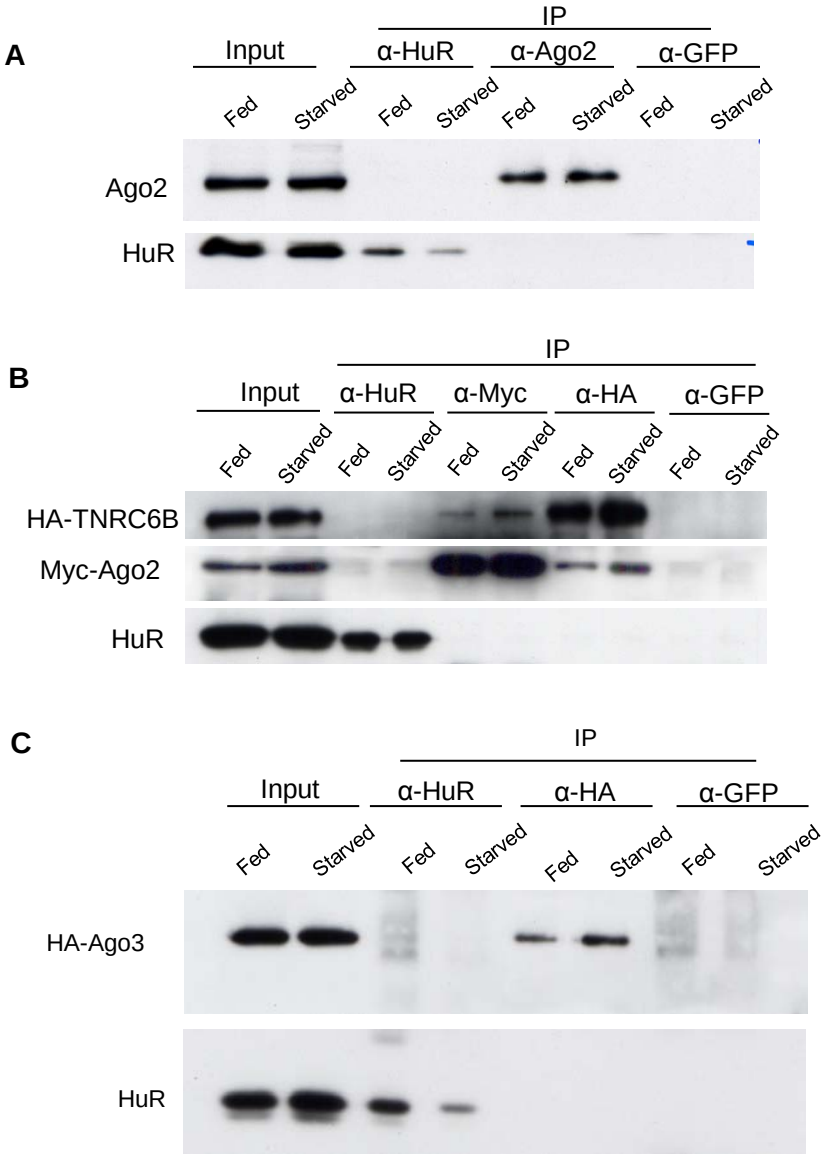


Supplementary Figure S3





Supplementary Figure S4



Supplementary Figure S5