Appendix

miRISC and the CCR4-NOT complex silence mRNA targets independently of 43S ribosomal scanning

Duygu Kuzuoğlu-Öztürk, Dipankar Bhandari, Eric Huntzinger, Maria Fauser, Sigrun Helms and Elisa Izaurralde

Table of contents

Appendix Figure S1

Appendix Figure S2

Appendix Table S1

Appendix Table S2

Appendix Table S3

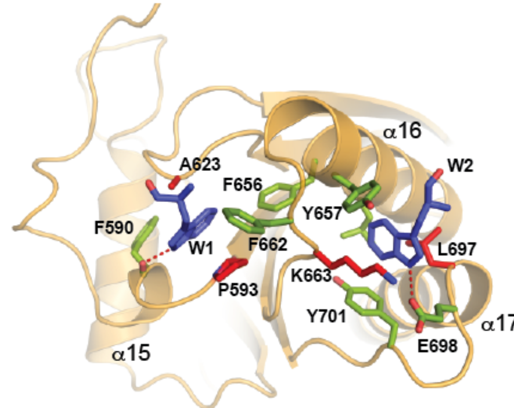
Appendix Table S4

Appendix Methods

Appendix References

Appendix Figure S1

A *Hs* AGO2



B

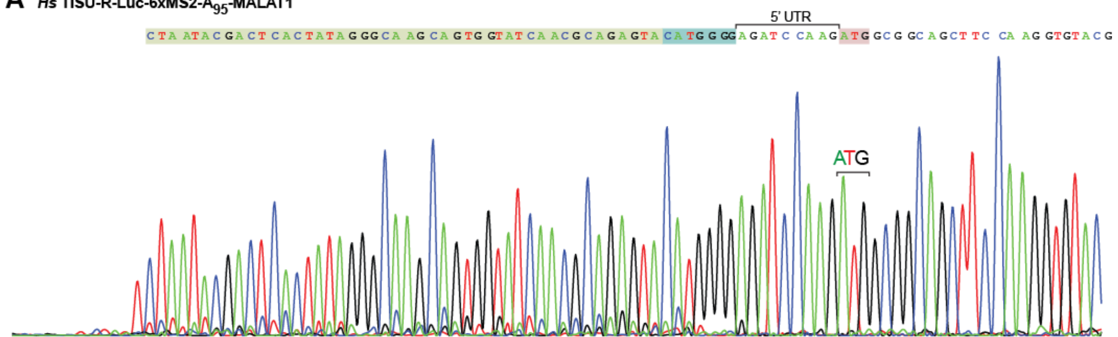
```
                            α15       β24                                   β25          β26
Hs AGO2
                     580       590 *     600         610                     620 *        630
Hs AGO2     577   VNNILLPQGRPPVFQQPVIFLGADVTHPPAGDGKK.....PSIAAVVGSMDAHPNRYCATVRVQ   635
Hs AGO1     572   INNILVPHQRSAVFQQPVIFLGADVTHPPAGDGKK.....PSITAVVGSMDAHPSRYCATVRVQ   630
Hs AGO3     575   INNILVPHQRPSVFQQPVIFLGADVTHPPAGDGKK.....PSIAAVVGSMDAHPSRYCATVRVQ   633
Hs AGO4     566   INNVLVPHQRPSVFQQPVIFLGADVTHPPAGDGKK.....PSIAAVVGSMDGHPSRYCATVRVQ   624
Dm AGO1     698   INSILVPSIRPKVFNEPVIFLGADVTHPPAGDNKK.....PSIAAVVGSMDAHPSRYAATVRVQ   756
Dm AGO2     943   INHKIKDDPRL.PMMKNTMYIGADVTHPSPDQREI.....PSVVGVAASHDPYGASYNMQYRLQ  1000
Nc QDE2     642   TNHNIK.TPIPLLAKGKTMVVGYDVTHPTNLAAGQSPASAPSIVGLVSTIDQHLGQWPAMVWNN   704
Sp AGO1     558   INCSLIPKS.NPLGNVPTLILGGDVYHPGVGATGV......SIASIVASVDLNGCKYTAVSRSQ   614
Hs PIWI1    615   ELWRVD......IPLKLVMIVGIDCYHDMTAGRRS.......IAGFVASINEGMTRWFSRCIFQ   665
Hs PIWI2    728   ELWGVD......IPLKQLMVIGMDVYHDPSRGMRS.......VVGFVASINLTLTKWYSRVVFQ   778
Hs PIWI3    637   ALWKVE......TDVQRTMFVGIDCFHDIVNRQKS.......IAGFVASTNAELTKWYSQCVIQ   687
Hs PIWI4    606   ELWAVE......IPLKSLMVVGIDVCKDALSKDVM.......VVGCVASVNPRITRWFSRCILQ   656

                                      α16                     β27         α17
Hs AGO2
                   640                650                660 *     670          680
Hs AGO2     636   QHRQEI..........IQDLAAMVRELLIQFYKS..T.RFKPTRIIFYRDGVSEGQFQQVLHHE   686
Hs AGO1     631   RPRQEI..........IEDLSYMVRELLIQFYKS..T.RFKPTRIIFYRDGVPEGQLPQILHYE   681
Hs AGO3     634   RPRQEI..........IQDLASMVRELLIQFYKS..T.RFKPTRIIFYRDGVSEGQFRQVLYYE   684
Hs AGO4     625   TSRQEISQELLYSQEVIQDLTNMVRELLIQFYKS..T.RFKPTRIIYYRGGVSEGQMKQVAWPE   685
Dm AGO1     757   QHRQEI..........IQELSSMVRELLIMFYKS..TGGYKPHRIILYRDGVSEGQFPHVLQHE   808
Dm AGO2     1001  RGALEE..........IEDMFSITLEHLRVYKEY..R.NAYPDHIIYYRDGVSDGQFPKIKNEE  1051
Nc QDE2     705   PHGQES.........MTEQFTDKFKTRLELWRSNPANNRSLPENILIFRDGVSEGQFQMVIKDE   759
Sp AGO1     615   PRHQEV..........IEGMKDIVVYLLQGFRAM..T.KQQPQRIIYFRDGTSEGQFLSVINDE   665
Hs PIWI1    666   DRGQEL..........VDGLKVCLQAALRAWNSC..N.EYMPSRIIVYRDGVGDGQLKTLVNYE   716
Hs PIWI2    779   MPHQ I..........VDSLKLCLVGS KK YEV..N.HCLPEKIVVYR GVSDGQLKTVANYE   829
Hs PIWI3    688   KTGEEL..........VKELEICLKAALDVWCKN..E.SSMPHSVIVYRDGVGDGQLQALLDHE   738
Hs PIWI4    657   RTMTDV..........ADCLKVFMTGALNKWYKY..N.HDLPARIIVYRAGVGDGQLKTLIEYE   707

                     α17               β28          β29   α18         β30
Hs AGO2
                   690     * 700          710          720         730         740
Hs AGO2     687   LLAIREACIKLEKDYQ.PGITFIVVQKRHHTRLFCTDKNERVGKSGNIPAGTTVDTKITHPTEF   749
Hs AGO1     682   LLAIRDACIKLEKDYQ.PGITYIVVQKRHHTRLFCADKNERIGKSGNIPAGTTVDTNITHPFEF   744
Hs AGO3     685   LLAIREACISLEKDYQ.PGITYIVVQKRHHTRLFCADRTERVGRSGNIPAGTTVDTDITHPYEF   747
Hs AGO4     686   LIAIRKACISLEEDYR.PGITYIVVQKRHHTRLFCADKTERVGKSGNVPAGTTVDSTITHPSEF   748
Dm AGO1     809   LTAIREACIKLEPEYR.PGITFIVVQKRHHTRLFCAEKKEQSGKSGNIPAGTTVDVGITHPTEF   871
Dm AGO2     1052  LRCIKQACDKVG..CK.PKICCVIVVKRHHTRFFPSGDVTTSNKFNNVDPGTVVDRTIVHPNEM  1112
Nc QDE2     760   LPLVRAACKLVYPAGKLPRITLIVSVKRHQTRFFPTDPKHIHFKSKSPKEGTVVDRGVTNVRYW   823
Sp AGO1     666   LSQIKEACHSLSPKYN.PKILVCTTQKRHHARFFIKNKSD.GDRNGNPLPGTIIEKHVTHPYQY   727
Hs PIWI1    717   VPQFLDCLKSIGRGYN.PRLTVIVVKKRVNTRFF....AQSGGRLQNPLPGTVIDVEVTRPEWY   775
Hs PIWI2    830   IPQLQK.CFEAFENYQ.PKMVVFVVQKKISTNLY....LAAPQNFVTPTPGTVVDHTITSCEWV   887
Hs PIWI3    739   AKKMST.YLKTISPNN.FTLAFIVVKKRINTRFF....LKHGSNFQNPPPGTVIDVELTRNEWY   796
Hs PIWI4    708   VPQLLSSVAESSSNTS.SRLSVIVVRKKCMPRFF....TEMNRTVQNPPLGTVVDSEATRNEWY   766

                  β31          β32                               α19             α20
Hs AGO2
                  750       760       770                  780       790       800
Hs AGO2     750   DFYLCSHAGIQGTSRPSHYHVLWDD......NRFSSDELQILTYQLCHTYVRCTRSVSIPAPAY   807
Hs AGO1     745   DFYLCSHAGIQGTSRPSHYYVLWDD......NRFTADELQILTYQLCHTYVRCTRSVSIPAPAY   802
Hs AGO3     748   DFYLCSHAGIQGTSRPSHYHVLWDD......NCFTADELQLLTYQLCHTYVRCTRSVSIPAPAY   805
Hs AGO4     749   DFYLCSHAGIQGTSRPSHYQVLWDD......NCFTADELQLLTYQLCHTYVRCTRSVSIPAPAY   806
Dm AGO1     872   DFYLCSHQGIQGTSRPSHYHVLWDD......NHFDSDELQCLTYQLCRTYVRCTRSVSIPAPAY   929
Dm AGO2     1113  QFFMVSHQAIQGTAKPTRYNVIENT......GNLDIDLLQQLTYNLCHMFPRCNRSVSYPAPAY  1170
Nc QDE2     824   DFFLQAHASLQGTARSAHYTVLVDEIFRADYGNKAADTLEQLTHDMCYLFGRATKAVSICPPAY   887
Sp AGO1     728   DFYLISHPSLQGVSVPVHYTVLHDE......IQMPPDQFQTLCYNLCYVYARATSAVSLVPPVY   785
Hs PIWI1    776   DFFIVSQAVRSGSVSPTHYNVIYDN......SGLKPDHIQRLTYKLCHIYYNWPGVIRVPAPCQ   833
Hs PIWI2    888   DFYLLAHHVRQGCGIPTHYVCVLNT......ANLSPDHMQRLTFKLCHMYWNWPGTIRVPAPCK   945
Hs PIWI3    797   DFFIVSQSVQDGTVTPTHYNVIYDT......IGLSPDTVQRLTYCLCHMYYNLPGIIRVPAPCH   854
Hs PIWI4    767   DFYLISQVACRGTVSPTYYNVIYDD......NGLKPDHMQRLTFKLCHLYYNWPGIVSVPAPCQ   824

                     α20                              α21      α22
Hs AGO2
                   810       820       830       840       850       860
Hs AGO2     808   YAHLVAFRARYHLVDKEHDSAEGSHTSGQSNGRDHQALAKAVQVHQDTLRTMYFA           862
Hs AGO1     803   YARLVAFRARYHLVDKEHDSGEGSHISGQSNGRDPQALAKAVQVHQDTLRTMYFA           857
Hs AGO3     806   YAHLVAFRARYHLVDKEHDSAEGSHVSGQSNGRDPQALAKAVQIHQDTLRTMYFA           860
Hs AGO4     807   YARLVAFRARYHLVDKDHDSAEGSHVSGQSNGRDPQALAKAVQIHHDTQHTMYFA           861
Dm AGO1     930   YAHLVAFRARYHLVEKEHDSGEGSHQSGCSEDRTPGAMARAITVHADTKKVMYFA           984
Dm AGO2     1171  LAHLVAARGRVYLTGTNRFLDLKKE..........YAKRTIVPEFMKKNPMYFV          1214
Nc QDE2     888   YADLVCDRARIHQ.KELFDALDENDSVKTDD...FARWGNSGAVHPNLRNSMYYI           938
Sp AGO1     786   YAHLVSNLARYQDVTADDTFVETSEAS......MDQEVKPLLALSSKLKTKMWYM           834
Hs PIWI1    834   YAHKLAFLVGQSIHREP.........................NLSLSNRLYYL           861
Hs PIWI2    946   YAHKLAFLSGHILHHEP.........................AIQLCENLFFL           973
Hs PIWI3    855   YAHKLAYLVGQSIHQEP.........................NRSLSTRLFYL           882
Hs PIWI4    825   YAHKLTFLVAQSIHKEP.........................SLELANHLFYL           852
```

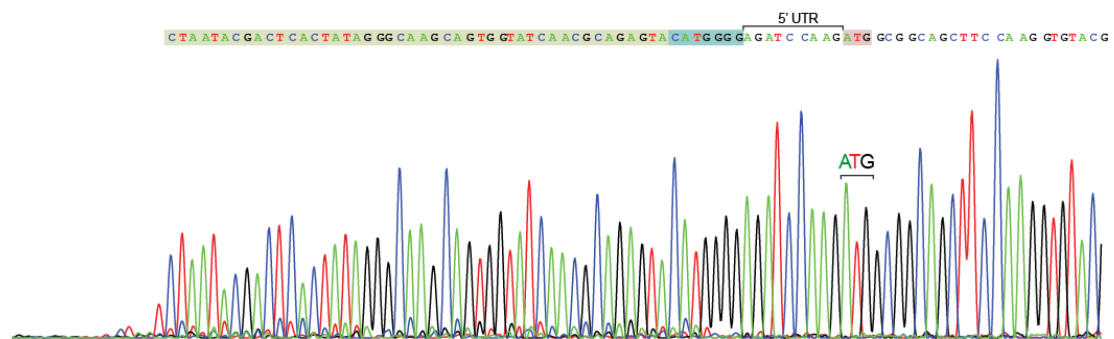
Figure S1. The W-binding pockets are conserved in the AGO proteins that interact with GW182/TNRC6 proteins.

A Ribbon representation of *Hs* AGO2 (PDB ID: 4OLB) showing the position of the tryptophan-binding pockets. Residues lining the pockets are shown as green sticks or as red sticks when the residues were mutated. The tryptophan residues are shown as blue sticks.

B Structure-based sequence alignment of the PIWI domain of human AGO1, AGO2, AGO3 and AGO4, *Drosophila melanogaster* (*Dm*) AGO1 and AGO2, *Neurospora crassa* (*Nc*) QDE2, *Schizosaccharomyces pombe* (*Sp*) AGO1 and the human PIWI-like clade. Residues lining the tryptophan-binding pockets 1 and 2 are shaded in green and orange, respectively. Residues forming the catalytic tetrad are highlighted in red. Residues mutated in this study are marked with a red asterisk. Residues lining the W-binding pockets are conserved in *Hs* AGO1, AGO3 and AGO4 as well as in *Dm* AGO1, which is the AGO protein dedicated to the miRNA pathway in *Dm* (Okamura *et al*, 2004; Schirle & MacRae, 2012). By contrast, these residues are less conserved in the PIWI clade of Argonaute proteins and in *Dm* AGO2, which do not interact with GW182 (Behm-Ansmant *et al*, 2006; Miyoshi *et al*, 2009). To abolish the interaction of *Hs* AGO2 and *Dm* AGO1 with GW182 proteins, we substituted a proline residue in pocket 1 with glycine to prevent hydrophobic stacking interactions with tryptophan residues and an alanine with a bulky phenylalanine to block tryptophan binding (mutant P1). In pocket P2, we substituted a leucine residue with a bulky tyrosine to block the pocket and a lysine residue with alanine to prevent hydrophobic stacking interactions (mutant P2).

Appendix Figure S2





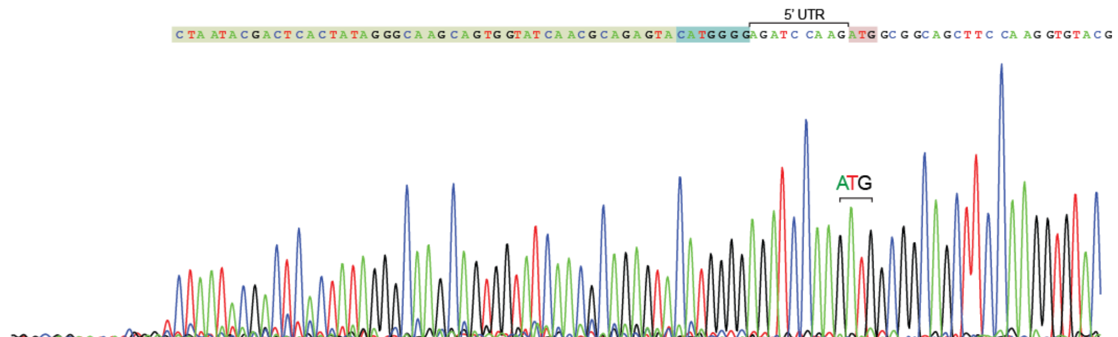


Figure S2. Partial sequencing chromatograms of 5′-RACE amplified cDNA fragments for the indicated reporters.

Appendix Figure S3

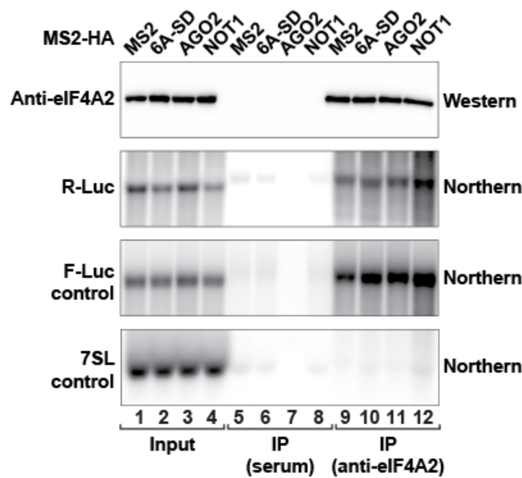


Figure S3. eIF4A2 binding to mRNAs is not influenced by silencing.

Lysates from HEK293T cells expressing R-Luc-MS2-A_{95}-MALAT1 reporter and the indicated MS2-HA tagged proteins were immunoprecipitated using anti-eIF4A2 antibody as described in Fig 4F–H. The RNAs coimmunoprecipitating with eIF4A2 were analyzed by northern blot. The panel shows a representative western and northern blots of input and IP fractions. For the western blot, 2% of the input and 6% of the IP fraction were analyzed. For the northern blots 2% of the input and 40% of the IP were analyzed.

Appendix Table S1. Mutants used in this study.

Protein	**Name of the construct**	**Mutations**
Dm AGO1 NM_166020.1	P1	P714G + A744F
	P2	L819Y
	P1+2	P714G + A744F + L819Y
Hs AGO2	P1	P593A + A623F
	P2	L697Y + K663A
	P1+2	P593A + A623F + L697Y + K663A
Dm DCP1 NM_137998.2	DCP1* (GSSG)	R70G + L71S + N72S + T73G
Hs DCP2 AY135173.1	DCP2*	E148Q
Hs NOT1 EF553522.1	NOT1 Mut	F1101S + N1105A + K1114A + R1138A + N1144A + F1145A + K1276A + F1281A + E1284A
Hs DDX6 NM_004397.3	DDX6 E236Q	E236Q
	DDX6 Mut1	R94E + F101D + Q322A + N324A + R375A
	DDX6 Mut2	Q209A + H312A + T316A + R320A + R335A + K341A + K342A
Hs TNRC6A AY035864.1	TNRC6A-SD	Residues 1272 – 1709

Appendix Table S2. Reporters used in this study.

Organism	Name of the Reporter	Vector
Dm	F-Luc-5BoxB-poly(A)	pAc5.1C
	F-Luc-5BoxB-A_{95}-C_7-HhR	pAc5.1C
	F-Luc-A_{95}-C_7-HhR	pAc5.1C
	F-Luc-V5-5BoxB-A_{95}-C_7-HhR	pAc5.1C
	8nt-F-Luc-5BoxB-A_{95}- C_7-HhR	pAc5.1C
	8nt-F-Luc-V5-5BoxB-A_{95}- C_7-HhR	pAc5.1C
	8nt-F-Luc-5BoxB-poly(A)	pAc5.1C
	F-Luc-poly(A)	pAc5.1C
	F-Luc-*par6*-poly(A)	pAc5.1C
	8nt-F-Luc-*par6*-poly(A)	pAc5.1C
	R-Luc- A_{95}-HhR	pAc5.1C
	F-Luc-*Nerfin-1*-poly(A)	pAc5.1C
	8nt-F-Luc-*Nerfin-1*-poly(A)	pAc5.1C
	F-Luc-*cg5281*-poly(A)	pAc5.1C
	HA-GST	pAc5.1C
	8nt-HA-GST	pAc5.1C
	F-Luc-Met_{31}-5BoxB-poly(A)	pAc5.1C
	F-Luc(Leu)-V5-5BoxB-A_{95}-C_7-HhR*	pAc5.1C
	8nt-F-Luc(Leu)-V5-5BoxB-A_{95}-C_7-HhR*	pAc5.1C
Hs	R-Luc-5BoxB-poly(A)	pCI-neo
	R-Luc-5BoxB-A_{95}-MALAT1	pCI-neo
	R-Luc-poly(A)	pCI-neo
	R-Luc-6xMS2	pCI-neo
	R-Luc-6xMS2-A_{95}-MALAT1	pCI-neo
	R-Luc-A_{95}-MALAT1	pCI-neo
	TISU-R-Luc-6xMS2- A_{95}-MALAT1	pCI-neo
	TISU-HA-R-Luc-6xMS2-MALAT1	pCI-neo
	TISU-R-Luc-6xMS2-poly(A)	pCI-neo
	CAA-R-Luc-6xMS2-A_{95}- MALAT1	pCI-neo
	CGG-R-Luc-6xMS2bs-A_{95}- MALAT1	pCI-neo
	β-Globin-GAPDH (Control)	pcDNA3.1
	β-Globin-6xMS2-poly(A)	pcDNA3.1
	F-Luc	pEGFP-N3
	EGFP-HCV-F-Luc	pT7
	R-Luc-8x*let7*-poly(A)	pCI-neo
	R-Luc-Mut (*let7*-binding sites mutated)	pCI-neo
	TISU-R-Luc-8x*let7*-poly(A)	pCI-neo
	TISU-R-Luc-Mut (*let7*-binding sites mutated)	pCI-neo
	R-Luc-8x*let7*-A_{95}-MALAT1	pCI-neo
	R-Luc-Mut-A_{95}-MALAT1 (*let7*-binding sites mutated)	pCI-neo

* F-Luc(Leu)-V5 contains substitutions of all in-frame Met with Leu.

Appendix Table S3. Sequence of 5'-UTRs used in this study.

Organism	Reporter	Sequence
Dm	109nt-F-Luc	auaucacuaccguuugaguucuugugcuguguggauacuccucccgacacaaagccgcuccaucagccagcagucgucuaauccagagaccccggaucgggguaccaac**AUG**GCC
	8nt-F-Luc	auaucacu**AUG**GCC*
Hs	216nt-R-Luc	agaucacuagaagcuuuauugcgguaguuuaucacaguuaaauugcuaacgcagucagugcuucugacacaacagucucgaacuuaagcugcagugacucucuuaagguagccuugcagaaguuggucgugaggcacugggcagguguccacucccaguucaauuacagcucuuaaggcuagaguacuuaauacgacucacuauaggcuagccacc**AUG**GCU
	TISU-R-Luc	agauccaag**AUG**gcggcaGCU*
	CAA-R-Luc	agauccaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaagccacc**AUG**GCU
	CGG-R-Luc	agauccuagguugaaaguacuuugacggcggcggucaaucuuacggcggcggcggacauagauacggcggcgguagaaacuacggcggcggcggauuagaauaguaaagccacc**AUG**GCU

The first AUG is indicated in bold and underlined. *These 5'-UTRs were confirmed by 5'-RACE (Clontech), see Figs EV2A, EV3A and Appendix Fig S2.

Appendix Table S4. Antibodies used in this study.

Antibody	**Source**	**Catalog Number**	**Dilution**	**Monoclonal/ Polyclonal**
Anti-HA-HRP (For western blots)	Roche	12013 819 001	1:5,000	Monoclonal
Anti-HA (for immunoprecipitations an immunofluorescence)	Covance	MMS-101P		Mouse Monoclonal
Anti-GFP (for Western blotting)	Roche	11 814 460 001	1:2,000	Mouse Monoclonal
Anti-GFP (for immunoprecipitations)	In house			Rabbit polyclonal
Anti-tubulin	Sigma Aldrich	T6199	1:10,000	Monoclonal
Anti-mouse IgG-HRP	GE Healthcare	NA931V	1:10,000	Sheep Polyclonal
Anti-rabbit IgG-HRP	GE Healthcare	NA934V	1:10,000	Donkey Polyclonal
Anti-*Hs* DDX6	Bethyl Laboratories	A300-461A	1:3,000	Rabbit polyclonal
Anti-*Hs* PABP	Abcam	ab21060	1:5,000	Rabbit polyclonal
Anti-*Dm* GW182	In house		1:2,000	Rabbit polyclonal
Anti-*Dm* NOT1	Kind gift from E. Wahle		1:1,000	Rabbit polyclonal
Anti-*Dm* AGO1	Abcam	Ab5070	1:1,000	Rabbit polyclonal
Anti-*Dm* Me31B	In house		1:3,000	Rabbit polyclonal
Anti-*Hs* TNRC6A	Bethyl Laboratories	A302-329A	1:1000	Rabbit polyclonal
Anti-*Hs* eIF4A2 (for immunoprecipitations)	Abcam	Ab31218		Rabbit polyclonal
Anti-*Hs* eIF4A2 (for Western blotting)	In house		1:1,000	Rabbit polyclonal
Anti-*Hs* NOT2	Bethyl	A302-562A	1:2,000	Rabbit polyclonal
Anti-*Hs* NOT3	Abcam	Ab55681	1:2,000	Rabbit monoclonal
Anti-V5	AbD Serotec	MCA1360GA	1:5,000	Mouse Monoclonal
Anti-4E-T	Bethyl	A300-706A	1:1,000	Rabbit polyclonal
Alexa Fluor 594-labelled goat anti-mouse	Invitrogen	A-11005	1:1,000	Polyclonal

Appendix Methods

DNA constructs

The plasmids for expression of the β-globin-6xMS2 and the control β-globin-GAP mRNAs in human cells were kindly provided by Dr J. Lykke-Andersen and were described previously (Lykke-Andersen *et al*, 2000). The plasmid for expression of the R-Luc-5BoxB-poly(A) reporter in human cells was kindly provided by Dr W. Filipowicz and was described previously (Pillai *et al*, 2004). The reporter containing the MALAT1 3'-end was generated as described in Bhandari *et al*, 2014. All constructs were fully sequenced to confirm the presence of the mutations and the absence of additional mutations. Protein mutants used in this study are listed in Appendix Table S1.

Coimmunoprecipitation assays in S2 cells

The interaction of AGO1 (wild-type or mutants) with endogenous miRNAs and GW182 was tested as described previously (Rehwinkel et al, 2005; Eulalio *et al*, 2008). Briefly, 2.5×10^6 cells were seeded per well in 6-well plates and transfected with 1.5 µg HA-tagged proteins (MBP and AGO1 wild-type or mutants). Three days after transfection, the cells from 8 wells were pooled and resuspended in 2 ml of NET buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 and 10% glycerol) supplemented with protease inhibitors (Roche-EDTA free protease-inhibitor cocktail tablet). The cells were lysed by sonication (3 times 30 sec followed by 30 sec on ice) and incubated for 15 min on ice. Cell debris were removed by 15 min centrifugation at 16,000 *g* at 4°C. Aliquots for western (2.5%) and northern (5%) blot analysis were taken and the remaining cell lysate was incubated with anti-HA antibody (8 µg, Covance) for 1 hour at 4°C. Next, protein G-Sepharose beads

(GammaBind G-Sepharose, GE Healthcare) were added to the lysate (100 µl of a 50% slurry) and incubated for an additional hour. Beads were washed three times with NET buffer. An aliquot of beads (20%) was directly resuspended in SDS-PAGE sample buffer for western blot analysis. The remaining beads were resuspended in 200 µl of buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA pH 8.0 and 0.5% SDS and treated with proteinase K (1.5 mg/ml) for 2.5 hours at 50°C. RNA isolation and analysis was performed as described by Rehwinkel et al, 2005. miR-2a and $tRNA^{Ala}$ were detected using radioactively labeled antisense oligonucleotide probes (miR-2a: GCTCATCAAAGCTGGCTGTGATA, $tRNA^{Ala}$: CTCACATGCTAAGCGAGCGCTCTAC).

Coimmunoprecipitation assays in HEK293T cells

To test the interaction of AGO2 (wild-type or mutants) with endogenous miRNAs and GFP-tagged or endogenous TNRC6s, HEK293T cells (4×10^6/10-cm dish) were seeded one day before transfection using Turbofect (Thermo Scientific). The transfection mixtures contained plasmids expressing λN-HA tagged MBP (4 µg) or AGO2 [either wild-type (9 µg) or mutant (12 µg)] and 8 µg of plasmid expressing GFP-tagged TNRC6s. Cells were harvested 48 hours after transfection. Cells corresponding to 4 dishes were pooled, washed with PBS and lysed on ice for 15 min in 1 ml of NET buffer. Immunoprecipitations were performed as described for S2 cells. The oligonucleotide probes used for detection of miR-16 and $tRNA^{Ala}$ were as follows: CGCCAATATTTACGTGCTGCTA (miR-16) and CTCTACCGCTTGAGCTAATTCCCC ($tRNA^{Ala}$).

RNA immunoprecipitation in HEK293T cells

HEK293T cells (4×10^6/10-cm dish) were seeded 24 hours before transfection with Lipofectamine 2000. The transfection mixtures contained 5 μg of pEGFP-N3-F-Luc transfection control reporter and any one of the following reporters: pCIneo-R-Luc-6xMS2-A_{95}-MALAT1 (10 μg), pCIneo-TISU-R-Luc-6xMS2-MALAT1 (8 μg), pCIneo-CAA-R-Luc-6xMS2-A_{95}-MALAT1 (15 μg) or pCIneo-CGG-R-Luc-6xMS2-A_{95}-MALAT1 (8 μg). Cells were washed with ice-cold PBS and lysed in 500 μl of NET buffer for 15 min on ice. The cell debris were removed by a 15 min centrifugation at 16,000 *g* at 4°C. Aliquots (5%) were taken for western and northern blot analysis. The remaining lysate was incubated with 3 μg of anti-eIF4A2 antibody (Abcam, ab31218) for 2 hours at 4°C. Next, protein G-Sepharose beads were added to the lysate (100 μl of a 50% slurry) and incubated for additional 2 hours. After 2 hours incubation, beads were washed 3 times with NET buffer and resuspended in 1 ml NET buffer without detergent. An aliquot (20%) of the bead suspension was directly resuspended in SDS-PAGE sample buffer for western blot analysis. The remaining beads were used for RNA isolation using TriFast (Peqlab Biotechnologies). At this stage, samples were spiked with 4 μg of double stranded RNA. RNA samples were treated with DNase I (Turbo DNA-free kit, Ambion, AM1907). Input and precipitated fractions were analyzed by western and northern blotting. Note that the protein G-Sepharose beads used for RNA immunoprecipitation were preincubated with yeast RNA (250 μg of yeast RNA /100 μl of 50% slurry) and washed with PBS before use.

Tethering assays and knockdowns

For tethering assays in S2 cells, the transfection mixtures contained 0.1 μg of Firefly luciferase reporter plasmid (or 0.3 μg for the reporter containing a 8-nt 5'-UTR), 0.4

µg of *Renilla* luciferase transfection control and 4 ng of plasmids expressing the λN-HA tagged proteins. In the experiment described in Fig 2M, the transfection mixtures contained in addition 0.95 µg of plasmids expressing GFP or GFP-DCP1 GSSG mutant (Chang et al, 2014). For tethering assays in human cells, HEK293T cells (0.8 × 10^6/well in six-well plates) were transfected using Lipofectamine 2000 (Life technologies). The transfection mixtures contained 0.25 µg of *Renilla* luciferase reporter plasmids (0.5 µg for the reporters containing the MALAT 3' end), 0.25 µg of the pEGFP-N3-F-Luc transfection control and 0.1–1 µg of plasmids expressing λN-HA or HA-MS2 tagged proteins [0.3 µg for TNRC6A-SD and AGO2; 0.1 µg for DDX6 (wild-type or mutant) and 1 µg for NOT1].

In the experiment described in Fig 2N and Fig 5E–G, the transfection mixtures contained 0.5 µg of the control plasmid (β-globin-GAP), 0.5 µg of the plasmid encoding the β-globin-6xMS2, plasmids expressing the MS2-HA tagged proteins (0.2 µg for TNRC6A-SD, 0.1 µg for AGO2 and 2 µg for CNOT1). In Fig 2N, the transfection mixtures contained in addition plasmids expressing HA-tagged DCP2 (either wild-type or catalytically inactive DCP2* mutant; 1 µg). Me31B knockdowns in S2 cells and DDX6 depletion in HeLa cells were performed as described previously (Eulalio *et al*, 2007; Chen *et al*, 2014).

Polysome profiling in HEK293T cells

HEK293T cells (9 × 10^6/15-cm dish) were seeded one day before transfection with Lipofectamine 2000 (Life Technologies). The transfection mixtures contained 20 µg of the R-Luc-6xMS2-$(A)_{96}$-MALAT1 reporter, 4 µg of the pEGFP-N3-F-Luc transfection control and 6 µg of a plasmids expressing MS2-HA or MS2-HA-DDX6. Cells were treated with cycloheximide 48 hrs after transfection at a final

concentration of 50 µg/ml for 30 min. Cells were washed once with ice-cold PBS (containing 50 µg/ml cycloheximide), and lysed in 300 µl of ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Triton X-100, 2 mM DTT, 1 U/µl RiboLock RNase inhibitor, 0.5% Na-deoxycholate and 50 µg/ml cyclohexamide for 10 min on ice. The cell debris were removed by a 10 min centrifugation at 10,000 *g* at 4°C. The supernatants were layered on top of a linear 10–50% (w/v) sucrose gradient (prepared using Gradient Master 107ip Biocomp) in 10 mM Tris-HCl (pH 7.4), 75 mM KCl, 1.5 mM $MgCl_2$, and 50g/ml cycloheximide. Centrifugation was carried out in a Beckmann, SV55Ti rotor at 41,000 r.p.m. for 2 hours at 4°C.

Fractions were collected using a density-gradient fractionation system (Teledyne Isco), and polysome profiles were monitored by absorbance of light at 254 nm. Fractions were spiked with 10 µg of double-stranded RNA and treated with proteinase K (0.1 mg/ml, in 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.5% SDS) for 30 min at 42°C. Total RNA was isolated by two rounds of phenol:chloroform extraction followed by one chloroform extraction. RNA samples were treated with DNase I and analyzed by northern blotting.

Immunofluorescence in HeLa cells

Immunostainings of HeLa cells performed as described by Lazzaretti *et al*, (2009).

Knock-down in HeLa cells

HeLa cells were seeded in 6-well plates and transfected using Lipofectamine 2000 (Life technologies) for luciferase assays. DDX6 knockdowns and complementation assays were performed as described in Chen et al., 2014. Briefly, HeLa cells were

transfected with 4 µg of plasmids expressing control or DDX6 shRNA. One day after transfection, cells were selected in medium containing 1.5 µg/ml puromycin. After twenty-four hours of selection, cells were seeded in 6-well plates in medium without puromycin. Twenty-four hours after reseeding, cells were re-transfected with the transfection mixtures contained 0.8 µg of plasmids expressing the relevant shRNA, 0.5 µg of the R-Luc and 0.2 µg of the F-Luc control reporter together with λN-HA-tagged proteins as indicated in the figure. 72 hours after the second transfection, cells were harvested and luciferase activities were measured.

5′ RACE

RNA samples were harvested from HEK293T or S2 cells expressing the indicated reporters. 5′ RACE was performed according to manufacturer's protocol (Clontech, SMARTer™ RACE cDNA Amplification Kit) using the universal primer and R-Luc (ggaaggttcagcagctcgaaccaagcggtgaggtac) or F-Luc (gataaataacgcgcccaacaccggttaaaagaattgaagagagttttc) reverse primers. The 5′ RACE products were run on an a 1% agarose gel, visualized by ethidium bromide staining and purified using the QIAquick Gel Extraction kit (Qiagen). A fraction of the purified cDNA was sent directly for sequencing, whereas another fraction was cloned in the TOPO cloning vector using the TOPO-TA cloning kit (Thermo Fischer Scientific). After transformation of *E. coli,* DNA plasmids were isolated from single colonies and send for sequencing.

Appendix References

Bhandari D, Raisch T, Weichenrieder O, Jonas S, Izaurralde E (2014) Structural basis for the Nanos-mediated recruitment of the CCR4-NOT complex and translational repression. *Genes Dev* 28: 888 – 901

Cope CL, Gilley R, Balmanno K, Sale MJ, Howarth KD, Hampson M, Smith PD, Guichard SM, Cook SJ (2014) Adaptation to mTOR kinase inhibitors by amplification of eIF4E to maintain cap-dependent translation. *J Cell Sci* 127: 788 – 800

Lykke-Andersen J, Shu MD, Steitz JA (2000) Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. *Cell* 103: 1121 – 1131

Miyoshi K, Okada TN, Siomi H, Siomi MC (2009) Characterization of the miRNA-RISC loading complex and miRNA-RISC formed in the Drosophila miRNA pathway. *RNA* 15: 1282 – 1291

Okamura K, Ishizuka A, Siomi H, Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev* 18: 1655 – 1666