GW182 interaction with Argonaute proteins is essential both for miRNA-mediated translational repression and mRNA decay

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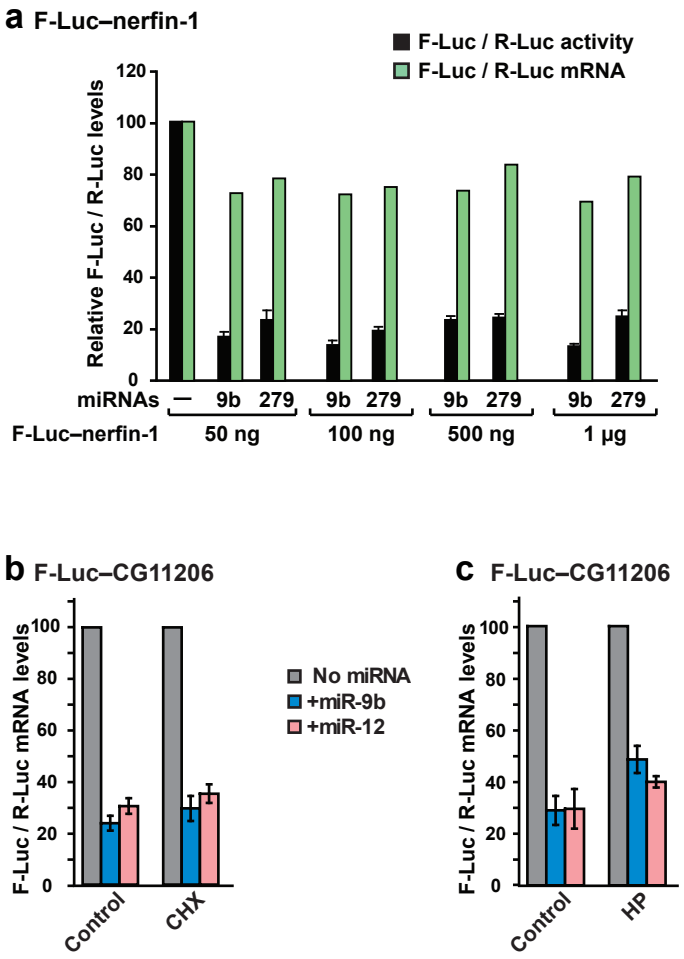
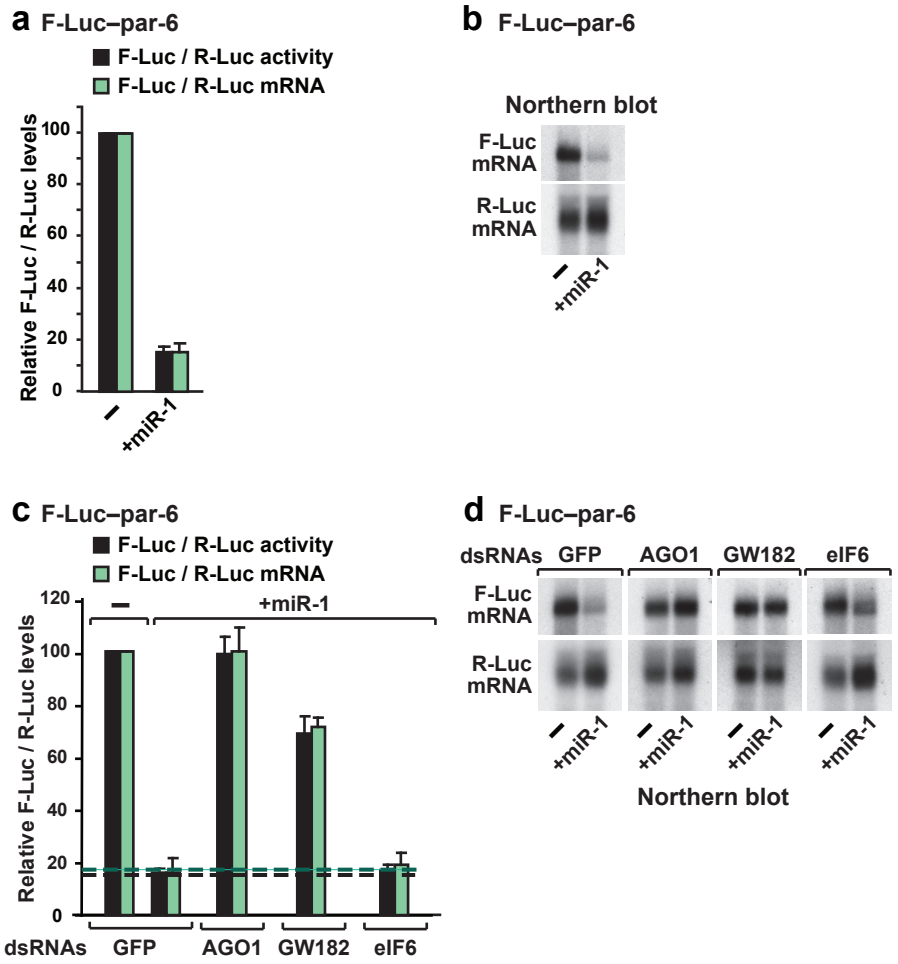
Supplementary Figure 1



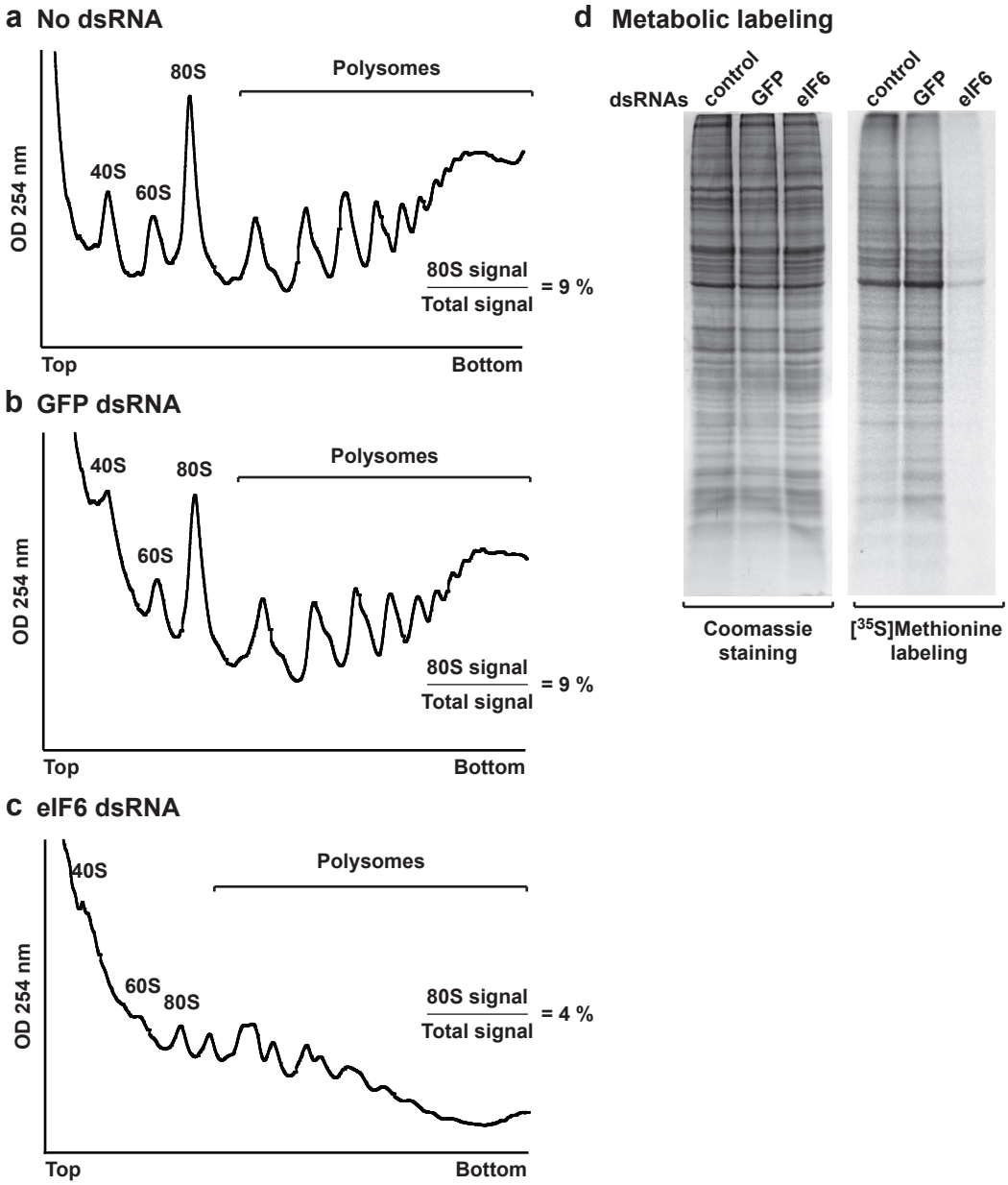
Supplementary Figure 1 Different modes of miRNA-mediated gene silencing. (**a**) S2 cells were co-transfected with a mixture of three plasmids: plasmids expressing primary miRNAs (miR-9b or miR-279) or the corresponding empty vector (–), a plasmid expressing *Renilla* luciferase (R-Luc), and increasing amounts of the F-Luc–nerfin-1 reporter as indicated. Firefly luciferase activity (black bars) and mRNA levels (green bars) were normalized to that of the *Renilla* luciferase transfection control. For each condition, the normalized values of F-Luc activity and mRNA levels were set to 100 in cells transfected with the empty vector (*i.e.* in the absence of the miRNAs, only shown for the lowest amount of reporter). (**b,c**) S2 cells were co-transfected with a mixture of three plasmids: the F-Luc–CG11206 reporter, plasmids expressing primary miRNAs (colored bars) or the corresponding empty vector (gray bars), and a plasmid expressing *Renilla* luciferase (R-Luc). Cells were treated with cycloheximide (CHX, 10 µg/ml; SIGMA) or hippuristanol (HP, in DMSO; 4 µM final concentration) 7 hours before isolating the RNA samples. In panel (**c**) control cells were treated with corresponding amounts of DMSO. Firefly luciferase mRNA levels were normalized to that of the *Renilla* luciferase. The normalized values were set to 100 in the absence of miRNAs (gray bars). Mean values ± standard deviations from three independent experiments are shown.

Supplementary Fig. 2.



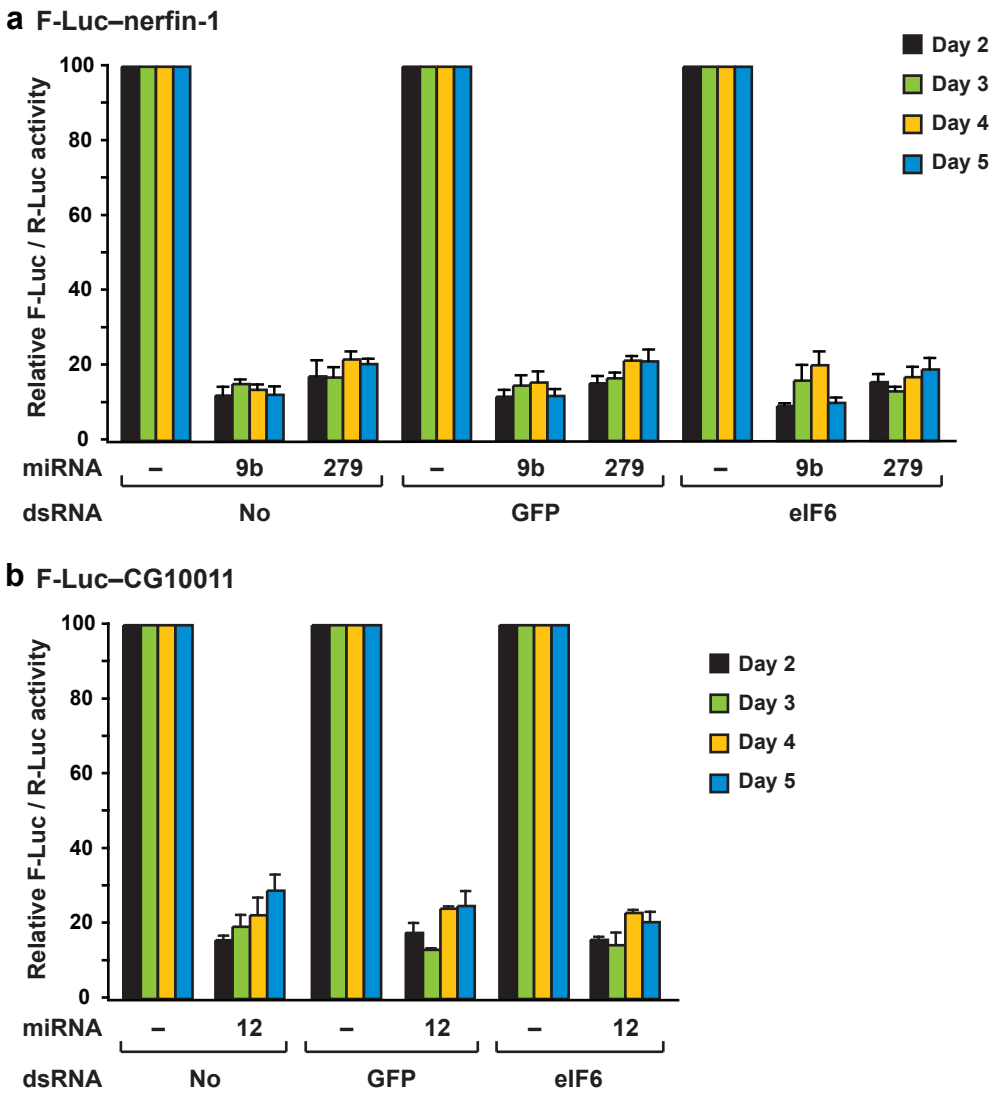
Supplementary Figure 2 Depleting eIF6 does not prevent miRNA-mediated mRNA decay. (**a–d**) S2 cells were co-transfected with a mixture of three plasmids: the F-Luc–par-6 reporter, a plasmid expressing primary miR-1 (+) or the corresponding empty vector (–); and a plasmid expressing *Renilla* luciferase (R-Luc). In panel (**c**) cells were treated with the indicated dsRNAs on days 0 and 4, and transfected on day 6. Samples were analyzed as described in Figure 1. (**b,d**) Northern blot analysis of representative RNA samples isolated from S2 cells shown in panels (**a,c**), respectively.

Supplementary Fig. 3.



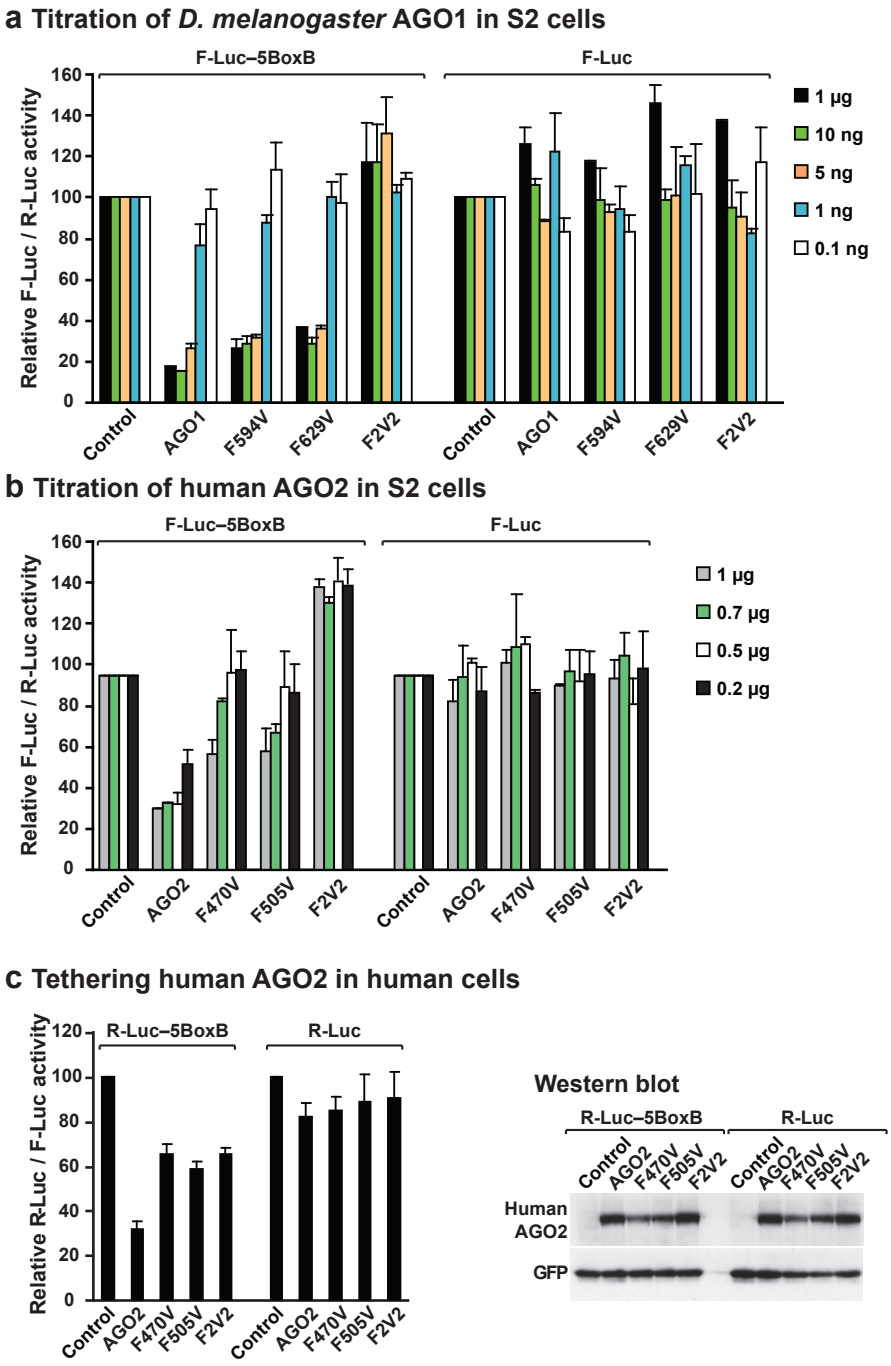
Supplementary Figure 3 Depletion of eIF6 reduces 80S ribosome and polysome formation. (**a–d**) S2 cells were treated with a dsRNA targeting endogenous eIF6 on day 0 and day 4. GFP dsRNA served as a negative control. (**a–c**) On day 9 cells were lysed and fractionated on 15%–45% sucrose gradients. As a control, extracts from untreated cells were also analyzed. The absorbancy at 254 nm of each fraction was quantitated and normalized to the total intensity across all fractions. (**d**) An aliquot of cells collected on day 9 was pulse labeled with [^{35}S]methionine for 1 h. Total cell extracts were analyzed by SDS-PAGE followed by fluorography.

Supplementary Fig. 4.



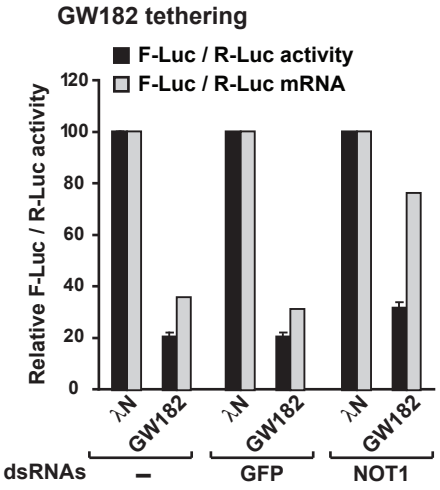
Supplementary Figure 4 Depleting eIF6 does not prevent miRNA-mediated gene silencing. (**a,b**) S2 cells were co-transfected with a mixture of three plasmids: the indicated F-Luc reporters, a plasmid expressing primary miRNAs or the corresponding empty vector (–); and a third plasmid expressing *Renilla* luciferase (R-Luc). One day after transfection cells were treated with the indicated dsRNAs. Firefly and *Renilla* luciferase activities were measured 2, 3, 4 and 5 days after addition of dsRNAs. Firefly luciferase activity was normalized to that of the *Renilla* luciferase transfection control. For each condition, the normalized values of F-Luc activity were set to 100 in cells transfected with the empty vector [(–); *i.e.* in the absence of the miRNAs].

Supplementary Fig. 5.



Supplementary Figure 5 Activity of *D. melanogaster* AGO1 and human AGO2 mutants in tethering assays. (**a**) S2 cells were transfected with the F-Luc–5BoxB or F-Luc reporters, a plasmid expressing *Renilla* luciferase, and decreasing amounts of plasmids expressing the λN-HA-peptide (Control) or λN-HA fusions of wild-type AGO1 or AGO1 mutants as indicated. Firefly luciferase activity was normalized to that of the *Renilla* and set to 100 in cells expressing the λN-HA-peptide alone for each condition (Control bars). (**b**) A similar experiment as described in panel (**a**) was repeated with wild-type or mutated human λN-HA-AGO2 protein in S2 cells. (**c**) HeLa cells were transfected with the R-Luc-5BoxB or R-Luc reporters described by Pillai *et al*. (2004), a plasmid expressing firefly luciferase, and a plasmid expressing GFP. Vectors expressing the λN-HA-peptide (Control) or λN-HA fusions of wild-type AGO2 or AGO2 mutants were included in the transfection mixtures as indicated. For each reporter, *Renilla* luciferase activity was normalized to that of the firefly and set to 100 in cells expressing the λN-HA-peptide alone (Control bars). Protein expression levels were analyzed by Western blotting.

Supplementary Fig. 6.



Supplementary Figure 6 GW182 represses translation of bound mRNAs. S2 cells were treated with GFP or NOT1 dsRNAs on days 0 and 4. On day 6 cells were transfected with the F-Luc–5BoxB reporter, a plasmid expressing *Renilla* luciferase, and 5 ng of vectors expressing the λN-HA-peptide or λN-HA-GW182. F-Luc activities (black bars) and mRNA levels (gray bars) were quantitated and normalized to that of the *Renilla* control. In cells expressing the λN-HA-peptide alone, these values were set to 100 for each knockdown.

Supplementary Table 1 - List of constructs and oligonucleotides used in this study.

Contruct	Plasmid	Cloning sites	5' oligos	3' oligos
F-Luc-nerfin-1	pJLuc	gift from Steve Cohen		
miR-9b	pAc5.1A	EcoRI-XhoI	5' CGGAATTCCAGGTCAATCGTCAGAAACAATTTAAG	5' CCGCTCGAGAGACGCTGCCTCTTAGCCTTTGGCGAG
miR-279	pAc5.1A	EcoRI-XhoI	5' CCGGAATTCAAGAGACGCCGCTTATCAACGCTA	5' CCGCTCGAGCTCTTTTGTGCACTCGAACAGGTG
F-Luc-CG11206	pAc5.1B-F-Luc	EcoRI-SalI	5' CCGGAATTCGAGTGAAAAGCTAGCTAGATAGGA	5' ACGCGTCGACAGAGTTTTGATCATGGGTTTATTC
miR-12	pAc5.1A	EcoRI-XhoI	5' CGGAATTCTATTTTCATTTTCGTTTTCCCTTAAATTTG	5' CCGCTCGAGCTGGCCATTCGCCTCCTTTAGGCTCTCCAC
F-Luc-par6	pJLuc	gift from Steve Cohen		
miR-1	pAc5.1A	EcoRI-XhoI	5' CCGGAATTCCCCCTTTTTCCCTGCAATTACCGT	5' CCGCTCGAGATTTAATGCCTGTGTGGAATGGTA
Dm AGO1	pAc5.1B-λN-HA	Rehwinkel et al. (18)	5' GGGAATTCTATGTACCCATACGATGTTCCAGATTACGCTATGTCCACGGAGCGTGAGCTGGC	5' CCGCTCGAGTTAGGCAAAGTACATGACCTTCTTGG
Dm AGO1 F594V	pAc5.1B-λN-HA		mutagenesis oligos : 5' CGAGGATGCGCTGCGTAATGTCACCCAGCAGCTGCAGAAGATC	5' GATCTTCTGCAGCTGCTGGGTGACATTACGCAGCGCATCCTCG
Dm AGO1 F629V	pAc5.1B-λN-HA		mutagenesis oligo: 5' CGGATCAAGTGGAACCCATGGTCCGTTACCTGAAGATCACC	5' GGTGATCTTCAGGTAACGGACCATGGGTTCCACTTGATCCG
Dm AGO1 F2V2	pAc5.1B-λN-HA		mutagenesis oligos: same as for F594V and F629V	same as F594V and F629V
olgos used to generate AGO1 siRNA resistant version			5' CCGATTCGCAGCGCGTCAAGTTCACCAAAGAAATTAAAGGCCTGAAGATCGAGATCACCCACTGCGG	5' CCGCAGTGGGTGATCTCGATCTTCAGGCCTTTAATTTCTTTGGTGAACTTGACGCGCTGCGAATCGG
Human AGO2	pAc5.1B-λN-HA	EcoRI-NotI	5' CGGAATTCTATGTACTCGGGAGCCG	5' TTCCTTTTTTGCGGCCGCCTCAAGCAAAGTACATGGTG
Human AGO2-F470V	pAc5.1B-λN-HA	EcoRI-NotI	mutagenesis oligos: 5' CACGGAAGTCCATCTGAAGTCCGTCACAGAGCAGCTCAGAAAGATC	5' GATCTTTCTGAGCTGCTCTGTGACGGACTTCAGATGGACTTCCGTG
Human AGO2-F505V	pAc5.1B-λN-HA	EcoRI-NotI	mutagenesis oligos: 5' GCGGACAGCGTGGAGCCCATGGTCCGGCACCTGAAGAACACGTATG	5' CATACGTGTTCTTCAGGTGCCGGAcCATGGGCTCCACGCTGTCCGC
Human AGO2-F2V2	pAc5.1B-λN-HA	EcoRI-NotI	mutageneis oligos: same as for F470V and F505V	same as F470V and F505V
GW182	pAc5.1B-λN-HA	HindIIII-NotI	5' CCCAAGCTTGATGGCTTACCCATACGATGTTCCAGATTACGCTATGCGTGAAGCCCTTTTTTCCC	5' ATAAGAATGCGGCCGCTTAATCATCAACAATGGAATAAACGAG
GW182(1-592)	pAc5.1B-λN-HA	HindIII-EcoV	same as GW182	5' CTCCCAAGCTTGATGGATGGTTGGCGTCGACATG
Renilla-V5	pAc5.1A-R-Luc-V5	KpnI-XhoI	5' CGGGGTACCAACATGACTTCGAAAGTTTATGA	5' CCCTCGAGTTGTTCATTTTTGAGAACTCGCTC
Renilla	pAc5.1A-R-Luc	KpnI-XhoI	5' CGGGGTACCAACATGACTTCGAAAGTTTATGA	5' CCCTCGAGTCATTGTTCATTTTTGAGAACTCGCTC
Human AGO2	pCI-Neo	gift from Witec Filipowicz		
Human AGO2-F470V	pCI-Neo		mutants generated using the same oligonucleotides as above	
Human AGO2-F505V	pCI-Neo			
Human AGO2-F2V2	pCI-Neo			

T7 oligonucleotides for dsRNA synthesis				
GFP			5' TTAATACGACTCACTATAGGGAGGATGGTGAGCAAGGGCGAGGAG	5' TTAATACGACTCACTATAGGGAGGCTTGTACAGCTCGTCCATGCCG
AGO1			5' TTAATACGACTCACTATAGGGAGACATTAAAAAGCTGACCGATATGC	5' TTAATACGACTCACTATAGGGAGATTGACGTTGATCTTCAGACACAG
GW182			5' TTAATACGACTCACTATAGGGAGGAGGAGGTAACGGGTCAAGCAATATA	5' TTAATACGACTCACTATAGGGAGGCCGGCTATTCCTATACCTCCCGTT
eIF6			5' TAATACGACTCACTATAGGGCCACGTAAATACAGAGAAAATG	5' TAATACGACTCACTATAGGGCCCTGGTTGCTCAGCACGGCGT
NOT1			5' TAATACGACTCACTATAGGGAGAGCTCACTCAGCATCGCCATCG	5' TAATACGACTCACTATAGGGAGAGTAGGCGAAGGCCGACACAAT