Supplemental Data

Importance of Translation and Non-Nucleolytic

Ago Proteins for On-Target RNA Interference

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

DNA and RNA used for transfection

A summary of reporter plasmids and the mRNAs they encode is provided in Tables S2 and S3. Plasmids pCL-6E1, pCL-2E1, pCL-2P, pMIR125b, pMIR125bΔ, and pCMV-Luc have been described previously [10, 11]. Plasmids pCL-G_P, pCL-G_M, and pCL-$4G_M$ are identical to pCL-6E1 except that the six copies of E1 have been replaced by one copy of an element perfectly complementary to siEGFP (G_P: CGGCAAGCTGACCCTGAAGTTC) or 1-4 copies of an element partially complementary to siEGFP (G_M: CGGCAAGCTATACCTGAAGTTC), respectively. The spacing between 3' UTR elements was 8-17 bp for pCL-6E1, 8 bp for pCL-2E1, 6 bp for pCL-2P, and 6-10 bp for pCL-$4G_M$. Plasmid pCL-2P-UTR encodes a firefly luciferase transcript bearing two copies of element P 69-124 nucleotides downstream of the translation termination codon, whereas plasmid pCL-2P-ORF is identical but for a point mutation in that termination codon that causes ribosomes to translate 45 additional codons before terminating at another stop codon just downstream of the two copies of element P. Plasmid pRL was constructed by replacing the firefly luciferase coding region of pCMV-Luc with the Renilla luciferase coding region of phRL-TK (Promega).

Plasmids pNHA-Ago1, pNHA-Ago2, pNHA-Ago3, and pNHA-Ago4 were constructed from plasmid pCI-λNV5 [25] by inserting tandem DNA fragments encoding a hemagglutinin (HA) epitope tag (YPYDVPDYA), a hexaglycine linker, and human Ago1, Ago2, Ago3, or Ago4, respectively [13], downstream of a sequence encoding a high-affinity variant of the boxB-binding domain of lambda N (MNARTRRRERRAEKQAQWKAAN). In the resulting hybrid proteins, this boxB-binding domain was joined to the amino terminus of each Ago protein via a lengthy spacer that contained the HA tag within it and ended with six flexible glycine residues. Plasmid pHA-Ago2 was constructed from pNHA-Ago2 by deleting the lambda N-related gene segment. Plasmid pNHA-Ago2-D597A was constructed by mutating the active-site Asp-597 codon of pNHA-Ago2 (GAC → GCC). Plasmid pNHA-Ago2-F470V/F505V, used as a negative control [22] (data not shown), was constructed by mutating codons 470 (TTC → GTC) and 505 (TTC → GTC) of pNHA-Ago2 to render the encoded Ago2 protein nonfunctional in tethered repression assays. Plasmid pTR+10boxB was constructed from plasmid phRL-TK by inserting two tandem copies of a β-globin 5boxB DNA fragment encoding five boxB elements [26] into the Renilla luciferase 3' UTR.

siRNA duplexes used for transfection included siEGFP (5'-GAACUUCAGGGUCAGCU UGCCG-3' + 5'-GCAAGCUGACCCUGAAGUUCAU-3') [27], siAgo1 (5'-UUCUUGAGCAC

CUCUUCUCUU-3' + 5'-GAGAAGAGGUGCUCAAGAAUU-3'), siAgo2 (5'-UUCAGAUGG ACUUCCGUGCUU-3' + 5'-GCACGGAAGUCCAUCUGAAUU-3'), siAgo3 (5'-UUACCAA UCUGCUAAUUUCUU-3' + 5'-GAAAUUAGCAGAUUGGUAAUU-3'), siAgo4 (5'-AUUGC UAUUAGUUCUGGCCUU-3' + 5'-GGCCAGAACUAAUAGCAAUUU-3') [13], and siGL2 (5'-UCGAAGUAUUCCGCGUACGUU-3' + 5'-CGUACGCGGAAUACUUCGAUU-3').

Transfection and reporter assays

293T and HeLa cells were grown in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS); HCT116 cells were grown in modified McCoy's 5A medium (Invitrogen) containing 10% FBS. Lipofectamine 2000 (Invitrogen) was used for all transfections except when DharmaFECT 2 (Dharmacon) was used to compare repression by siEGFP in 293T, HeLa, and HCT116 cells. Firefly and Renilla luciferase activity was measured in a Tecan SpectraFluor Plus instrument by using the Dual-Glo luciferase assay system (Promega) according to the manufacturer's instructions. Reporter mRNAs were assayed by Northern blot analysis of equal amounts of total cytoplasmic RNA, as previously described [10, 11]. Northern blot band intensities were quantified by using a Molecular Dynamics Storm 820 Phosphorimager.

In the miR-125b repression assays, 293T cells were transiently transfected with a DNA mixture (1 μg) that contained a plasmid bearing a firefly luciferase reporter gene (50 ng), a plasmid encoding Renilla luciferase as an internal standard (pRL, 75 ng), and a plasmid encoding or not encoding miR-125b (pMIR125b or pMIR125bΔ, respectively; 875 ng; [10]). In the siEGFP repression assays, 293T, HeLa, or HCT116 cells were transiently transfected with a DNA/RNA mixture that contained a plasmid bearing a firefly luciferase reporter gene (50 ng), a plasmid encoding Renilla luciferase as an internal standard (75 ng), and either siEGFP or siGL2 (negative control) (20 nM final concentration). Assays of luciferase protein and mRNA were performed on extracts prepared 36 hr (Table 1; Figures 1, S1, S2, and S3), 42 hr (Figures 3, 4, S8, and S9), or 60 hr (Table S1) after transient transfection, as described above. To test the effect of diminished levels of endogenous Ago proteins, 293T cells were transfected with siAgo1, siAgo2, siAgo3, siAgo4, or siGL2 (negative control) (20 nM final concentration) 36 hr prior to transient transfection with the aforementioned plasmids. Measurements of repression by miR-125b and siEGFP were repeated at least six times.

Normalized levels of firefly luciferase protein in transfected cells were calculated by dividing the enzymatic activity of firefly luciferase by that of Renilla luciferase (internal standard) after subtracting from each any background activity observed in extracts of untransfected cells. Normalized levels of firefly luciferase mRNA were calculated from the ratio of firefly luciferase mRNA to Renilla luciferase mRNA, as determined by Northern blotting. Repression ratios for protein production or mRNA concentration were calculated by dividing the normalized concentration of firefly luciferase protein or mRNA in the absence of an si/miRNA by its normalized concentration in the presence of an si/miRNA. Repression ratios for translation efficiency (protein synthesis per mRNA molecule) were calculated by dividing the repression ratio for protein production by the repression ratio for mRNA concentration. Repression ratios per element for a reporter containing *n* copies of the same element were determined by calculating the *n*th root of the total repression ratio. Reported error ranges correspond to standard deviations.

The sensitivity and precision of the tethering assays was improved by using a Renilla luciferase reporter bearing ten copies of boxB, as five copies would have allowed Ago2 to repress luciferase production by only a factor of two [19, 20]. 293T cells were transiently transfected with a DNA mixture (1 μg) that contained a plasmid encoding a human Ago protein (6-500 ng), plasmid pTR+10boxB (500 ng), plasmid pCMV-Luc (20 ng), and plasmid pUC19. After 36 hr, protein and RNA extracts were prepared and assayed as described above. The effect of each tethered Ago paralog on reporter gene expression was determined at the protein and mRNA level by measuring Renilla luciferase activity (normalized to firefly luciferase activity) and by Northern blot analysis of RL+10boxB mRNA (normalized to firefly luciferase mRNA). Repression ratios for protein production, cytoplasmic mRNA concentration, and translation efficiency were calculated from the ratio of the normalized concentration of Renilla luciferase protein and mRNA in the absence versus the presence of the transfected N-HA-tagged Ago gene. Each experiment was repeated at least three times.

Immunoblot assays

Protein extracts of transfected cells were fractionated by electrophoresis on 12% polyacrylamide-SDS gels and electrotransferred to nitrocellulose. After blocking with 5% dry milk in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T), the blot was probed for 1 hr at room temperature with monoclonal anti-HA antibodies (diluted 1:10,000 in PBS-T, Sigma), polyclonal anti-Ago2 antibodies (1:1,500, Upstate/Millipore), or monoclonal anti-actin antibodies (1:3,000, Sigma), incubated with a secondary antibody conjugated to horseradish peroxidase (1:20,000 in PBS-T, Bio-Rad), and detected with an Immun-Star HRP chemiluminescence kit (Bio-Rad) and a FluorChem 8900 imager (Alpha Innotech). Alternatively, the immunoblot was probed with monoclonal anti-Ago1 or anti-Ago2 antibodies provided by Gunter Meister, as previously described [28].

Microarray data

The relative concentration of Ago mRNAs in various human tissues was calculated from data in the GeneAtlas Version 2 microarray database of the Genomics Institute of the Novartis Research Foundation (http://symatlas.gnf.org/SymAtlas/) [24]. dChip software [29] was used for normalization and model-based analysis of those data, which were obtained with Affymetrix HG-U133A GeneChip arrays.

Real-time RT-PCR

DNase I-treated total RNA from 293T or HCT116 cells (1 μg) was reverse transcribed with an iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Real-time PCR was performed on a DNA Engine Opticon 2 (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad) and primer pairs specific for each of the four human Ago mRNAs or for 18S rRNA (internal standard) [13, 30]. The PCR mixtures were heated to 95°C for 3 min and then subjected to 40 amplification cycles (15 sec at 95°C, 30 sec at 58°C, 30 sec at 72°C), and the data were analyzed with Opticon Monitor 3.1 software (Bio-Rad).

Supplemental references

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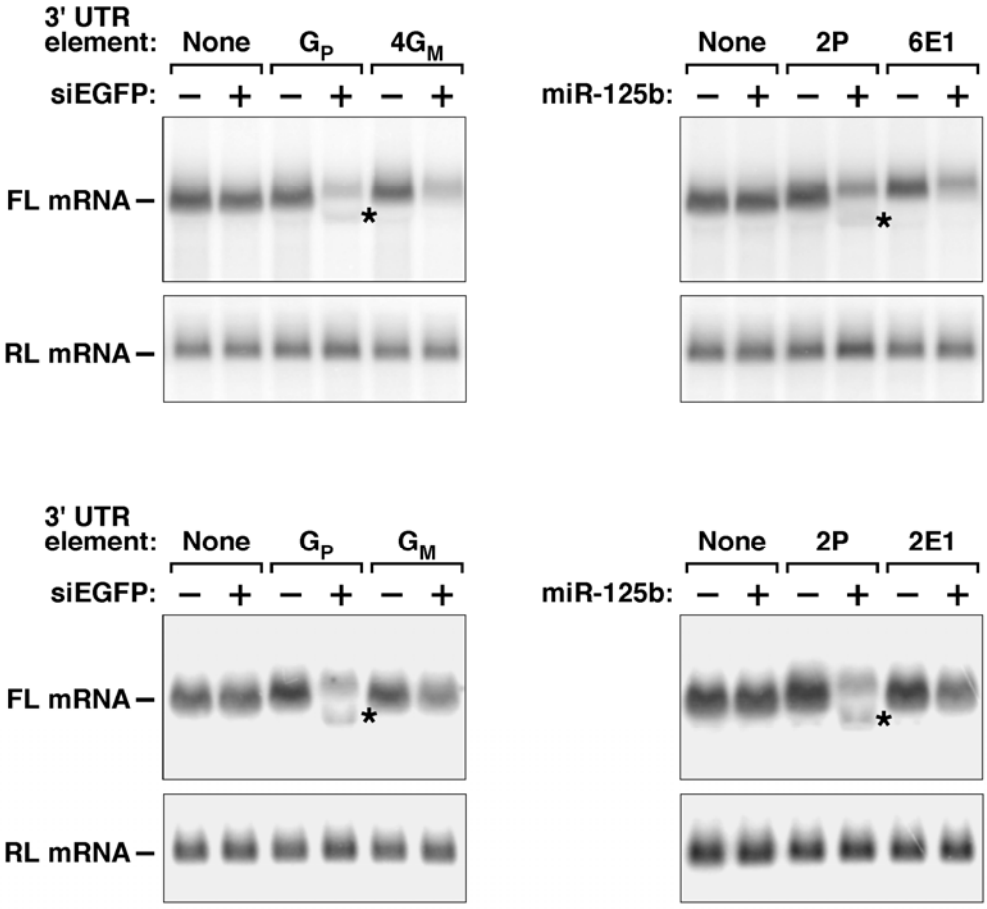

Figure S1. Influence of siEGFP and miR-125b on reporter mRNA concentration.

RNA extracted from 293T cells 36 hr after transient transfection was analyzed by Northern blotting to determine the effect of siEGFP and miR-125b on the cytoplasmic concentration of a firefly luciferase (FL) reporter mRNA containing element G_P (one copy), G_M (one or four copies), P (two copies), or E1 (two or six copies) in the 3' UTR. Renilla luciferase (RL) mRNA served as an internal standard. The bands marked by asterisks correspond in length to the 5' products of mi/siRNA-guided endonucleolytic cleavage of the reporter mRNAs [11].

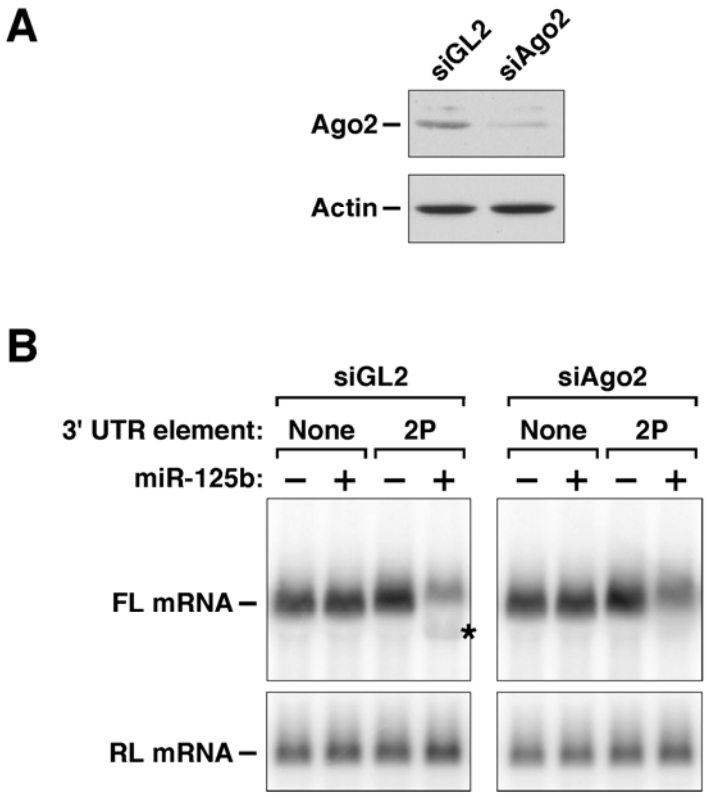

Figure S2. Effect of Ago2 deficiency on the ability of miR-125b to reduce the cellular concentration of FL+2P mRNA.

(A) Diminished abundance of Ago2. 293T cells were transiently transfected with siRNA complementary to Ago2 mRNA or GL2 mRNA (negative control), and levels of Ago2 and actin were examined 72 hr later by immunoblot analysis of protein extracts.

(B) Changes in reporter mRNA concentration. 293T cells were transiently transfected with siRNA complementary to Ago2 mRNA or GL2 mRNA (negative control). After 36 hr, the same cells were transiently cotransfected with a firefly luciferase reporter gene bearing two copies of element P (FL+2P) in the 3' UTR, a gene encoding or not encoding miR-125b, and a Renilla luciferase (RL) gene (internal standard). After an additional 36 hr, cytoplasmic RNA was isolated and analyzed by gel electrophoresis and blotting, using radiolabeled probes complementary to FL and RL mRNA.

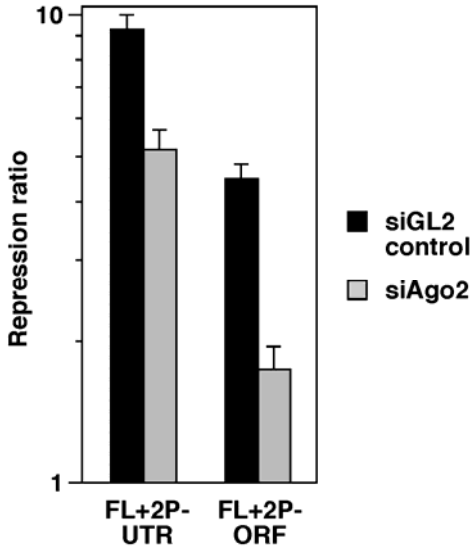

Figure S3. Effect of Ago2 deficiency on the downregulation of FL+2P-UTR and FL+2P-ORF by miR-125b.

293T cells were transiently transfected with siRNA complementary to Ago2 mRNA, resulting in a 75% reduction in the cellular concentration of Ago2 protein, or with siRNA complementary to GL2 mRNA (negative control), and the effect on downregulation of FL+2P-UTR and FL+2P-ORF by miR-125b was compared by measuring luciferase activity. Error bars represent the standard deviation of multiple measurements.

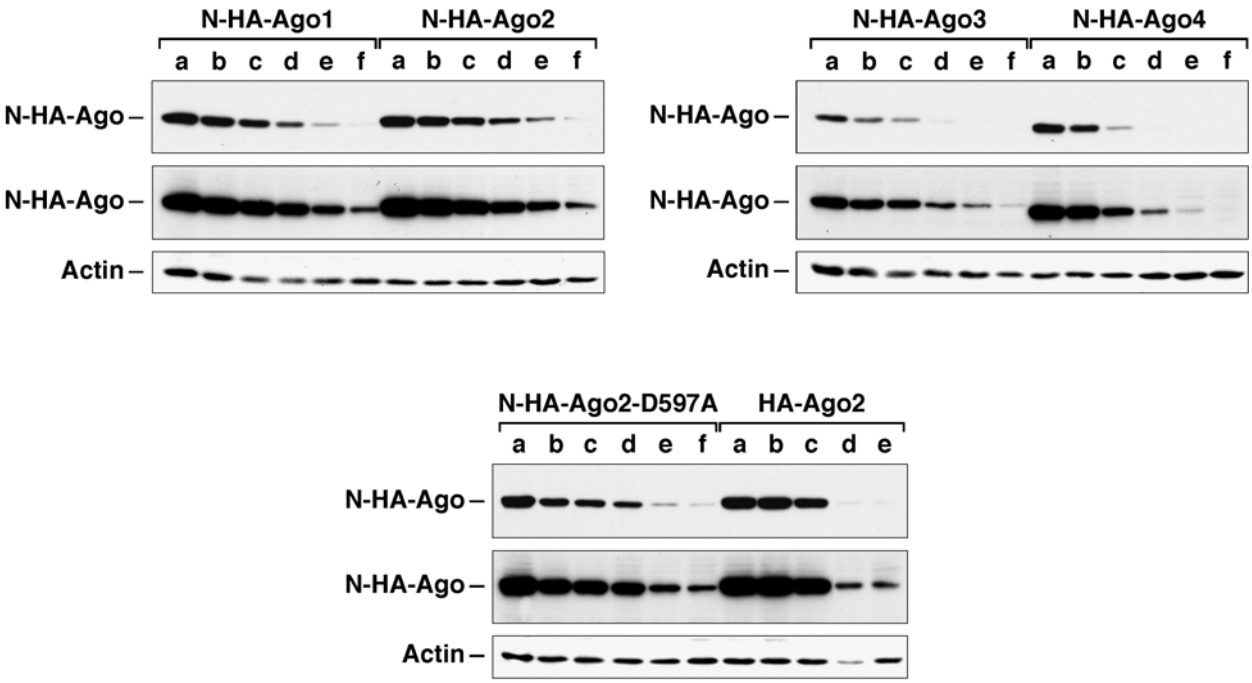

Figure S4. N-HA-Ago protein concentrations in transfected cells.

293T cells were transiently transfected with various amounts of plasmid DNA encoding N-HA-Ago1, N-HA-Ago2, N-HA-Ago3, N-HA-Ago4, N-HA-Ago2-D597A, or HA-Ago2, and the cellular concentration of the epitope-tagged Ago protein was examined 36 hr later by immunoblot analysis of protein extracts with anti-HA and anti-actin antibodies. Both a short (above) and a long (below) exposure are shown for the blots probed with anti-HA antibodies. For N-HA-Ago3 and N-HA-Ago4, all six concentrations of the tethered protein were tested for their effect on reporter gene expression, whereas for N-HA-Ago1 and N-HA-Ago2, only concentrations b, c, d, e, and f were tested, and for the untethered negative control (HA-Ago2), only concentrations c, d, and e were tested (see Figure 2A). The contributions of translational repression and accelerated mRNA decay to downregulation by the various tethered Ago proteins (Figure 2B) were determined at equivalent N-HA-Ago concentrations (corresponding to a relative concentration of 2, as graphed in Figure 2A).

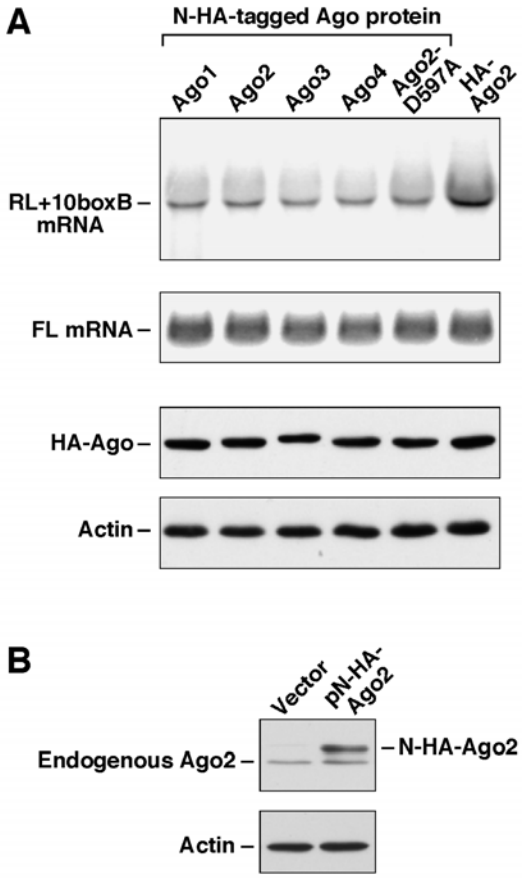

Figure S5. Influence of tethered Ago proteins on mRNA concentration.

(A) Reduction in RL+10boxB mRNA caused by tethering Ago proteins, as determined by Northern blot analysis of the cytoplasmic RNA samples described in Figure 2B.

(B) Relative concentration of N-HA-Ago2 and endogenous Ago2 in transfected 293T cells. The protein samples described in Figure 2B were analyzed by immunoblotting with anti-Ago2 and anti-actin antibodies.

	Relative mRNA concentration			
Tissue or cells	Ago1	Ago2	Ago3	Ago4
Lymph node	106	75	56	99
Globus pallidus	67	85	54	73
Liver	60	95	59	70
PB-CD14+ monocytes	140	38	47	96
Prefrontal cortex	138	134	102	111
Whole blood	132	93	58	116
Thymus	111	91	95	61
Pituitary	102	117	113	56
Amygdala	96	99	66	76
Fetal brain	91	98	57	76
BM-CD33+ myeloid cells	162	67	44	110
Testis-interstitial	125	122	93	71
Temporal lobe	85	94	54	66
Spinal cord	96	82	45	69
Adrenal gland	108	96	77	54
PB-CD56+ NK cells	130	59	49	71
Trachea	98	92	61	60
Trigeminal ganglion	120	114	95	51
Occipital lobe	112	95	61	67
Whole brain	108	99	61	67
PB-BDCA4+ dendritic cells	145	55	48	75
Cerebellum	120	95	59	72
Thalamus	102	79	46	64
Testis-seminiferous tubule	121	105	69	69
Lung	98	118	51	78
Kidney	86	92	60	46
Tonsil	106	78	53	56
Testis	113	108	60	71
Salivary gland	108	108	56	70
Prostate	105	110	77	48
Testis-Leydig cells	121	117	66	69
Caudate nucleus	93	77	37	59
Cingulate cortex	132	96	57	70
Tongue	94	99	62	44
Cerebellum-peduncles	131	104	59	68
PB-CD8+ T cells	150	89	58	70
Skeletal muscle	96	126	61	59
Parietal lobe	85	111	53	53
Atrioventricular node	122	114	58	68
Hypothalamus	123	116	51	75
Fetal thyroid	115	102	73	42
Adrenal cortex	121	107	64	55
Superior cervical ganglion	105	173	78	66
Bone marrow	96	113	45	62
Testis-germ cells	141	121	76	56
Dorsal root ganglion	137	136	62	74
Pancreas	109	119	53	60
Heart	99	156	59	68
Medulla oblongata	117	126	64	56
BM-CD34+ cells	129	74	49	51
Pancreatic islets	119	94	60	45
Pons	111	99	39	62
Olfactory bulb	108	122	68	42
Bronchial epithelial cells	72	108	48	37
Uterus corpus	97	115	45	53
PB-CD4+ T cells	141	82	57	45
Fetal lung	113	131	55	57
Ovary	128	161	65	65
721 B lymphoblasts	118	88	63	29
Appendix	94	146	54	54
Ciliary ganglion	119	148	57	62
Placenta	95	138	53	49
Thyroid	108	129	53	51
Uterus	115	139	61	50
Subthalamic nucleus	144	79	39	54
Cardiac myocytes	114	87	30	54
Fetal liver	77	155	44	49
Adipocyte	149	137	51	59
PB-CD19+ B cells	141	73	33	46
Skin	135	137	35	64
BM-CD105+ endothelial cells	83	157	38	46
Smooth muscle	136	93	36	43
BM-CD71+ early erythroid cells	87	228	27	73
Mean	112	108	58	63

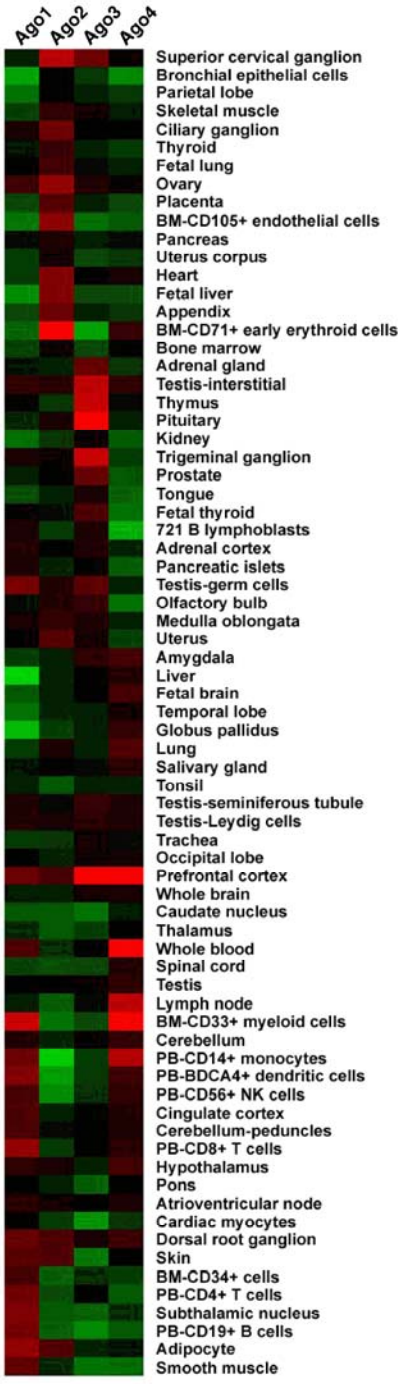

Figure S6. Differential expression of Ago proteins in human tissues.

Gene expression profiling data, available from http://symatlas.gnf.org [24], were used to compare the concentrations of mRNAs encoding the four Ago paralogs in 73 human tissues. Black, average expression; red, high expression; green, low expression.

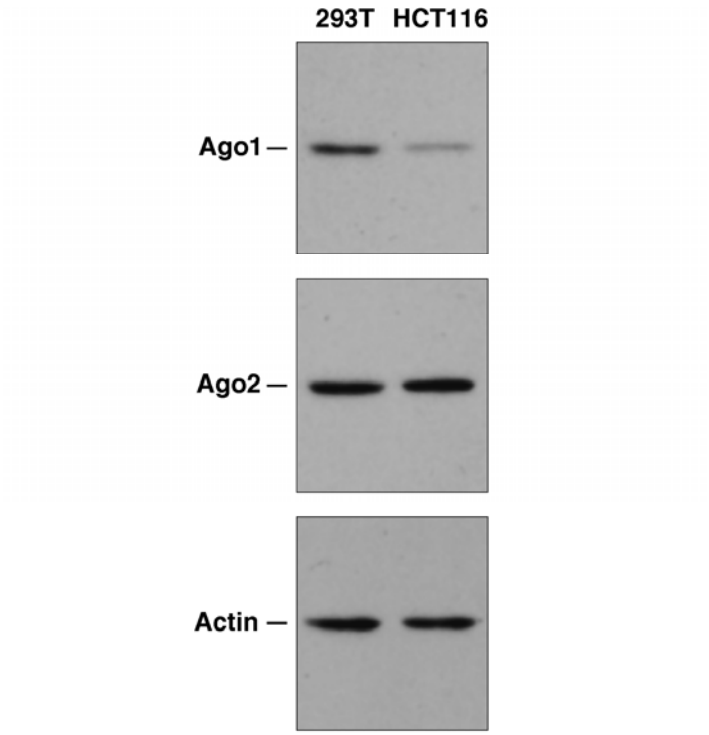

Figure S7. Relative concentration of Ago1 and Ago2 proteins in 293T versus HCT116 cells.

The concentration of Ago1 and Ago2 was compared in 293T versus HCT116 cells by immunoblot analysis of protein extracts with monoclonal antibodies specific for Ago1, Ago2, or actin. In 293T cells, the concentration of Ago1 was 2.1 ± 0.3 times that in HCT116 cells, whereas the concentration of Ago2 in the two cell lines was almost identical (0.9 ± 0.1).

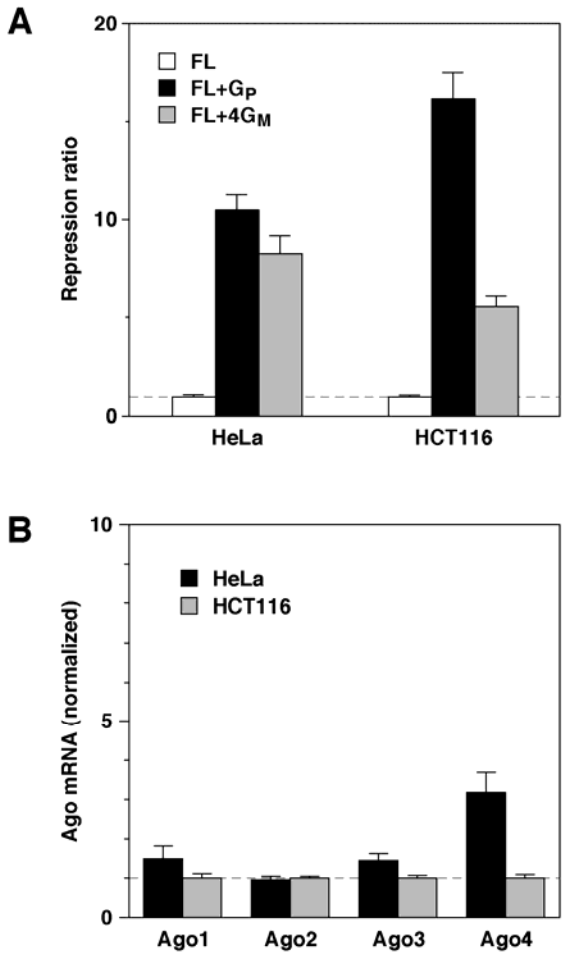

Figure S8. Differential specificity of RNA interference in HeLa and HCT116 cells.

(A) Specificity of RNA interference. The ability of siEGFP to repress luciferase production from reporter genes containing no complementary elements (FL), one perfectly complementary element (FL+G_P) or four imperfectly complementary elements (FL+$4G_M$) was compared in HeLa cells and HCT116 cells 42 hr after transient transfection with each of those reporter genes and a Renilla luciferase gene (internal standard).

(B) Relative concentration of Ago mRNAs. Quantitative RT-PCR was used to compare the concentrations of messages encoding each of the four Ago proteins in HeLa cells and HCT116 cells. The abundance of each transcript was normalized to its level in HCT116 cells.

Error bars represent the standard deviation of multiple measurements.

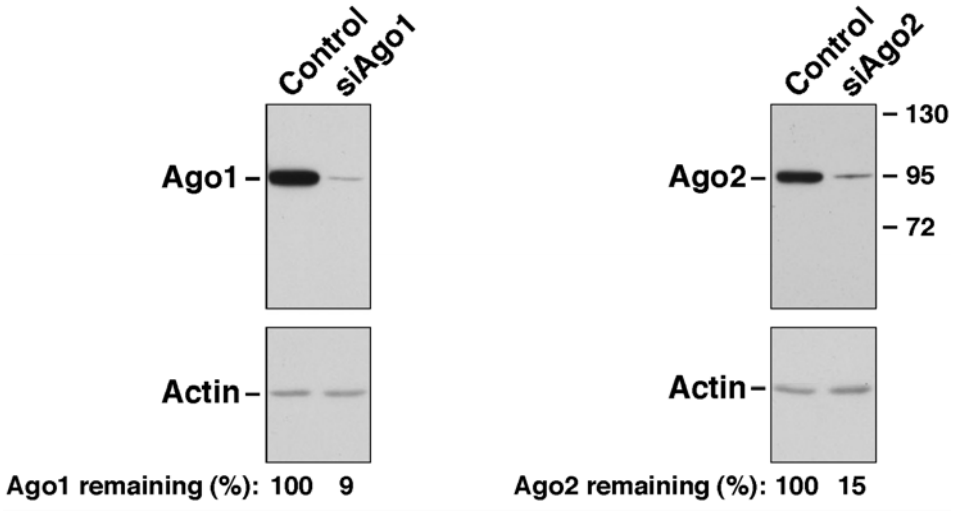

Figure S9. Depletion of Ago1 and Ago2 proteins by RNA interference.

293T cells were transfected with an siRNA specific for Ago1 or Ago2 mRNA or with a nonspecific siRNA (siGL2) that served as a negative control. The cellular concentration of Ago1 or Ago2 was examined 42 hr later by immunoblot analysis of protein extracts with anti-Ago1 (top left), anti-Ago2 (top right), or anti-actin (bottom) monoclonal antibodies. Calibration is in kilodaltons.

Table S1. Contributions of impaired translation and diminished mRNA concentration to repression by siEGFP or miR-125b 60 hours after transfection.

			Repression ratio				
3' UTR element	Copies	mi/siRNA	Protein production	mRNA concentration		Translation efficiency	
				Total	Per element	Total	Per element
None	0	0	1.00 ± 0.06	1.00 ± 0.07		1.00 ± 0.09	
G_P	1	siEGFP	12.89 ± 1.07	3.81 ± 0.26	3.81 ± 0.26	3.38 ± 0.36	3.38 ± 0.36
G_M	4	siEGFP	11.45 ± 0.65	2.50 ± 0.25	1.26 ± 0.04	4.59 ± 0.53	1.46 ± 0.05
None	0	miR-125b	1.00 ± 0.06	1.00 ± 0.06		1.00 ± 0.08	
P	2	miR-125b	8.98 ± 0.43	3.48 ± 0.21	1.87 ± 0.05	2.58 ± 0.20	1.61 ± 0.07
E1	6	miR-125b	10.43 ± 0.51	2.86 ± 0.23	1.19 ± 0.02	3.65 ± 0.34	1.24 ± 0.02

The experiment described in Table 1 was repeated, except that the effect of siEGFP or miR-125b on reporter gene expression was determined at the protein and mRNA level 60 hr (rather than 36 hr) after transient transfection of 293T cells. That similar results were obtained in both experiments (as well as 24 hr after transfection (data not shown)) indicates that sufficient time was provided for mRNA and protein levels to reach steady state.

Table S2. Reporter plasmids and and encoded mRNAs.

Figure or Table	Reporter plasmid	Encoded mRNA
Table 1	pCMV-Luc	FL
	pCL-G_P	FL+G_P
	pCL-G_M	FL+G_M
	pCL-$4G_M$	FL+$4G_M$
	pCL-2P	FL+2P
	pCL-2E1	FL+2E1
	pCL-6E1	FL+6E1
	pRL (control)	RL
Table S1	As in Table 1	
Figure 1	pCL-2P-UTR	FL+2P-UTR
	pCL-2P-ORF	FL+2P-ORF
	pRL (control)	RL
Figure 2	pTR+10boxB	RL+10boxB
	pCMV-Luc (control)	FL
Figure 3	pCMV-Luc	FL
	pCL-G_P	FL+G_P
	pCL-$4G_M$	FL+$4G_M$
	pRL (control)	RL
Figure 4	pCMV-Luc	FL
	pCL-G_P	FL+G_P
	pCL-$4G_M$	FL+$4G_M$
	pCL-2P	FL+2P
	pCL-6E1	FL+6E1
	pRL (control)	RL
Figure S1	As in Table 1	
Figure S2	pCMV-Luc	FL
	pCL-2P	FL+2P
	pRL (control)	RL
Figure S3	As in Figure 1	
Figure S5	As in Figure 2	
Figure S8	As in Figure 3	

Table S3. Characteristics of reporter plasmids.

Reporter plasmid	Luciferase	RNA element(s)	Copies	Location	Distance from termination codon to RNA element(s)	Promoter	Poly(A) signal
pCMV-Luc	FL	None	–	–	–	CMV	SV40
pCL-G_P	FL	G_P (siEGFP perfect match)	1	3' UTR	136 nt	CMV	SV40
pCL-G_M	FL	G_M (siEGFP partial match)	1	3' UTR	136 nt	CMV	SV40
pCL-4G_M	FL	G_M (siEGFP partial match)	4	3' UTR	136 nt	CMV	SV40
pCL-2P	FL	P (miR-125b perfect match)	2	3' UTR	135 nt	CMV	SV40
pCL-2E1	FL	E1 (miR-125b partial match)	2	3' UTR	136 nt	CMV	SV40
pCL-6E1	FL	E1 (miR-125b partial match)	6	3' UTR	136 nt	CMV	SV40
pCL-2P-UTR	FL	P (miR-125b perfect match)	2	3' UTR	69 nt	CMV	SV40
pCL-2P-ORF	FL	P (miR-125b perfect match)	2	ORF	–	CMV	SV40
pRL	RL	None	–	–	–	CMV	SV40
pTR+10boxB	RL	boxB (N-HA-Ago tethering site)	10	3' UTR	39 nt	HSV-TK	SV40