

Biochemical isolation of Argonaute protein complexes by Ago-APP

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During microRNA (miRNA)-guided gene silencing, Argonaute (Ago) proteins interact with a member of the TNRC6/GW protein family. Here we used a short GW protein-derived peptide fused to GST and demonstrate that it binds to Ago proteins with high affinity. This allows for the simultaneous isolation of all Ago protein complexes expressed in diverse species to identify associated proteins, small RNAs, or target mRNAs. We refer to our method as “Ago protein Affinity Purification by Peptides” (Ago-APP). Furthermore, expression of this peptide competes for endogenous TNRC6 proteins, leading to global inhibition of miRNA function in mammalian cells.

Argonaute | small RNAs | microRNAs | GW proteins | RNAi

To repress gene expression, microRNAs (miRNAs) guide Argonaute (Ago) proteins to distinct target sites on mRNAs (1, 2). Ago proteins contain four different domains with distinct functions. The N domain is important for small RNA loading, the PAZ domain binds the 3' and the MID domain the 5' end of the small RNA. The PIWI domain is structurally similar to RNase H, and indeed some Ago proteins can function as small RNA-guided endonucleases. In humans, only Ago2 is catalytically active whereas Ago1, Ago3, and Ago4 are inactive (3, 4). Recent structural and biochemical experiments identified small structural elements that affect the endonucleolytic activity of Ago proteins (5–9). For miRNA-guided repression independently of Ago-mediated RNA cleavage, Ago proteins recruit a member of the TNRC6/GW182 protein family (also referred to as GW proteins and TNRC6A-C in human), which coordinate all downstream silencing events including binding to the poly(A)-binding proteins on the poly(A) tail of the mRNA, recruiting deadenylase complexes such as PAN2–PAN3 or the CCR4–NOT complex and translational repression (10–13). GW (glycine-tryptophan) proteins are characterized by an unstructured, tryptophan (Trp)-rich N-terminal half that serves as an Ago-binding domain (Fig. 1A). In particular, two Trp of a GW protein bind into two specific pockets on the Ago surface (10, 14, 15). We have recently identified a short TNRC6B-derived peptide that efficiently interacts with Ago proteins (Fig. 1A, T6B peptide) (15, 16). We hypothesized that such a peptide might be a powerful tool for the isolation of endogenous Ago proteins from cell or tissue lysates. We find that this peptide precipitates all four endogenous human Ago proteins efficiently, and we refer to this method as “Ago protein Affinity Purification by Peptides” (Ago-APP). Furthermore, it also allows for an accurate quantification of Ago protein levels in different primary tissues. Strikingly, Ago-APP binds Ago proteins that are involved in miRNA-guided gene silencing from any animal lysate. Furthermore, in plants, where GW proteins are not conserved, the T6B peptide mimics interactions with components of the RNA-guided DNA methylation pathway, and indeed we have efficiently isolated Ago-associated small RNAs within a length frame indicative for this pathway. Finally, transfection of the T6B peptide leads to a strong repression of endogenous miRNA pathways. Taken together, we have developed

and characterized a novel highly efficient tool to study small RNA pathways in many different cell types, tissues, and species.

Results and Discussion

Ago-APP Efficiently Precipitates All Four Human Ago Protein Complexes from Cell Lysates. To test our hypothesis that Ago-APP might be a powerful tool to pull down Ago proteins, we expressed FLAG/HA (FH)-tagged Ago proteins in HEK293 cells and performed glutathione S-transferase (GST)–T6B peptide-mediated pull-down assays (Fig. 1B). Ago-APP isolated all four FH-Ago proteins efficiently but not FH-HIWI, -HIWI2, or -HILI, three Argonaute proteins of the PIWI clade that do not interact with GW proteins (17) (Fig. 1B). Next we analyzed endogenous Ago2 pull-down efficiency and compared it to conventional anti-Ago2 immunoprecipitation (Fig. 1C). Both an anti-Ago2 antibody and the T6B peptide efficiently precipitated endogenous Ago2, whereas a control antibody or GST alone did not (*Upper*). As expected, the T6B peptide pulled down endogenous Ago1 as well (*Lower*). Furthermore, we analyzed associated miRNAs by Northern blotting (Fig. 1D). As exemplified by a Northern blot against let-7a, the T6B peptide precipitated miRNA-loaded and presumably functional Ago complexes. It has recently been reported that Ago-free miRNA pools may exist in cells (18, 19). To analyze these findings in more detail, we set out to deplete all Ago proteins from HeLa cell lysate and analyzed codepletion of let-7a and miR-30a

Significance

Small RNA-guided gene-silencing pathways regulate fundamental cellular processes. Small RNAs such as microRNAs (miRNAs) directly bind to a member of the Argonaute (Ago) protein family. In animals, Ago proteins interact with a member of the GW protein family (referred to as TNRC6A–C). Based on an Ago-interacting TNRC6 peptide, we have developed a method allowing for the efficient isolation and characterization of Ago protein complexes from any animal organism. We refer to this method as “Ago protein Affinity Purification by Peptides.” Our approach also allows for the identification of Ago-bound small RNAs as well as mRNAs. Expression of this peptide in living cells leads to global miRNA inactivation, thus providing a powerful tool to study miRNA function on various levels.

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The authors declare no conflict of interest.

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Data deposition: The small RNA sequencing data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE70553).

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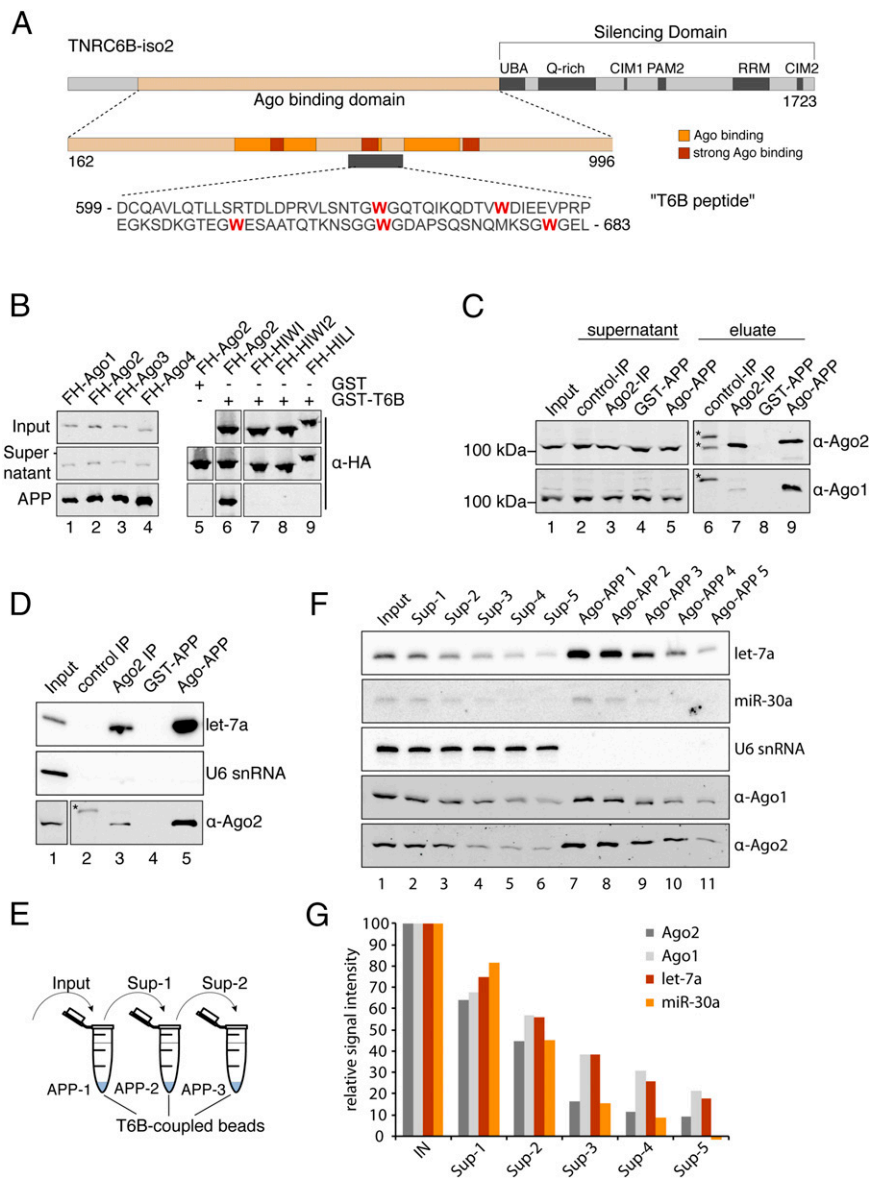


Fig. 1. Precipitation of Ago complexes by Ago-APP. (A) Schematic representation of the TNRC6B domain organization and its Ago-interacting regions. The position and amino acid sequence of the peptide used for Ago-APP are shown. (B) Ago-APP from HEK293 cells overexpressing FH-Ago1-4 (lanes 1-6) and FH-tagged human PIWI proteins (lanes 7-9). (C and D) Comparison of Ago2-immunoprecipitation (IP) and Ago-APP in terms of Ago enrichment (C) and coprecipitation of miRNAs (D). A monoclonal antibody against human Ago2 and an unrelated antibody were used for IPs. Recombinant GST-T6B and GST alone were used for Ago-APPs. Asterisks indicate unspecific bands. (E) Schematic representation of an Ago protein and miRNA codepletion experiment using Ago-APP. (F) Codepletion of Ago proteins and associated miRNAs. Supernatants of Ago-APPs were repeatedly incubated with T6B-coupled beads as shown in E. Ago1 and Ago2 protein levels were analyzed by Western blotting. The levels of the highly abundant let-7a and the weakly expressed miRNA-30a were analyzed by Northern blotting. A probe against U6 snRNA was used to control for equal RNA loading. (G) Quantification of Western and Northern blot signals shown in F. Background signals of the blots were deduced to obtain the protein and miRNA signal intensities of input and supernatant samples.

(Fig. 1 E and F). After repeated incubation steps, we observed a clear codepletion of let-7a and miR-30a together with Ago1 and Ago2 (Fig. 1G for signal quantification), suggesting that, at least in our cell lysates, mature miRNAs are nearly quantitatively bound by Ago proteins and that Ago-free mature miRNA reservoirs may only be present in trace amounts corresponding to miRNA biogenesis intermediates. However, it should be noted that cell lysates might not fully resemble the conditions within living cells. Furthermore, our biochemical experiments are semiquantitative.

Specific Isolation and Characterization of Ago Protein Complexes Using Ago-APP. For further specificity examination, human, mouse, *Drosophila*, and *Arabidopsis* cell/tissue lysates were subjected to

Ago-APP and analyzed by mass spectrometry (Fig. 2A). All four human and mouse Ago proteins were precipitated from the lysates. In *Drosophila*, we found only Ago1 in our pull-downs, which is the major Ago protein required for miRNA function in this species. In *Arabidopsis*, GW proteins function only in RNA-directed DNA methylation (RdDM), where an RNA polymerase V subunit binds to Ago4 by Trp interactions resembling those of animal GW and Ago proteins (20). Strikingly, Ago-APP mimics this interaction and Ago4, -6, and -9 are efficiently pulled down (Fig. 2A, lane 5; Fig. 2F). To further validate that Ago-APP enriches Ago proteins equally well, we compared Ago protein levels in total lysates with Ago proteins isolated by Ago-APP (Fig. 2B). For Ago quantification, we used state-of-the-art label-free mass spectrometry [selected

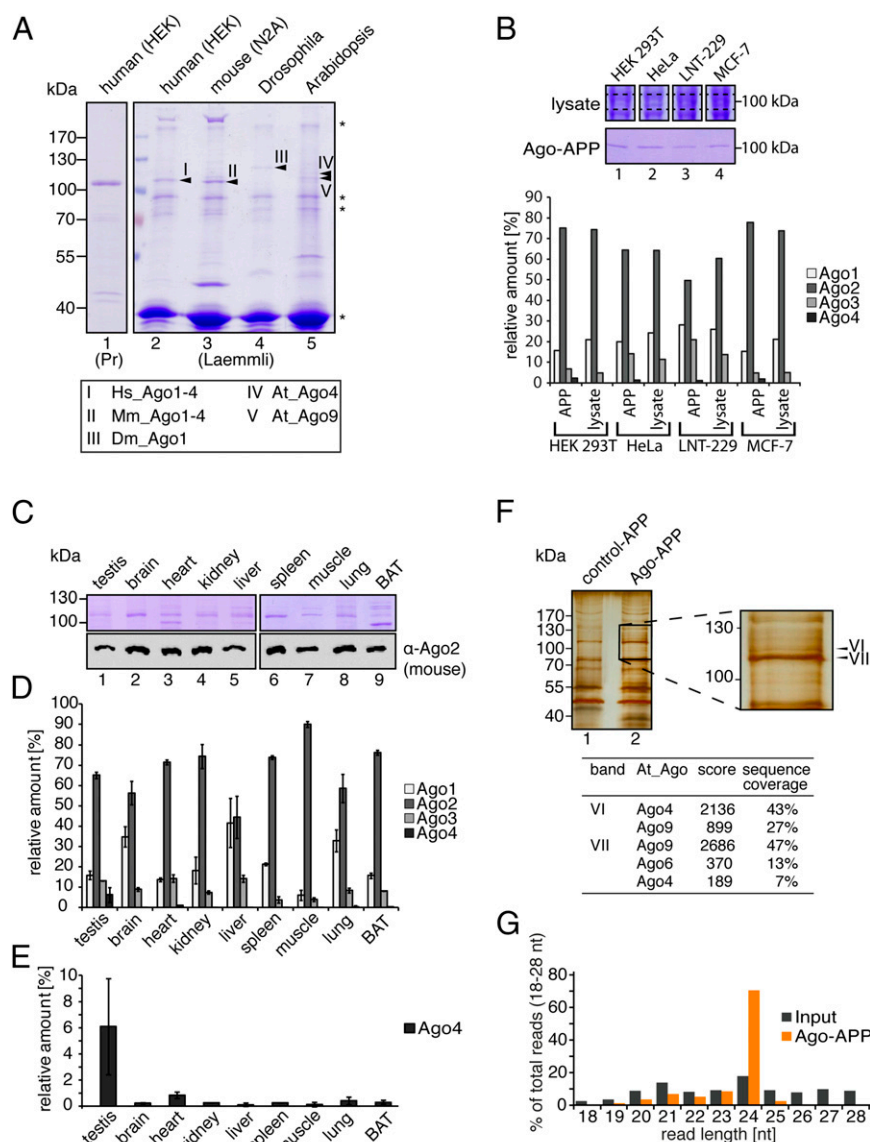


Fig. 2. Isolation and characterization of Ago complexes from different species. (A) Ago-APP and mass-spectrometric analysis of different protein extracts. Samples were eluted with PreScission protease (lane 1, Pr) or Laemmli buffer (lanes 2–5), which simultaneously eluted GST-T6B and aggregates of it (asterisks). (B) Total lysates from the indicated cell lines were separated by SDS-PAGE with (Lower) and without (Upper) prior enrichment by Ago-APP. For total lysates without affinity purification, the area around 100 kDa was cut from the gel as indicated. The relative amounts of Ago1–4 were determined by SRM with stable isotope-labeled peptides. The proportion of one Ago protein related to the total Ago pool is shown. (C) Ago-APP was used to purify endogenous Ago1–4 from different murine tissues. The samples were stained with Coomassie (Upper) and analyzed by Western blotting (Lower). BAT, brown adipose tissue. (D) Mass-spectrometric analysis of the Ago-APPs conducted in C. The relative amounts of Ago1–4 were determined as in B. The proportion of one Ago paralogue related to the total Ago pool is shown. Error bars represent the SD of identical samples that were quantified with two different paralogue-specific peptides. (E) Relative amount of Ago4 in different mouse tissues, derived from the same samples/measurements described in C and D. (F) Ago-APP from an *Arabidopsis* callus with egg cell-like character and mass-spectrometric identification of the purified Ago proteins. A GST-tagged peptide lacking all five tryptophans was included as negative control. (G) Length comparison of small RNA sequencing reads of *Arabidopsis* input and Ago-APP samples.

reaction monitoring (SRM)] (*Materials and Methods*). Indeed, the levels of the individual Ago proteins are similar in total lysates and in Ago-APP-enriched samples, suggesting that Ago-APP is not biased toward a specific Ago protein. Of note, Ago4 is in rather low abundance and can therefore be quantified only after Ago-APP enrichment (Fig. 2B).

To further validate the broad applicability of Ago-APP outside of cell lines, we also performed pull-down experiments from lysates prepared from different mouse tissues. Ago proteins were efficiently isolated as indicated by Western blotting against mouse Ago2 (Fig. 2C). Considering the opportunity to simultaneously isolate all four mammalian Ago proteins,

we thought to use our approach to determine the relative amounts of Ago proteins within various mouse tissues (Fig. 2D and E). Relative Ago protein levels have only been estimated before (21, 22). Such earlier quantifications relied on antibodies with different affinities and thus might not be accurate. Furthermore, Ago protein levels have not been quantified from primary tissues or organs. We find that Ago2 is the most prominent Ago protein in all tissues analyzed, followed by Ago1 and Ago3. Ago4 is found mainly in testes, which is consistent with the testes phenotype of Ago4 knockout mice (23) (Fig. 2E).

The striking observation that Ago-APP selectively isolates RdDM-pathway active Ago proteins prompted us to analyze

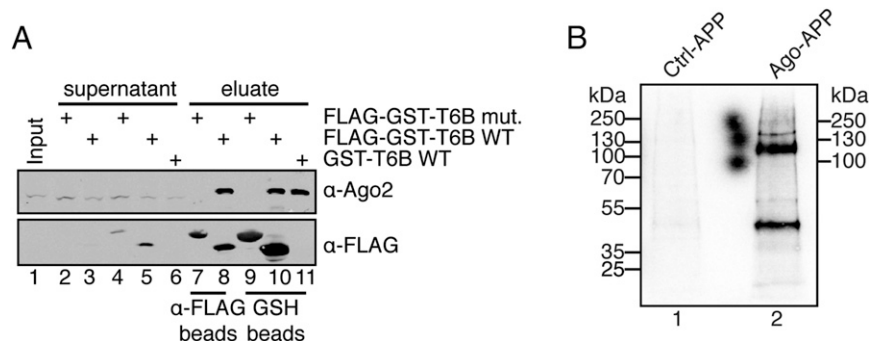


Fig. 3. Ago-APP can be used for simultaneous PAR-CLIP of human Ago1–4. (A) Ago-APPs using differently tagged T6B versions (lanes 3, 5, 6, 8, 10, 11) and a mutated control peptide (lanes 2, 4, 7, 9). (B) PAR-CLIP using Ago-APP (lane 2) as well as a mutated control peptide (lane 1) from HEK293 lysates.

isolated plant Ago complexes in more detail (Fig. 2F). Lysates from egg cell-like callus material (24) were subjected to Ago-APP, and precipitated proteins were analyzed. Similar to our previous experiment (Fig. 24), the RdDM Ago proteins Ago4 and -9 were readily identified. In addition, the third RdDM Ago protein in *Arabidopsis thaliana*, Ago6, was also detected (Fig. 2F). siRNAs involved in RdDM are 24 nt long (25). Using small RNA cloning and deep sequencing from our Ago-APP samples, we find that 24-nt-long siRNAs are selectively enriched by Ago-APP, demonstrating that Ago-APP is a powerful tool to study RdDM mechanisms in various plant species or tissues (Fig. 2G). Furthermore, mass spectrometry analysis could be used to identify novel components involved in RdDM in various plant tissues or species.

Ago-APP Is Compatible with PAR-CLIP and Can Be Used to Identify Ago-Associated miRNA Targets. In animals, Ago complex isolation is widely used for the identification of associated miRNA target RNAs (26–30). Thus, we asked whether Ago-APP is applicable for miRNA target identification. A common method is based on UV cross-linking of Ago proteins to target mRNAs and the

subsequent cloning and sequencing of target RNA fragments (HITS-CLIP and PAR-CLIP) (26, 27). However, the protocol is highly dependent on effective immunoprecipitation of the cross-linked Ago protein complexes. To provide a tool that can be conveniently used in such CLIP protocols, we fused the GST-T6B peptide to FLAG, allowing for anti-FLAG affinity purifications as commonly used in CLIP protocols (Fig. 3A). Both anti-FLAG coupled agarose or control glutathione beads isolated endogenous Ago2 efficiently from HeLa cell lysate (Fig. 3A, lanes 8 and 10). We next performed PAR-CLIP experiments using HEK293 cells (Fig. 3B). UV₃₆₅-cross-linked Ago proteins were isolated, and the associated RNA was radiolabeled and separated by SDS-PAGE. Whereas Ago-APP recovered endogenous Ago proteins with radiolabeled RNA, the control peptide did not, indicating that our approach is highly useful for miRNA target identification using PAR-CLIP.

T6B Peptide Efficiently Inhibits miRNA Function in Vivo. The T6B peptide occupies the TNRC6 protein-binding pocket on Ago and thus might be a valuable tool to block global miRNA function

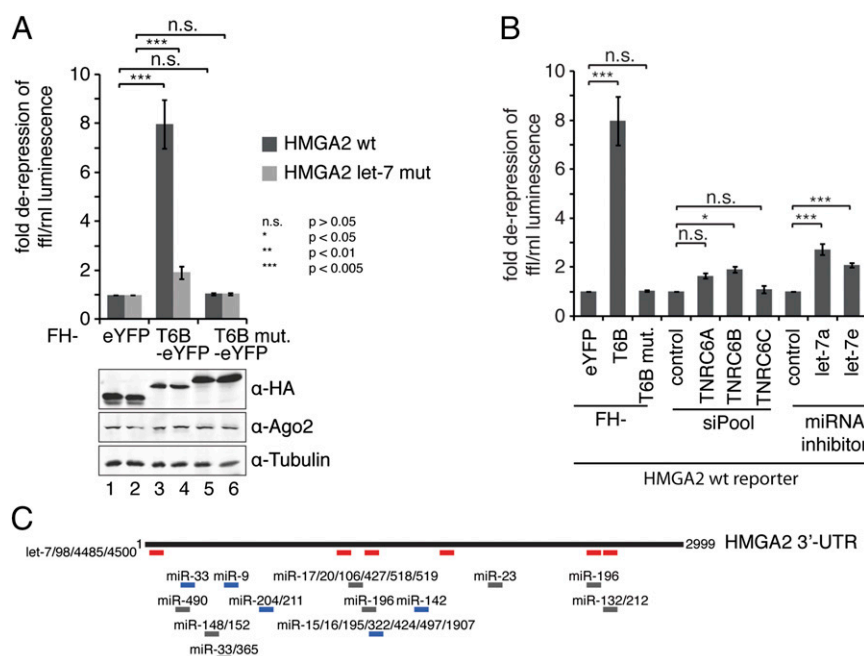


Fig. 4. T6B peptide expression inhibits endogenous miRNA-guided gene silencing. (A) Luciferase assays conducted on the HMGA2 3'-UTR to monitor T6B-mediated de-repression. Reporter de-repression was monitored in HeLa cells overexpressing the FH- and GFP-tagged T6B peptide (wild type and mutant). (B) Comparison of T6B-mediated de-repression with TNRC6A-C knockdowns and miRNA antisense inhibitors. (C) Predicted miRNA binding sites in the HMGA2 3'-UTR.

in vivo (Fig. 4). For application of the T6B peptide in cells and to protect it from immediate degradation, we fused it to enhanced YFP (eYFP). A peptide with mutated Trp residues was used as control. The expression plasmids were transfected together with a luciferase reporter carrying the 3' UTR of HMGA2, and luciferase activity was measured 2 d after transfection. Whereas the mutated peptide had no effect on the expression of the reporter, the T6B peptide strongly increased luciferase activity, indicating that miRNA-guided gene silencing was effectively inhibited (Fig. 4A). Finally, we compared the peptide inhibition with common miRNA antisense inhibitors directed against let-7a or let-7e and to knock-downs of the individual TNRC6 proteins using specific siRNA pools (siPools) (31) (Fig. 4B). Both inhibitors against the let-7 miRNAs released repression by two- to threefold. Depletion of individual TNRC6 proteins had even weaker effects, suggesting redundancy as observed before (31). Strikingly, our peptide fused to eYFP led to a seven- and eightfold increase in luciferase activity, suggesting highly efficient inhibition of the miRNA pathway. The fact that the individual miRNA inhibitors show weaker effects suggests that the HMGA2 3' UTR is under the control of not only the let-7 family but also various other miRNAs (Fig. 4C).

In summary, we have developed a novel Ago family protein purification strategy with several unique features and advantages compared with common methods. First, Ago-APP is highly efficient due to high affinity of the peptide to Ago. Second, not only one, but all Ago family proteins involved in mammalian and insect miRNA function can be isolated and even depleted from lysates, which is not possible with available antibodies. This fact is important for the identification of miRNA targets because Ago2 immunoprecipitation is mainly used, and other Ago-protein-specific targets, if they exist, may be recovered by

our new approach. Third, Ago proteins and thus miRNAs and associated targets can be isolated and identified from any given animal species. Fourth, our peptide can be used as an inhibitor of global miRNA regulation. Furthermore, it can be induced from stably integrated lentiviral constructs, for example, to shut down the entire miRNA regulation in cell lines that are difficult to transfect or even whole animals at defined time points.

Materials and Methods

Ago-APP. Per 50 μ L of glutathione-sepharose 4 Fast Flow (GE Healthcare) or anti-FLAG-M2 agarose (Sigma-Aldrich), a minimum of 100 μ g (FLAG)-GST-tagged peptide (TNRC6B 599–683) was coupled to the beads for 3 h at 4 °C. Excess peptide was removed by washing with PBS three times, and lysate was added to the peptide-coupled beads. After incubation for 3 h at 4 °C, the beads were washed four times with NET buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, 1 mM NaF; supplemented with 0.5 mM DTT and 1 mM AEBSF before use) and once with PreScission cleavage buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) or PBS. The beads were eluted by PreScission cleavage (~4 μ g PreScission in one bead volume of PreScission cleavage buffer overnight at 4 °C) or by addition of 2 \times Laemmli sample buffer and incubation at 95 °C for 5 min.

Detailed methods and materials are available in *SI Materials and Methods*.

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