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The Requirement for GW182 Scaffolding Protein Depends on whether Argonaute is Mediating Translation, Transcription, or Splicing

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Abstract

GW182 and argonaute 2 (AGO2) are core proteins of the RNA interference (RNAi) complex. GW182 is a scaffolding protein that physically associates with AGO2 and bridges its interactions with other proteins. A fundamental problem in biology is how scaffolding proteins adapt or contribute to differing functional demands within cells. Here we test the necessity for human GW182 proteins (paralogs TNRC6A, TNRC6B, and TNRC6C) for several mechanisms of small duplex RNA-mediated control of gene expression, including translational silencing by miRNAs, translational silencing by siRNAs, transcriptional silencing, transcriptional activation, and splicing. We find that GW182 is required for transcriptional activation and for the activity of miRNAs, but is dispensable for the regulation of splicing, transcriptional silencing, and the action of siRNAs. AGO2, by contrast, is necessary for each of these processes. Our data suggests that GW182 does not alter AGO2 to make it active. Instead, GW182 organizes protein complexes around AGO2. Sometimes this higher level of organization is necessary and sometimes it is not. AGO2 and GW182 offer an example for how a partnership between a scaffolding protein and a functional protein can be powerful but not obligatory.

Abstract

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BRIEFS:

AGO2 can act independently of TNRC6.

Introduction

GW182 was first observed as an autoantigen residing in cytoplasmic bodies and associated with mRNA.^{1,2} GW182 was subsequently discovered to bind argonaute (AGO) protein and play a critical role in translational repression during RNA interference (RNAi).^{3,4,5} Studies indicated that GW182 bound to AGO2 and provided a bridge between AGO2 and proteins involved in decapping and the degradation of mRNA.^{6–13}

There are three GW182 paralogs in human cells, trinucleotide repeat containing 6A (TNRC6A), TNRC6B, and TNRC6C. The TNRC6A paralog is also known as human GW182. GW182 is a multidomain protein (Fig. 1A) containing an N-terminal domain that can bind one or more copies of AGO2.^{14–17} The AGO binding domain contains many glycine-tryptophan (GW) repeats that serve as anchors for protein:protein interactions and these GW repeats are essential for association with AGO2. GW182 also contains C-terminal domains that can function independently of AGO to interact with additional proteins and silence bound transcripts.¹⁸

Inside cells, RNAi proteins and small RNAs form a programmable ribonucleoprotein complex that facilitates sequence-selective recognition of RNA targets and the control of gene expression.¹⁹ The small RNA dictates the sequence-selectivity of recognition while AGO protects the small RNA and promotes efficient search and recognition of target sequences.²⁰ The best known examples of RNA-mediated control of gene expression are inhibition of translation by micro RNAs (miRNAs) (Figure 1B) and small interfering RNAs (siRNAs) (Figure 1C). miRNAs typically have many cellular targets and are not perfectly complementary to those targets while siRNAs typically have one target and are perfectly complementary.

siRNAs and miRNAs are well known to function in the cytoplasm. RNAi factors, including AGO2 and TNRC6, are also localized in mammalian cell nuclei^{21,22} and control gene transcription (Figure 1D) and splicing (Figure 1E).^{23,24} The control of transcription can involve both transcriptional silencing and transcriptional gene activation.^{25,26} These diverse functions (translation inhibition, transcriptional activation/silencing/splicing) pose different

challenges to efficient protein function. Understanding mechanism requires learning how AGO2 and GW182 paralogs collaborate in controlling gene expression and the extent to which they can function independently.

We take advantage of the diverse regulatory modes possible through the RNAi pathway to examine the necessity of TNRC6 paralogs for the regulation of gene expression. We find that expression of TNRC6 paralogs is necessary for examples of transcriptional activation and miRNA-mediated gene silencing. Expression of the TNRC6 is not necessary for RNA-mediated transcriptional silencing, splicing, or the action of an siRNA. These data suggest that the AGO2:TNRC6 interaction is not obligatory for all modes of gene regulation through the RNAi machinery.

Experimental Methods

RNA synthesis.

RNA oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Double-stranded RNAs were prepared by mixing the two RNA strands and annealing them in 2.5× PBS solutions. Stock solutions (20 μM) were prepared for transfection in cell cultures.

Cell culture and transfection.

HeLa cells and Hela pLUC/705 cells (generously provided by Dr. Ryszard Kole) were cultured in minimum essential medium eagle (MEM, Sigma-Aldrich) supplemented with 1% MEM nonessential amino acids (Sigma-Aldrich) and 10% FBS (Sigma-Aldrich). A549 cells (ATCC) were cultured in F-12K media containing 10% FBS. T47D cells (ATCC) were maintained in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS), 0.5% nonessential amino acids (NEAA), 0.4 units/mL bovine insulin, 10 mM HEPES and 1 mM sodium pyruvate.

Oligofectamine RNAiMAX (Invitrogen) was used for all RNA duplex transfections. For forward transfection experiment (unless otherwise noted), cells were plated into a six-well plate (Costar) 24 hours before transfection. Lipid was added into OPTI-MEM (Invitrogen) and duplex RNA was added in a final volume of 1.25 mL. For reverse transfection, cells were detached and seeded into 6 well plates in 1 mL culture media. At the same time, lipid (Invitrogen) and duplex RNA were added into OPTI-MEM in a final volume of 1 mL. For double transient transfection experiments, the first transfection was performed and 2 days later the second transfection was carried out. Media was changed 24 hours later and every two days. Cells were typically harvested 2 days after transfection for RNA analysis by quantitative polymerase chain reaction (qPCR) analysis and 4 days after transfection for protein analysis by western blot.

Western blot and qPCR analysis.

Cyclooxygenase 2 (COX2), progesterone receptor (PR) and ataxin-3 (ATX-3) protein expression was analyzed by Western blot analysis. Protein from whole cell lysate was separated using 4 to 20% acrylamide precast gels (BioRad). The following primary

antibodies were used: anti-ATX-3 monoclonal antibody (MAB5360, Millipore), anti-PR monoclonal antibody (Cell Signal. Tech.), anti-Cox2 monoclonal antibody (Cayman Chemical) and anti- β -actin monoclonal antibody (Sigma). Protein bands were quantified using ImageJ using actin levels as an internal control. The percentage of inhibition or activation was calculated as a relative value to a control sample that was treated with a duplex RNA (siGL2) that lacked complementarity to transcribed human genes.

Total RNA was extracted using TRIzol (Life Technologies), and 2 μ g of RNA was subjected to DNase I (Worthington Biochemical Corp.) treatment. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). After an appropriate dilution of the cDNA sample, qPCR was performed on a CFX96 real-time PCR system (BioRad) using iTaq SYBR Green Supermix (Bio-Rad). Data were normalized relative to levels of GAPDH mRNA. The following qPCR primer sets were used for TNRC6A: 5'-AGCAAGCACAGGTACATCAG-3' (forward) and 5'-CAG TTG TGG CTG GAG TAG AAG-3' (reverse); TNRC6B: 5'-TGA CCC TGA ATC TGA CCC CTA TG -3' (forward) and 5'-TGC TGA AGT GCT ATG AAC GTT GG -3' (reverse); TNRC6C: 5'-CTG GAG GTC TAA GCA TTG GGC-3' (forward) and 5'-TCA GGG TCA TTC TCA GGG TCA A -3' (reverse). GAPDH primers were obtained from Applied Biosystems. Ago2 primers were purchased from ThermoFisher (Taqman gene expression assay (EIF2C2), Hs00293044_m1). The qPCR cycles are as follows: 50 °C for 2 min, 95 °C for 3 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Cell viability assay.

Four days after transfection, Hela cells were trypsinized and suspended in 1 mL culture media. Cells were mixed together with equal amount volume of trypan blue (Sigma) and counted by cell counter (TC20™ Automated Cell Counter, Bio-Rad). The percentage of live cells was calculated as a relative value to a control sample.

Mass spectrometry.

In solution fractionation mass spectrometry was adapted to measure the protein copy number per cell. Cell lysate was prepared using the FASP (Filter Aided Sample Preparation) method.²⁷ An Ultracel-10K (Millipore, UFC501024) filter was used for protein purification and trypsin digestion.

Mass spectrometry of the trypsinized peptides was performed by UT Southwestern proteomic core. An Ultimate 3000 RSLC nano-LC (Thermo Fischer Scientific) in-line connected to an Orbitrap Fusion Lumos (Thermo Fisher Scientific) is for MS analysis. Briefly, the sample was fractionated into 10 injections and peptides were loaded onto a reverse-phase column (Easy Spray column, either 75 μ m $\times$ 50 cm or 75 μ m $\times$ 75 cm, 2 μ beads). Peptides were loaded with solvent A (0.1% trifluoroacetic acid, 2% acetonitrile in water) and were separated with a linear gradient from 0% solvent A (2% acetonitrile, 0.1% formic acid in water) to 28% solvent B (0.1% formic acid, 80% acetonitrile, 10% trifluoroethanol, 10% water) at a flow rate of 250 nL/min over 60 minutes (for 50 cm column) or 90 minutes (for 75 cm column), followed by a wash reaching 99% solvent B for 5 min (50 cm column) or 25 min (75 cm column).

The mass spectrometer was operated in data-dependent, positive ionization mode, automatically switching between MS and MS/MS acquisition for the 10 most abundant peaks in a given MS spectrum. Full-scan MS spectra ($m/z = 400$ -1600) were acquired in the Orbitrap at a target value of 4E5 with maximum ion injection time of 50 ms, and a resolution of 120,000 at 200 m/z . The 10 most intense ions fulfilling a predefined criterion (AGC target 1E4 ions, maximum ion injection time of 100 ms, isolation window of 1.6 m/z , fixed first mass of 110 m/z , intensity threshold of 5E3, charge state = 2-7, peptide match preferred, exclude isotopes on, dynamic exclusion time of 25 s) were subjected to tandem MS scans in the ion trap using HCD with a stepped collision energy of 33 $\pm$ 5%. Raw files were processed using MaxQuant and used the latest human database from Uniprot. Then using the LFQ data as described to calculate the proteins copy number per cell.²⁸

In-gel complex identification MS was used to compare the relative abundance of proteins. Whole cell lysate of HeLa and A549 was prepared using FASP method lysis buffer (4% SDS, 100 mM Tris/HCl pH 7.6, 0.1 M DTT). 25 μ L of cell lysate was loaded into 4-20% SDS page gel and ran 30 mins under 50 V. Gel between 75-250 KD was cut and sent for MS. The data were analyzed with Andromeda (a peptide search engine) integrated into the MaxQuant environment and used the latest Human database from Uniprot. Relative abundance of protein was calculated (relative ratio to TNRC6A) based on two replicates.

Results

Experimental Design.

Double stranded RNA (dsRNA) can modulate gene expression through several different mechanisms.^{19,23,24} In the cytoplasm, fully complementary duplex RNAs can silence translation by recruiting AGO2 to induce cleavage of target transcripts.^{29,30} Alternatively, partially complementary miRNAs can bind to sequences within the 3'-untranslated region and can block translation by a variety of mechanisms that do not involve direct AGO2-mediated cleavage of the target mRNA.¹⁹

miRNAs and RNAi proteins are also found in cell nuclei.^{21,22} In the nucleus, dsRNA can recognize sequences near splice sites to redirect alternative splicing³¹. dsRNAs also have the capacity to interact with AGO2, recognize transcripts that overlap gene promoters, and either activate³² or repress expression of the parent genes³³. Activation is most easily observed when basal expression is low, while inhibition is observed when basal expression is high.

These diverse mechanisms may differ in their requirement for the mammalian GW182 paralogs TNRC6A-C. To test this hypothesis we designed siRNAs to inhibit expression of TNRC6A, TNRC6B, or TNRC6C and transfected these duplex RNAs into cells alone or in combination. In a second transfection, we then introduced a second dsRNA that was already known to modulate translation, splicing, or transcription. We then evaluated the effect of reduced TNRC6 levels on gene expression. For comparison the same procedure was followed to test the effect of silencing AGO2 on translation, splicing, or transcription. While there are four AGO variants in human cells (AGO1-AGO4), AGO2 appears to be the most important for RNAi and the one whose silencing has the most impact on gene expression.²⁴

Requirement for TNRC6 paralogs during translational silencing by fully complementary RNA.

For our first study we selected the simplest and best characterized mechanism of duplex RNA action – silencing of translation by a fully complementary duplex siRNA (Figure 2A).³⁰ We silenced TNRC6A, TNRC6B, TNRC6C individually and in double and triple combinations (Supplementary Figure 1).

We then attempted to inhibit of *ataxin-3* (*ATX-3*) expression using a duplex RNA that is fully complementary to a target sequence within the coding region of mRNA (Figure 2BC). *ATX-3* was chosen because it is a ubiquitously expressed gene whose silencing would not be expected to significantly impact cultured cells. Regardless of the combination of anti-TNRC6 siRNAs used, we observed no reversal of gene silencing upon reducing expression of one or more TNRC6 paralogs. By contrast, inhibition of AGO2 expression reversed RNA-mediated silencing of *ATX-3*. This result suggests that the TNRC6 paralogs are not necessary for translational silencing by fully complementary RNA.

Requirement for TNRC6 paralogs during translational silencing by miRNAs.

We chose to evaluate the control of SIRT1 expression and induction of apoptosis by miR-34a (Figure 3A) because it is a well characterized miRNA activity.^{34,35} Also, in contrast to systems that use luciferase reports, SIRT1 is an endogenous gene and allows a better understanding of natural miR-mediated gene regulation. The SIRT1 3'-untranslated region (3'-UTR) is partially complementary to miR-34a (Figure 3B). The mismatches do not block binding of the miRNA to its target but do prevent AGO2-mediated cleavage of the target mRNA.³⁶

We examined the effect of inhibiting the expression of the TNRC6 paralogs and observed that silencing of TNRC6A or all three TNRC6 variants protected cells from decreased proliferation upon transfection of the miR-34a mimic (Figure 3C). Since miR-34a triggers apoptosis, which in turn causes the levels of many proteins and RNAs to change, measuring the level of SIRT1 was not a reliable assay for activity. Reversal of the proliferation block suggest that, in contrast to translational silencing by fully complementary duplex RNAs, silencing by miRNAs requires TNRC6A. This observation is consistent with many observations that miRNA-mediated silencing involves proteins that bind to TNRC6 and reduce RNA stability or translation.³⁻⁶

Requirement of TNRC6 paralogs during RNA-mediated regulation of alternative splicing.

dsRNAs that are complementary to sequences near splice junctions can affect alternative splicing.³¹ We examined the effect of inhibiting expression of the TNRC6 paralogs using a model system consisting of a chromosomally integrated fusion of luciferase and an intervening region derived from human β -globin intron 2 (Figure 4A).³⁷ This model system had previously be used to evaluate the effect of both antisense oligonucleotides³⁷, duplex RNAs³¹, and single-stranded silencing RNAs³⁸ on alternative splicing. While this model can be evaluated by monitoring luciferase activity, we chose to directly monitor the change in splicing by agarose gel electrophoresis because we can visualize and quantitate the variation of both the intron 2-included and intron-2 excluded spliceforms.

We observed that, regardless of whether TNRC6A, TNRC6B, or TNRC6C were silenced alone or in combination, inhibiting TNRC6 expression did not effect the RNA-mediated control over alternative splicing (Figure 4B-D). By contrast, silencing AGO2 expression mostly abolished RNA-mediated alternative splicing. These data suggest that AGO2, but not the TNRC6 paralogs, are necessary for RNA-mediated alternative splicing.

Requirement for TNRC6 paralogs during RNA-mediated transcriptional silencing.

Duplex RNAs can bind to transcripts that overlap gene promoters and silence gene expression.^{23,24,39} Previous work has shown that this is an *in cis* effect where the small RNA recruits AGO2 to proximity of the protein complexes that regulate basal transcription. The progesterone receptor (*PR*) gene is one system where RNA-mediated transcriptional silencing has been well characterized.^{33,39,40} Several different duplex RNAs that are complementary to the region immediately upstream of the farthest upstream transcription start site have been shown to block *PR* expression in T47D breast cancer cells where basal transcription is high. Silencing involves binding to a noncoding transcript that overlaps the promoter in an antisense orientation (Figure 5A).

Several lines of evidence support the conclusion that RNA-mediated silencing of *PR* expression is due to inhibition of transcription. These lines of evidence include: 1) Inhibition of *PR* RNA expression; 2) Inhibition of *PR* expression when monitored by nuclear run-on analysis; 3) Lack of complementarity of inhibitory duplex RNAs to *PR* mRNA; 4) Physical association of inhibitory RNA to the promoter transcript; and 5) Different patterns of inhibition of *PR*-A and *PR*-B isoforms than is observed during standard translation silencing by duplex RNAs or antisense peptide nucleic acid (PNA) oligomers.

We examined RNA-mediated transcriptional silencing of *PR* protein after reducing levels of TNRC6A, TNRC6B, or TNRC6C alone or in combination. Regardless of which TNRC6 paralogs were silenced, we observed little or no effect on transcriptional silencing by the subsequent introduction of a duplex RNA known to block *PR* transcription (Figure 5BC). By contrast, inhibiting AGO2 expression readily reversed RNA-mediated transcriptional silencing of *PR*. These data suggest that AGO2, but not the TNRC6 paralogs, are necessary for RNA-mediated transcriptional silencing at the *PR* locus.

The data appear to show a slight reversal of silencing when TNRC6A, B, and C are silenced simultaneously, but not when TNRC6 A and B are silenced. As shown below, TNRC6C is expressed at extremely low levels or undetectable levels. Therefore, rather than ascribing a function to TNRC6C in transcriptional silencing that TNRC6A or B lack, the most reasonable explanation is that the slight reversal of silencing is an off-target effect do to the introduction of the three silencing RNAs into T47D cells.

Requirement for TNRC6 paralogs during RNA-mediated transcriptional activation.

The *COX-2* locus offers a well characterized system for examining RNA-mediated transcriptional activation in which duplex RNAs recognize a sense transcript that overlaps the *COX-2* promoter (Figure 6A).³² The *COX-2* gene is also a convenient model for studying the mechanism of RNA-mediated transcriptional activation because it possesses a high dynamic range between the activated and basal expression states. Fully complementary

dsRNAs, dsRNAs containing a central mismatch (12nc) designed to eliminate any potential for AGO2 mediated cleavage,³⁶ or a mimic of miRNA-589 can activate *COX-2* transcription when basal expression is low. Activation is robust and up to 30-fold higher levels of COX-2 protein can be produced after addition of promoter-targeted RNAs.³⁹

Several lines of evidence support a mechanism for COX-2 activation that involves elevated transcription.^{33,39–41} These lines of evidence include: 1) Increase in mRNA levels; 2) Increase in pre-mRNA levels; 3) Increased recruitment of RNA polymerase 2 at the COX-2 promoter; 4) Increase of histone marks associated with gene activation; 5) The activating RNAs lack complementary to COX-2 mRNA (COX-2 is controlled by a TATA box and the mRNA is well-defined); and 6) Reversal of gene activation upon silencing of WDR5, a protein known to be involved in gene activation that we recently demonstrated to interact with TNRC6.

We compared the effects of silencing TNRC6 paralogs alone or in combination and observed that inhibiting expression of TNRC6A and TNRC6B in tandem or all three paralogs together reversed activation of COX-2 expression upon addition of a promoter-targeted RNA that contained two central mismatches relative to its complementary target (Figure 6BC). Inhibition of AGO2 also reversed activation. Inhibiting expression of *TNRC6A*, *TNRC6B*, or *TNRC6C* individually did not affect activation. These results suggest that TNRC6 protein is necessary for RNA-mediated gene activation, but that TNRC6A and TNRC6B are redundant and the entire cellular pool of all TNRC6 paralogs must be reduced. The requirement for TNRC6 expression during gene activation is consistent with our previous mass spectrometry showing interactions between TNRC6A and various transcriptional activators and mediator subunits.⁴² The observation is also consistent with previous observations using RNA immunoprecipitation to detect the binding of both AGO2 and TNRC6A to the COX-2 promoter transcriptions.

Quantitative estimates of TNRC6 and AGO proteins per cell.

TNRC6 has the potential to bind multiple AGO proteins through its GW-rich AGO binding domain¹⁶ and has even been hypothesized to form phase separated condensates containing many AGO:TNRC6 interacting to enhance silencing of a target mRNA.¹⁷ TNRC6 can also bind the CCR4-NOT and PAN complexes by binding to NOT1 and PAN3 respectively.¹² In nuclei, TNRC6A can associate with factors involved in protein degradation, DNA repeat, and transcriptional control.⁴² The association between TNRC6 and other proteins has the potential to greatly expand the potential for small RNA:AGO:mRNA recognition to control gene expression. However, depending on the relative amounts of AGO and TNRC6 proteins inside cells it is also possible that AGO might act independent of binding to TNRC6 as part of a much smaller RNA:protein complex.

To begin to understand what types of complexes might be possible we used two different mass spectrometry approaches to estimate the abundance of AGO and TNRC6 proteins per cell. Both approaches allow evaluation of TNRC6A, TNRC6B, and TNRC6C. There are four AGO proteins in human cells (AGO1-4). While AGO2 is the primary catalytic protein involved in RNAi²⁹, our previous mass spectrometry had revealed interactions between TNRC6A with AGO1, AGO2, and AGO3.⁴²

In one approach, we used gel electrophoresis to isolate proteins that were similar to the expected molecular weight of AGO and TNRC6 proteins. These proteins were treated with trypsin and analyzed by mass spectrometry. This experiment allowed us to calculate the ratios of protein abundance for AGO and TNRC6 proteins in HeLa and A549 cells (Figure 7A). In both cell lines, we detected TNRC6A and TNRC6B but did not detect TNRC6C. AGO2 was present at greater abundance than either TNRC6A or TNRC6B individually. AGO1 and AGO3 were detected at a 3 to 4-fold lower level than AGO2. Taken together the levels of TNRC6A and TNRC6B were similar to the combined amounts of AGO1, AGO2, and AGO3.

We also employed quantitative label-free proteomics methods developed by Mann and colleagues that uses the mass spectral signal of histones as a proteomic ruler to estimate the absolute number of protein molecules per cells.^{28,43} In this technique, all cellular proteins are analyzed and data on thousands of proteins is obtained. High sensitivity is achieved by examining multiple aliquots of proteins by mass spectrometry and combining the results. Evaluating fractions rather than just one large sample improves detection of proteins that have low to moderate expression by reducing the likelihood that highly abundant proteins will overload the detection apparatus.

Our quantitative proteomic ruler analysis of material from HeLa cells revealed that AGO2 was present at 47,800 copies (Figure 7B). These data are similar to the number of AGO2 proteins previously reported in skin cells⁴⁴ or HeLa cells⁴³, reinforcing confidence in the methodology for mass spectrometry. In contrast to the estimate of relative amounts estimated after fractionation where AGO2 appeared to be more abundant than AGO1 or AGO3 (Figure 6a), AGO3 was almost as abundant as AGO2 while AGO1 was less abundant. The different AGO ratios (Figure 7A versus Figure 7B) may reflect the different purification protocols required by for the two mass spectrometry methods and suggest that data from quantitative mass spectrometry should be interpreted conservatively. TNRC6A and TNRC6B were present at 3600 and 14574 copies respectively. As observed for the relative ratio mass spectrometry, AGO4 and TNRC6C were not detected, consistent with neither protein playing a major cellular role.

Discussion

Scaffolding proteins help assemble protein complexes for efficient activities.^{45–47} They can be responsible for the assembly of complex cellular machines or may even form macromolecular aggregates that act as virtual organelles. Although their importance is clear, scaffolding proteins may not always receive adequate attention because unlike enzymes, receptors, or channels, they do not directly affect chemical change, stimulate biological pathways, or regulate the passage of key cellular factors. This report examines the different requirements for AGO and TNRC6 proteins during RNAi and the relative abundances of the two families of proteins.

Mammalian GW182 proteins provide an important model for how scaffolding proteins build intricate protein complexes because they have the potential to connect RNA:AGO complexes to effector proteins that regulate gene expression. AGO2 has a single binding site capable of

interacting with TNRC6 through GW repeats.¹⁶ TNRC6 can interact with up to three AGO proteins simultaneously.¹⁶

By binding multiple AGO proteins, TNRC6 has the potential to increase binding affinity by promoting cooperative interactions. Such interactions and the cooperativity they encourage may explain the observation that miRNA activity is correlated with the number of available binding sites.^{48–50} Beyond increasing the affinity of binding by promoting cooperativity, GW182 also contributes to gene regulation by recruiting the CCR4-NOT complex for translational repression and deadenylation.⁵¹

What if promoting cooperativity or recruitment of translational repressors is not necessary for controlling expression at specific targets or classes of target? Would GW182 still be necessary? To address this question we examined the involvement of the human GW182 paralogs in several different RNAi pathways including miRNA-mediated gene silencing, siRNA-mediated gene silencing, transcriptional silencing, transcriptional activation, and regulation of alternative splicing.

We observed that, while AGO2 was indispensable for each regulatory mechanism, the requirement of the GW182 paralogs was varied. Both fully complementary duplex RNAs and mismatch-containing miRNAs recognize mRNA in the cytoplasm and silence gene translation. Our data confirm the expected outcome that miRNA mimics require expression of GW182. TNRC6A and TNRC6B are redundant for miRNA-mediated silencing. By contrast, silencing by fully complementary RNAs does not appear to require GW182.

Why would cytoplasmic silencing of mRNA have a different requirement for GW182 depending on whether the silencing agent was fully complementary? Fully complementary RNAs function by binding to a single site within mRNA and cleaving it through the AGO2 catalytic engine.²⁹ There is no reason to expect that this activity would benefit from the ability of GW182 to promote cooperative interactions or the recruitment of additional protein factors because only one AGO2:RNA ribonucleoprotein complex is involved. Our data confirms that GW182 is not necessary and supports the suggestion that AGO2/RNA is competent to act as an independent ribonucleoprotein. However, we do not conclude that GW182 is not present. GW182 and other proteins might also be present because of the strength of the AGO:GW182 interaction, but their presence would have little or no function impact on the action of the siRNA.

Antisense oligonucleotides are well known to modulate alternative splicing and this mechanism has been used to develop two recently approved drugs.^{53,54} Antisense oligonucleotides do not require AGO2 or GW182, setting a precedent for believing that modulating alternative splicing can be achieved simply through recognition of an RNA target by an oligonucleotide without the need for recruiting a complex assortment of protein factors.

Our data support this belief. We demonstrate that AGO2 is necessary for recruitment of RNA to splice sites, presumably because AGO2 is indispensable for protecting and facilitating recognition by the RNA guide strand. GW182 is not necessary for RNA-mediated alternative splicing. This data suggests that simply blocking the splice site is sufficient, there

is no need to use the potential of GW182 as a scaffold for recruiting additional proteins. The finding is also consistent with the fact that there was only one target site for the duplex RNA designed to modulate splicing. A simple mechanism for AGO:RNA-mediated alternative splicing is consistent with the relatively simple and straightforward mechanism used for splice modulation by antisense oligonucleotides. As with siRNAs, it is possible that GW182 is bound to AGO, but the association is not necessary to redirect splicing.

We also examined examples of RNA mediated transcriptional activation and RNA mediated transcriptional silencing. At the *COX-2* promoter, expression of GW182 is essential. TNRC6A and TNRC6B are redundant, both must be silenced to reverse RNA-mediated gene activation. We have previously used mass spectrometry to demonstrate that TNRC6A can associate with several different proteins involved in transcription including members of the mediator complex and proteins involved in histone modification.⁴² The requirement for GW182 during transcriptional activation suggests that the scaffolding function of GW182 is necessary to switch promoter activity from a low basal state to a highly expressed activated state. GW182 is needed to recruit other proteins for transcriptional activation.

By contrast to the need for GW182 for activation at the *COX-2* locus, we saw no requirement for RNA-mediated transcriptional gene silencing at the *PR* locus. This outcome may reflect different requirements for transcriptional silencing and transcriptional activation. For transcriptional activation, the recruitment of factors is necessary to move from a low basal state to a high expressing activated state. For transcriptional silencing, our result suggests that the delivery of an RNA-AGO2 complex is sufficient to disrupt the activation complex and block transcription. The scaffolding function of GW182 would not be necessary.

It is important to note that we have examined only one example of transcriptional silencing and transcriptional activation apiece. The universe of RNA targets inside cell nuclei is a large one and the requirement for GW182 may vary depending on the context. Our results provide examples, not definitive predictive guidelines for all cases of RNA-mediated control of transcription. Finally, we note that the human protein paralogs TNRC6A and TNRC6B were relatively abundant. TNRC6C protein was barely detectable. While we examined only two cell lines, our data suggest that TNRC6A and TNRC6B will play much larger roles than TNRC6C. When specific RNA species or proteins interact as complexes inside cells, knowledge of stoichiometry can make an important contribution to understanding mechanism. To complement measurements of protein molecules per cell, methods for analyzing RNA numbers include droplet digital PCR (ddPCR) or quantitative PCR.⁵⁴

We find that the amount of AGO (AGO1, AGO2, AGO3) and TNRC6 (TNRC6A and TNRC6B) variants have remarkably similar abundances inside cells (Figure 7). There does not appear to be the large excess of GW182 necessary to push AGO into exclusive AGO-GW182 complexes and it is likely that AGO protein is available to interact independently of GW182. It is likely, however, that a substantial percentage of AGO and TNRC6 proteins are forming AGO:TNRC6 complexes that are likely to be the nuclei of even large complexes that rely on the scaffolding function of TNRC6. Much remains to be learned about the roles

of AGO1 and AGO3 (AGO4 appears to be expressed at a much lower level) and how these proteins affect the stoichiometry and function of the AGO:GW182 partnership.

Summary

Scaffolding proteins facilitate the assembly of protein complexes that are more than the sum of their parts. For RNAi, the scaffolding protein GW182 enhances cooperativity and recruits proteins that can block translation in the cytoplasm or enhance transcription in the nucleus. AGO:RNA is the minimal functional complex, however, and we have observed that GW182 is not always necessary for function. Our data suggest that scaffolding complexes should not be viewed as invariant and that independent activities are possible. Quantification of cellular proteins by mass spectrometry offers a useful perspective towards understanding the action of scaffolding proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AGO	Argonaute
TNRC6	Trinucleotide repeat containing gene 6
GW	glycine tryptophan
RNAi	RNA interference
COX-2	Cyclooxygenase 2
ATX-3	Ataxin 3
PR	Progesterone receptor

REFERENCES

- [1]. Eystathiou T, Chan EKL, Tenebaum SA, Keene JD, Griffith K and Fritzler JJ (2002) A phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles. *Mol. Biol. Cell* 13, 1338–1351. [PubMed: 11950943]
- [2]. Eystathiou T, Jakymiw A, Chan EKL, Seraphin B, Cougot N, Fritzler MJ (2003) The GW182 protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies. *RNA* 9, 1171–1173. [PubMed: 13130130]

- [3]. Liu J, Rivas FV, Wohlschlegel J, Yates JR, Parker R, and Hannon GJ (2005) A role for the P-body component GW182 in microRNA function. *Nat. Cell. Biol* 7, 1261–1266. [PubMed: 16284623]
- [4]. Rehwinkel J, Behm-Ansmant I, Gatfield D, and Izaurralde E (2005) A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. *RNA* 11, 1640–1647. [PubMed: 16177138]
- [5]. Jakymiw A, Lian S, Eystathioy T, Li S, Satoh M, Hamel JC, Fritzler MJ, and Chan EKL (2005) Disruption of GW bodies impairs mammalian RNA interference. *Nat. Cell Biol* 7, 1267–1274. [PubMed: 16284622]
- [6]. Eulalio A, Huntzinger E, and Izaurralde E (2008) GQ182 interaction with argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat. Struc. Mol. Biol* 15, 346–353.
- [7]. Li S, Lian SL, Moser JJ, Frtzler ML, Fritzler MJ, Satoh M, and Chan EKL (2008) Identification of GW182 and its novel isoform TNGW1 as translational repressors in Ago2-mediated silencing. *J. Cell Sci* 121, 4134–4144. [PubMed: 19056672]
- [8]. Eulalio A, Tritschler F, and Izaurralde E (2009) The GW182 protein family in animal cells: New insights into the domains required for miRNA mediated gene silencing. *RNA* 15, 1433–1442. [PubMed: 19535464]
- [9]. Yao B, Li S, and Chan EKL (2013) Function of GW182 and GW bodies in siRNA and miRNA pathways. *Adv. Ex. Med. Biol* 768, 147–163.
- [10]. Pfaff J and Meister G (2013) Argonaute and GW182 proteins: an effective alliance in cellular silencing. *Biochem Soc. Trans* 41, 855–860. [PubMed: 23863144]
- [11]. Fabian MR, Cieplak MK, Frank F, Morita M, Green J, Srikumar T, Nagar B, Yamamoto T, Raught B, Duchaine TF, Sonenberg N (2011) miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT. *Nat. Struct. Mol. Biol* 18, 1211–1217. [PubMed: 21984185]
- [12]. Braun JE, Huntzinger E, Fauser M and Izaurralde E (2011) GW182 proteins directly recruit deadenylase complexes to miRNA targets. *Mol. Cell* 44, 120–133. [PubMed: 21981923]
- [13]. Checkulaeva M, Mathys H, Zipprich JT, Attig J, Colic M, Parker R and Filipowicz W (2011) miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. *Nat. Struc. Mol. Biol* 18, 1218–1226.
- [14]. Takimoto K, Wakiyama M and Yokoyama S (2009) Mammalian GW182 contains multiple argonaute-binding sites and functions in microRNA-mediated translational repression. *RNA* 15, 1078–1089. [PubMed: 19398495]
- [15]. Schirle NT and MacRae IJ (2012) The crystal structure of human argonaute2. *Science* 336, 1037–1040. [PubMed: 22539551]
- [16]. Elkayam E, Faehnle CR, Morales M, Sun J, Li H, Joshua-Tor L (2017) Multivalent recruitment of human argonaute by GW182. *Mol. Cell* 67, 646–658. [PubMed: 28781232]
- [17]. Sheu-Gruttadauria J and MacRae IJ (2018) Phase transitions in the assembly and function of human miRISC. *Cell* 173, 1–12. [PubMed: 29570990]
- [18]. Lazzaretti D, Tournier I, and Izaurralde E (2009) The C-terminal domains of human TNRC6A, TNRC6B, and TNRC6C silence bound transcripts independently of argonaute proteins. *RNA* 15, 1059–1066. [PubMed: 19383768]
- [19]. Jonas S and Izaurralde E (2016). Towards a molecular understanding of micrRNA-mediated gene silencing. *Nat. Rev. Genetics* 16, 421–433.
- [20]. Song G, Chen H, Sheng G, Wang Y, and Lou J (2018) Argonaute facilitates the lateral diffusion of guide from being pushed away by ribosome. *Biochemistry* 57, 2179–2183. [PubMed: 29589750]
- [21]. Robb GB, Brown KM, Khurana J and Rana TM (2005) Specific and potent RNAi in the nucleus of human cells. *Nat. Struct. Mol. Biol* 12, 133–137. [PubMed: 15643423]
- [22]. Gagnon KT, Li L, Chu Y, Janowski BA and Corey DR (2014) RNAi factors are present and active in human cell nuclei. *Cell Rep* 6, 211–221. [PubMed: 24388755]
- [23]. Weinberg MS and Morris KV (2016) Transcriptional gene silencing in humans. *Nucl. Acids Res* 44, 6505–6517. [PubMed: 27060137]

- [24]. Kalantari R, Chiang C-M, and Corey DR (2016a) Regulation of mammalian transcription and splicing by nuclear RNAi. *Nucl. Acids. Res* 44, 524–537. [PubMed: 26612865]
- [25]. Morris KV, Chan SW, Jacobsen SE and Looney DJ (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* 305, 1289–1292. [PubMed: 15297624]
- [26]. Li LC, Okino ST, Zhao H, Pookot D, Place RF, Urakami S, Enokida H and Dahiya R (2006) Small dsRNAs induce transcriptional activation in human cells. *Proc. Natl. Acad. Sci. USA* 103, 17337–17342. [PubMed: 17085592]
- [27]. Wisniewski JR, Zougman A, Nagaraj N, and Mann M (2009) Universal sample preparation method for proteome analysis. *Nat. Methods* 6, 359–362. [PubMed: 19377485]
- [28]. Wisniewski JR, Hein MY, Cox J, and Mann M (2014) A “proteomic ruler” for protein copy number and concentration estimation without spike-in standards. *Mol. Cell. Proteomics* 1312, 3497–3506. [PubMed: 25225357]
- [29]. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song J-J., Hammond SM, Joshua-Tor L, and Hannon GJ (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science* 305, 1437–1442. [PubMed: 15284456]
- [30]. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K and Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498. [PubMed: 11373684]
- [31]. Liu J, Hu J and Corey DR (2012) Expanding the action of duplex RNAs into the nucleus: redirecting alternative splicing. *Nucl. Acids Res* 40, 1240–1250. [PubMed: 21948593]
- [32]. Matsui M, Chu Y, Zhang H, Gagnon KT, Shaikh S, Kuchimanchi S, Manoharan M, Corey DR and Janowski BA (2013) Promoter RNA links transcriptional regulation of inflammatory pathway genes. *Nucl. Acids Res* 41, 10086–10109. [PubMed: 23999091]
- [33]. Janowski BA, Huffman KE, Schwartz JC, Ram R, Hardy D, Shames DS, Minna JD and Corey DR (2005) Inhibiting gene expression at transcription start sites in chromosomal DNA with antigene RNAs. *Nat Chem Biol* 1, 216–222. [PubMed: 16408038]
- [34]. Yamakuchi M, Ferlito M, Lowenstein CJ. (2008) miR-34a repression of SIRT1 regulates apoptosis. *Proc. Natl. Acad. Sci. U.S.A* 105, 13421–13426. [PubMed: 18755897]
- [35]. Matsui M, Prakash TP, and Corey DR (2016) Argonaute 2-dependent regulation of gene expression by single-stranded miRNA mimics. *Mol. Ther* 24, 946–955. [PubMed: 26903376]
- [36]. Wang Y, Juranek S, Li H, Sheng G, Tuschl T and Patel DJ (2008) Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. *Nature* 456, 921–926. [PubMed: 19092929]
- [37]. Kang SH, Cho MJ and Kole R (1998). Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. *Biochemistry* 37, 6235–6239. [PubMed: 9572837]
- [38]. Liu J, Hu J, Hicks JA, Prakash TP, and Corey DR (2015) Modulation of splicing by single-stranded silencing RNAs. *Nucl. Acid Therapeutics* 25, 113–120.
- [39]. Schwartz JC, Younger ST, Nguyen NB, Hardy DB, Monia BP, Corey DR and Janowski BA (2008) Antisense transcripts are targets for activating small RNAs. *Nat Struct. Mol. Biol* 15, 842–848. [PubMed: 18604220]
- [40]. Yue X, Schwartz JC, Younger ST, Chu Y, Gagnon KT, Elbashir S, Janowski BA, and Corey DR (2010) Regulation of transcription by small RNAs complementary to sequences downstream from the 3' termini of genes. *Nat. Chem. Biol* 6, 621–629. [PubMed: 20581822]
- [41]. Janowski BA, Huffman KE, Schwartz JC, Ram R, Nordsell R, Shames DS, Minna JD, and Corey DR (2006) Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. *Nat. Struc. Mol. Biol* 13, 787–792.
- [42]. Hicks JA, Li L, Matsui M, Chu Y, Volkov O, Johnson KC, and Corey DR (2017) Human GW182 paralogs are the central organizers for RNA-mediated control of transcription. *Cell Reports* 20, 1543–1552. [PubMed: 28813667]
- [43]. Hein MY, Hubner NC, Poser I, Cox J, Nagaraj N, Toyoda Y, Gak IA, Weisswange I, Mansfeld J, Buchholz F, Hyman AA, and Mann M (2015) A human interactome in three quantitative dimensions organized by stoichiometries and abundances. *Cell* 163, 712–723. [PubMed: 26496610]

- [44]. Wang D, Zhang Z, O'Loughlin E, Lee T, Houel S, O'Carroll, Tarakhovsky A, Ahn NG, and Yi R (2012) Quantitative functions of argonaute proteins in mammalian development. *Genes Dev* 26, 693–703. [PubMed: 22474261]
- [45]. Nusinov R, Ma B, Tsai C-J. (2013) A broad view of scaffolding suggests that scaffolding proteins can actively control regulation and signalling of multienzyme complexes through allostery. *Biochim. Biophys. Acta* 1834, 820–829. [PubMed: 23291467]
- [46]. Good MC, Zalatan JG, Lim WA (2011) Scaffold proteins: hubs for controlling the flow of cellular information. *Science* 332, 680–686. [PubMed: 21551057]
- [47]. Walther C and Ferguson SSG (2015). Role of intracellular scaffolding proteins in the regulation of endocrine G protein-coupled receptor signalling. *Mol. Endocrinol* 29, 814–830. [PubMed: 25942107]
- [48]. Doench JG, Petersen CP and Sharp PA (2003). siRNAs can function as miRNAs. *Genes Dev* 17, 438–442. [PubMed: 12600936]
- [49]. Pillai RS, Artus CG, Filipowicz W (2004) Tethering of human Ago proteins to miRNA mimics the miRNA-mediated repression of protein synthesis. *RNA* 10, 1518–1525. [PubMed: 15337849]
- [50]. Broderick JA, Salomon WE, Ryder SP, Aronin N, Zamore PD (2011) Argonaute protein identity and pairing geometry determine cooperativity in mammalian RNA silencing. *RNA* 17, 1858–1869. [PubMed: 21878547]
- [51]. Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E (2006) mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev* 20, 1885–1898. [PubMed: 16815998]
- [52]. Havens MA and Hastings ML (2016) Splice-switching antisense oligonucleotides as therapeutic drugs. *Nucl. Acids Res* 44, 6549–6563 [PubMed: 27288447]
- [53]. Corey DR (2017) Nusinersen, an antisense oligonucleotide drug for spinal muscular atrophy. *Nature Neurosci* 20, 497–499. [PubMed: 28192393]
- [54]. Dodd DW, Gagnon KT, and Corey DR (2013). Digital quantitation of potential therapeutic target RNAs. *Nucl. Acid Therapeutics* 23, 188–194.

SYNOPSIS:

AGO2 and the scaffolding protein TNRC6 form the core RNAi complex. This critical collaboration, however, is not necessary for all RNAi-mediated pathways controlling gene expression.

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Figure 1. Schematic representation of TNRC6 paralogs and models of the small RNA pathway. (A) Domain organization of human TNRC6A isoform 2, TNRC6B isoform 1 and TNRC6C. UBA, ubiquitin associated-like domain; Q-rich, region rich in glutamine; PAM2: PABP-interacting motif 2; RRM, RNA recognition motif. (B) TNRC6 and AGO2 interact with miRNA leading to mRNA degradation. (C) AGO2 with fully complementary siRNA leads to the mRNA cleavage. (D) TNRC6 paralog involved in the gene activation or inhibition target the promoter site. (E) Ago2 on the regulation of splicing by duplex RNA.

Figure 2. Effect of silencing TNRC6 paralogs on inhibition of ATX-3 expression by a fully complementary duplex RNA targeting ATX-3 mRNA.

(A) Schematic showing recognition of a target site within ATX-3 mRNA by a fully complementary duplex RNA. (B) Western analysis of ATX-3 expression. In a first transfection (TRF) cells are treated with duplex RNAs to silence TNRC6 paralogs alone or in combination. In a second transfection, a duplex RNA is added that is designed to silencing ATX3 expression. (C) Quantification of data in (B). siGL2 is a noncomplementary control duplex RNA. n=2.

Figure 3. Effect of silencing TNRC6 paralogs or AGO2 on the activity of miR-34a. (A) Schematic of apoptotic pathway regulated by miR-34a. (B) Predicted target site recognition by miR-34a at the SIRT1 3'-UTR. (C) Effect of silencing TNRC6 paralogs or AGO2 on cell proliferation upon addition of a mimic of miR-34a. n=2.

Figure 4. Effect of silencing TNRC6 paralogs or AGO2 on the regulation of splicing by a duplex RNA.

(A) Schematic of target site within a fusion of the luciferase gene and an intervening region derived from human β -globin intron 2. (B) RT-PCR followed by agarose gel electrophoresis to separate aberrant and correct splice products after HeLa Luc/705 cells were double transfected by siRNAs. In a first transfection (TRF) cells are treated with duplex RNAs to silence TNRC6 paralogs alone or in combination or AGO2. In a second transfection, a duplex RNA is added that is known to correct splicing. (C-D) Quantification of data from (B). siGL2 is a noncomplementary control. 709 is a duplex RNA³¹ fully complementary to sequences near the introduced splice site within the β -globin-derived intron. Experiments were performed in HeLa-derived pLuc/705 cells containing a chromosomally integrated luciferase fusion. n=2.

Figure 5. Effect of silencing TNRC6 paralogs and AGO2 on transcriptional inhibition of PR expression by a duplex RNAs.

(A) Model of RNA-mediated transcriptional silencing at the *PR* promoter. The AGO:RNA complex recognizes an antisense transcript that overlaps the promoter. (B) Western analysis of PR protein expression. In a first transfection (TRF) cells are treated with duplex RNAs to silence TNRC6 paralogs alone or in combination or AGO2. In a second transfection, a duplex RNA is added that is known to inhibit PR expression. (C) Quantification of data in

(B). PR9 is a duplex RNA that silences PR expression. siGL2 is a negative control noncomplementary RNA. n=2.

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Figure 6. Effect of silencing TNRC6 paralogs and AGO2 on transcriptional activation of *COX-2* expression by a duplex RNAs.

(A) Model of RNA-mediated gene activation at the *COX-2* promoter. A duplex RNA recognizes a sense transcript that overlaps the *COX-2* promoter. (B) Western analysis of COX-2 protein expression. In a first transfection (TRF) cells are treated with duplex RNAs to silence TNRC6 paralogs alone or in combination or *AGO2*. In a second transfection, a duplex RNA is added that is known to activate COX-2 expression. (C) Quantification of data in (B). 12nc is a duplex RNA that is complementary to the *COX-2* promoter with central

mismatches and activates its expression. siGL2 is a negative control noncomplementary RNA. n=2.

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Figure 7. Relative abundance of TNRC6 paralogs measured by mass spectrometry. (A) The relative ratio of the TNRC6 paralogs and AGO proteins, with the amount of TNRC6A used as the standard. n=2. (B) The copy number of TNRC6 paralogs and Ago proteins per cell. N.D., no peptides detected.