

GW182 SILENCES MICRORNA TARGETS BY DIVERGENT FUNCTIONAL DOMAINS
AND REGULATES MICRORNA STABILITY

By

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To my grandparents and parents for their selfless devotion to my life; to my wife for her deepest love and constant encourages to me; to my mentor for his tireless help during my PhD study

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Abstract of Dissertation Presented to the Graduate School
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MicroRNA (miRNA) is a relatively new class of short endogenous non-coding RNA. MiRNA can pair with the 3' untranslated region of mRNA. This interaction inactivates protein translation from the mRNA, and eventually triggers mRNA decay. Almost all important cellular processes are known to be regulated in part by miRNA. Aberrant expression of miRNA is often associated with a variety of diseases including cancer. The goal of my dissertation research was to understand the miRNA silencing mechanism, identify and characterize key protein players in this process, and elucidate how the mature miRNA level is regulated. These discoveries will expand our knowledge of this key post-transcriptional gene regulation pathway and offer the possibility of developing miRNA-mediated therapeutic approaches.

GW182 is an 182kDa protein with multiple glycine/tryptophan repeats (GW or WG) playing a central role in miRNA-mediated gene silencing. GW182 interacts with its functional partners, the Argonaute proteins (AGO), via multiple domains to exert its silencing activity. Using tethering functional assays, we identified two non-overlapping GW182 silencing domains, namely $\Delta 12$ and $\Delta 5$, primarily induced translational repression rather than mRNA decay. GW or WG repeats within these two domains

were critical for silencing activity. These data implicated a dynamic and diversified role of GW182 in miRNA-mediated gene silencing conveyed by its silencing domains and further established GW182 as a central player in silencing gene expression.

Furthermore, we identified a new role of GW182 in regulating miRNA stability aside from its silencing function. Depletion of GW182 reduced mature miRNA level at different time points and caused impaired miRNA export through secretory vesicles termed exosomes. A 3'-5' exoribonuclease complex was identified to be responsible for miRNA degradation. These findings revealed a novel role of GW182 in regulating mature miRNA stability by binding to Argonaute proteins. This work also supported the importance of GW182 in miRNA-mediated silencing homeostasis.

CHAPTER 1 INTRODUCTION

The protein GW182, was discovered in 2002 using a serum from an autoimmune patient with motor and sensory neuropathy (1), is characterized by a large number of glycine/tryptophan (GW and WG) repeats that are distributed in various motifs throughout its sequence. GW182 is an 182 kDa phosphoprotein with up to 60 copies of GW/WG motifs, some of which have been shown to bind to its functional partner in the Argonaute protein family (Ago1-4) and are critical in microRNA (miRNA) mediated silencing function. The GW182 protein family has three paralogs in mammals named TNRC6A, B, and C, all of which have been demonstrated to play key roles in small interference RNA (siRNA) and miRNA silencing (2-5).

The initial discovery of GW182 noted that it was primarily localized to distinct cytoplasmic foci with dynamic morphology and movement (1,6). Based on their unique GW content and cytoplasmic localization, these foci therefore were provisionally termed GW bodies (GWBs). A set of other proteins involved in RNA degradation were later shown to reside in GWBs linking this structure to the function of RNA turnover (7-10). (Figure 1-1). These bodies are generally acknowledged to be conserved among species since mammalian GWBs share a similar subset of protein homologs with yeast or *Drosophila* processing bodies (P-bodies). However, Ago2 and GW182 are examples of some key proteins that do not have homologs in yeast P-bodies (11). In addition, the size and number of GWBs increase during cell proliferation in mammalian cells (6), whereas as a reflection of their functional differences, the number and size of P-bodies in yeast increase in response to stress (7,10). The heterogeneity of GWBs is denoted by observations that not all foci have the same composition: some foci contain

undetectable or very low levels of Ago2, mRNA decapping activator DCP1a, and DEAD box RNA helicase rck/p54.

siRNAs and miRNAs partially share the same pathway in silencing their targets based on their Watson-Crick base pair matching for target recognition (Figure 1-2). miRNAs are usually derived from endogenous transcripts from their respective gene loci to first form primary miRNAs (pri-mRNAs) with 7-methylguanosine (m7G) caps and polyadenylated (An) 3'-ends flanking hairpin structures as shown in (Figure 1-2, right panel). Mammalian genes for miRNAs exist as single genes, gene clusters, and within introns of other genes. The RNase III endonuclease Drosha-DGCR8 complex processes pri-miRNAs into about 70-nucleotide hairpin structures referred as precursor-miRNAs (pre-miRNAs). After being transported into cytoplasm, pre-miRNAs further associate with Dicer, another RNase III endonuclease, resulting in a ~22 base pairs miRNA:miRNA* duplex (2,5,12,13). In contrast, siRNAs originate from long double-stranded RNAs that can be derived from viral replication, transposons, or convergent transcripts. The Dicer complex slices these long RNAs into 20~25 base pairs, perfectly matching a double-stranded RNA duplex. Synthetic siRNA duplexes of the same size can also knockdown target genes. siRNA or miRNA duplexes undergo a selective loading process to AGO based on the thermodynamic preference of the two strands. Guiding strands directly associate with AGO proteins and the passenger strand will be ejected and eventually degraded (12,14,15). Guiding strands form a perfect complementarity with their target mRNAs, following which, Ago2 cleaves the target mRNA by its endonuclease activity localized in its C-terminal PIWI (RNase H-like P-element induced wimpy testis) domain (16). miRNA-loaded AGO complexes are

tethered to 3'UTR of target mRNAs and form an imperfect complementarity but are usually strictly matched at miRNA positions 2 to 8, known as “seed sequence” (17). All 4 AGO proteins (Ago1-4) are involved in miRNA-mediated gene silencing and the GW182 protein family functions downstream as key repressors by inhibiting active translation, as well as trigger bound mRNA deadenylation and eventual degradation (4,5,13) (Figure 1-2).

Extensive mapping of AGO-interacting and repression domains on GW182 generated consensus that the GW182 N-terminal GW-rich domain primarily binds to AGO on multiple sites, whereas the C-terminal domain possesses intrinsic silencing activity (18-22). However, in addition to that, the *Drosophila* and human N-terminal effective domain, which in fact covers much of the middle region of GW182, also has silencing activity (22-24). Although the detailed molecular mechanism underlying translational repression remains elusive, increasing evidence has suggested that the conserved GW182 Pam2 motif (Poly-A binding protein (PABP)-interacting motif 2) functions as a major docking site to interact with PABP (25,26). In addition, GW182 can further recruit CCR4-NOT deadenylase complex to facilitate mRNA decay (27-29). This dynamic process may occur in GWBs and a general model of how they affect translational repression and mRNA degradation will be discussed in Chapter 4.

Although a number of studies have dissected the function of GW182, it needs to be pointed out that there are a few inconsistencies. These discrepancies may arise from differences in the selection of systems (e.g. using *Drosophila*, yeast, versus mammals) or methodologies employed (e.g. investigating miRNA and mRNA by qRT-PCR vs Northern blot). In this chapter we focus primarily on the role of GW182 and GWBs in

siRNA and miRNA silencing pathway based on a number of published reports from our laboratory over the past ten years (1,6,7,20,24,30-36) to help understand the rationale and development of my dissertation study.

Role of GW182 and GWBs in siRNA Silencing Pathway

In 1998, Fire and Mello systematically described that double stranded RNA can potently and specifically interfere gene expression in *Caenorhabditis elegans* (37). For this work, a Nobel Prize was awarded within less than a decade, indicating its fundamental implications and important prospects for the future of cell and molecular biology. Elucidating the mechanism of this post-transcriptional gene regulation is tremendously beneficial for basic research and has clear implications for clinical applications. GW182 appears to be a core protein in cellular process and our laboratory has been investing in studies of the relationships between GW182 and the siRNA pathway.

Disruption of GW Bodies Impaired Mammalian RNA Interference

Since the discovery of GW182 and GWBs in 2002, substantial efforts have elucidated their functional implications. In 2005, Jakymiw et al. and others simultaneously reported that GW182 bound to Ago2 (31,38) linking it to the then newly established siRNA and miRNA work (37,39). By using an Ago2 specific antibody, we demonstrated that Ago2 localized to discrete cytoplasmic foci that were co-stained with an anti-GW182 monoclonal antibody as well as the prototype human anti-GWB serum 18033 (31). Co-immunoprecipitation data using polyclonal human serum 18033 or mouse monoclonal anti-GW182 antibody 4B6 pulled down Ago2, demonstrating the strong interaction between Ago2 and GW182. Intriguingly, Cy3-3'-end-labeled lamin-A/C specific antisense siRNA duplex localized to GWB and co-immunoprecipitated with anti-

GW182 antibody implying the potential functional connections between GWBs and siRNA pathway.(31).

Our laboratory had previously demonstrated that knocking down the GWB scaffold protein GW182 by specific siRNA could affect the stability of GWBs (6). Therefore, a sequential knockdown strategy was performed to first knockdown the endogenous GW182 protein followed by a second transfection conducted with siRNA against lamin A/C 48 hours later. The results clearly demonstrated that depletion of GW182 caused the disappearance of the GWB foci and more importantly, abolished the knockdown of lamin A/C. In contrast, the knockdown of lamin A/C was affected when the cells were firstly transfected with a siRNA against luciferase, which was not a target in these cells. Similar results in reduced lamin A/C siRNA reporter activity were obtained when lamin A/C siRNA was co-transfected with siRNA to GW182 (32). GW182 paralogs TNRC6B and TNRC6C largely share conserved domains and have been shown to have functional redundancy (3,40). In fact, depletion of individual GW182 family members caused partial de-repression of silenced reporters (22), and tethering assays mapped similar silencing domains on all GW182 family proteins (18,19,22,41). It remained to be determined the distinctive functions of each GW182 paralogs, in particular cellular context.

In brief, GW182 binds to Ago2 both *in vivo* and *in vitro*. This protein complex serves as a core component of the human RISC complex that co-localizes to the GWBs with transfected siRNA. GWBs play a critical role in siRNA silencing function as disassembly of GWBs by either dominant-negative constructs or siRNA directly knocking down GWB scaffold protein GW182 leads to the loss of siRNA function. These

data provided strong evidence for linking between GW182 and GWBs to siRNA function.

Small Interfering RNA-Mediated Silencing Induced Target-Dependent Assembly of GW/P Bodies

To address the correlation between siRNA and GWBs, siRNA against endogenous lamin A/C or siRNA against luciferase was transfected into HeLa cells. The size and number of GWBs were monitored by indirect immunofluorescence using both prototype human serum 18033 and rabbit anti-Dcp1a antibody. Intriguingly, larger and greater numbers of GWBs were observed only in lamin A/C siRNA transfections but not in mock or luciferase siRNA that lacked endogenous target (34). Another siRNA against the endogenous target RAGE showed similar effects as lamin A/C siRNA induced higher numbers and increased the size of GWBs, but not by a synthetic “RISC free” chemically modified siRNA (34). siRNAs against endogenous targets do not appear to affect stress granules as detected by the marker TIAR, the T-cell intracellular antigen-1(TIA-1)-related protein. The mRNA-dependent effect in these experiments was further examined in 3T3 cells stably transfected with GFP vs untransfected 3T3 cells using a single siRNA to GFP. The transfection of the GFP siRNA led to a significant increase in both the size and number of GWBs in the stable GFP expressing 3T3 cells, but not in wildtype 3T3 lacking GFP transcripts (34). The protein levels of several GWB components, such as Ago2, DCP1a, rck/p54 and RNA binding protein Lsm4, did not increase upon lamin A/C siRNA transfection, an observation that is consistent with the concept that these proteins are recruited from free cytoplasmic forms into GWBs.

In summary, transfection of endogenous targeted functional siRNA can modulate the size and number of GWBs. GW182 and Ago2 were required for functional siRNA

silencing activity. Based on these data, it can be concluded that RNAi was a key regulatory mechanism for the assembly of GWBs.

Role of GW182 and GWBs in miRNA Silencing Pathway

The first described miRNA, *lin-4*, can negatively regulate its target gene LIN-14 during different developmental stages in *C.elegans* (39). The identification of *let-7* miRNA as the first miRNA conserved across species has opened a door for the remarkable advances in the field of miRNA research dating to 2000 (42). It is currently held that more than 50% of mRNAs are regulated by miRNAs involved in almost all known cellular pathways (2,4). GW182, discovered two years after *let-7* was described in mammals, has become a central player in the miRNA mediated silencing pathway (4,5). Our laboratory has been working on the role of GW182 in miRNA pathway including identification of novel GW182 isoform, elucidation of Ago2 binding domains and mRNA silencing domains for the past few years (20,24,35).

TNGW1 Is a Novel Isoform of GW182 and Distinct at both Transcriptional and Translational Levels

Human TNRC6A (the gene encoding GW182) is located on chromosome 16p11.2. Since the discovery of GW182 (1), homologs have been subsequently characterized in various species including *Drosophila* and *C. elegans* (43-46). However, the isoforms of GW182 were not explored until 2008 (35). Interestingly, both the NCBI database and University of California Santa Cruz Genome Browser predicted a novel isoform of GW182, later named as TNGW1 by Li et al. with a nucleic acid sequence identical to GW182 but having an additional N-terminal region containing trinucleotide repeats (TNRs). The mRNA for TNRC6A longer isoform TNGW1 contained 5 additional exons upstream of the putative AUG start codon of GW182 and the TNR repeat domain was

encoded by the fifth exon. To verify the presence of this isoform, reverse transcriptase PCR using primers flanking the unique N-terminal TNR domain were utilized to examine RNA from a number of different human cell lines and tissue samples. The amplified PCR products were submitted to direct DNA sequencing, which validated the correct length and an in-frame junction between the novel 5' exons of TNGW1 and GW182 as found in the various cells examined.

The next question was whether TNGW1 mRNA is expressed as a native protein. To address this question, a recombinant protein containing the TNR domain alone was generated to raise monoclonal and polyclonal antibodies as specific probes for the expression of this putative longer isoform of GW182. After confirming the specificity of these antibodies (i.e. cross reactivity with GW182 minus the TNR domain) by addressable laser bead immunoassay and Western blot, the differential expression of TNGW1 and GW182 in HeLa cells was examined by immunoprecipitation-Western blots. Antibodies specific for the TNGW1 TNR domain only recognized the slower migrating TNGW1 band whereas rabbit polyclonal and mouse monoclonal antibodies directed against GW182 recognized both the 210 and 182 kDa forms of GW182. This supported the notion that both TNGW1 and GW182 proteins were expressed in HeLa cells. Of note, the expression of GW182 was considerably higher than TNGW1.

We then asked, what is the relationship between these two proteins? They could be independently translated as distinct mRNAs, from the same mRNA with a different AUG start codon, or GW182 could simply be a post-translationally processed form of TNGW1. To distinguish these three possibilities, siRNA specifically targeted TNGW1 mRNA (referred as siTNR, thereafter) was applied to discriminate TNGW1 and GW182

mRNA. Forty-eight hours after transfection, only TNGW1 protein became undetectable whereas levels of the GW182 protein remained the same compared to the control or mock transfection (35). These results demonstrated that TNGW1 protein was derived from its own unique mRNA. It remained unclear whether GW182 was the post-translational product from TNGW1 because the GW182 band observed after siTNR treatment could be a stable product processed from TNGW1. However, the Western blot result from cells treated with siRNA targeted a common region of TNGW1 and GW182 (referred as siGW182) ruled out this possibility because the disappearance of both proteins suggesting both isoforms retained similar turnover rates. We concluded that each protein isoform is reasonably considered as a product translated from its individual mRNA. Collectively, TNGW1 and GW182 appear to be derived from distinct transcription and translation events (35).

Indirect immunofluorescence data implied that TNGW1 was either absent or expressed at much lower level compared to GW182 in a subset of GWBs. However, their silencing activity seemed to be identical. To determine the silencing effect induced by TNGW1 and GW182, a reporter tethering assay was adopted from Dr. Filipowicz's work (47). An N-terminal λ N-hemagglutinin (NHA) polypeptide tag was fused to TNGW1, GW182, or Ago2. The NHA tag binds strongly to the 5BoxB RNA secondary structure cloned in the 3'UTR of either Firefly luciferase (FL) or Renilla luciferase (RL) reporter. Co-transfection with NHA-tagged GW182 or Ago2 fragments with the RL-5BoxB reporter allowed discrimination of their repression activity. RL- or FL-no sites in their 3'UTR served as an internal control, respectively. A 46% repression was observed to the reporter when NHA-Ago2 was tethered to the 3'UTR of the reporter, which is

consistent with the original report describing this tethering assay (47). In comparison, tethered NHA-TNGW1 or -GW182 induced stronger repression (67.6% and 65.3%, respectively), which was 46.9% or 41.3% stronger than that induced by Ago2, respectively (35). Quantitative RT-PCR was also performed to analyze the stability of reporter mRNA level. Interestingly, tethered Ago2 induced 50.8% reporter mRNA degradation as compared to tethered TNGW1 and GW182 (24.5% and 23.7%, respectively). These data together indicate that tethered GW182 and TNGW1 possess stronger silencing activity than Ago2. The silencing activity in this experiment is primarily caused by interfering with translation with only mild effects on the reporter mRNA stability. In contrast, tethered Ago2 causes more reporter decay than GW182/TNGW1.

To further characterize the hierarchical relationship between Ago2 and GW182 isoforms, tethering assays were performed in siRNA-mediated Ago2 and GW182 knockdown cells, respectively. The repression effect induced by tethered Ago2 was completely abolished in GW182 knockdown cells. By contrast, the TNGW1 and GW182 repressions were not affected by introducing Ago2 siRNA. These data suggested that TNGW1/GW182 has a more direct and central role on repression than Ago2, which may carry miRNAs and secure miRNA:mRNA interactions.

The C-Terminal Half of Human Ago2 Bound to Multiple GW-Rich Regions of GW182 and Required GW182 to Mediate Silencing

Since the AGO-GW182 interaction is important, A series of deletion constructs were generated spanning the entire TNGW1 for initial mapping of the Ago2 binding domain(s) (20). An in vivo GST-pulldown assay was utilized to determine the interactions between different constructs. Most of our data was consistent with what Takimoto et al. published almost at the same time: there were three Ago2 binding sites

in N-terminal GW182. One was equivalent to the GW182 N-terminal $\Delta 1$ (aa 254-751) region and other two were in the GW182 middle $\Delta 12$ (aa 896-1219) region. An additional binding site localized in the GW182 C-terminal $\Delta 5$ (aa 1670-1962) was observed in our experiments but not in those of Takimoto et al. (all GW182 deletion fragments shown in Figure 2-1). Our subsequent experiments confirmed weak $\Delta 5$ -PIWI interaction compared to that of $\Delta 12$ -PIWI (24). Interestingly, all the Ago2-binding domains were enriched in the GW/WG motif. Therefore, the $\Delta 5$ -PIWI interaction might result from low affinity of C-terminal $\Delta 5$ to Ago2 due to smaller number and density of GW/WG motifs in this region (3). Taken together, our laboratory and others identified four Ago2 binding regions on GW182 protein and that the Ago2 PIWI domain interacted with Ago2.

A Renilla-5BoxB tethering reporter was also used to determine the silencing ability of Ago2 fragments and other Ago family proteins and, more importantly, evaluate their relationship to GW182. When tethered to the 3'UTR of the RL-5BoxB reporter, NHA-PIWI induced almost equally strong repression as the NHA-full length Ago2 (20). In contrast, NHA-PAZ did not repress Ago2. These data suggested that the silencing ability harbored by Ago2 was mainly within its C-terminal PIWI domain. Other Ago family proteins including Ago1 and Ago4 were tethered to the reporter to assess their repression abilities. Comparable repression effects were observed in Ago1 and 4 similar to that of tethered Ago2 or PIWI. Intriguingly, Ago3m that did not bind to GW182 as described previously, did not have repression activity in tethering assays. These observations, together with those of Li et al. (35), demonstrated that the repression induced by the AGO family is dependent on their interaction with GW182.

Summary

It has been ten years since the discovery of GW182, an amazing protein with many unique features that deserved attention. This discovery opened the door for numerous studies on miRNA silencing mechanisms, which were poorly understood dating to the identification of the first miRNA, Lin-4, in 1993 (39). Currently, it has been widely accepted that GW182, together with its paralogs, is a key component of the RISC associated miRNA silencing pathways. We therefore sought to further map the GW182 silencing domain(s) to gain a better understanding of this protein (Chapter 2). In the meanwhile, we used lentiviral based shRNA strategy to deplete GW182 in HEK293 cells to define its role in regulating AGO bound miRNA level (Chapter 3). My dissertation research defined two non-overlapping silencing domains on GW182, and demonstrated GW182 is important in regulating mature miRNA half-life.

This chapter is part of a submitted chapter entitled “Function of GW182 and GW bodies in siRNA and miRNA pathways.” in the book “Ten Year History of Progress in GW/P Body Research” Springer Press.

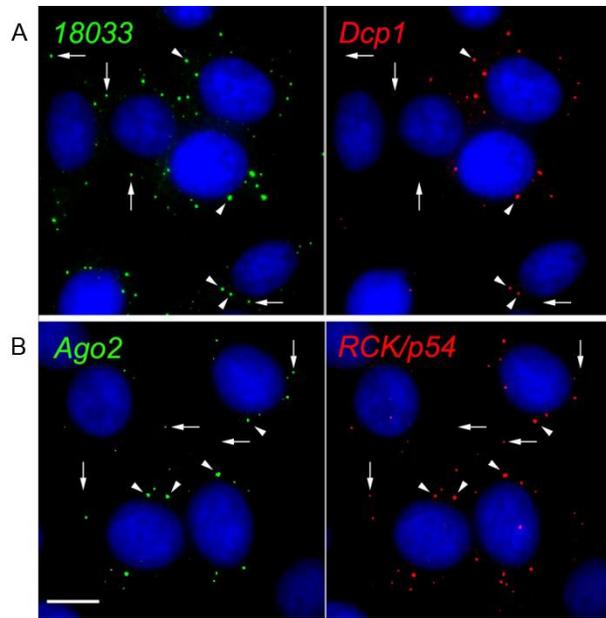
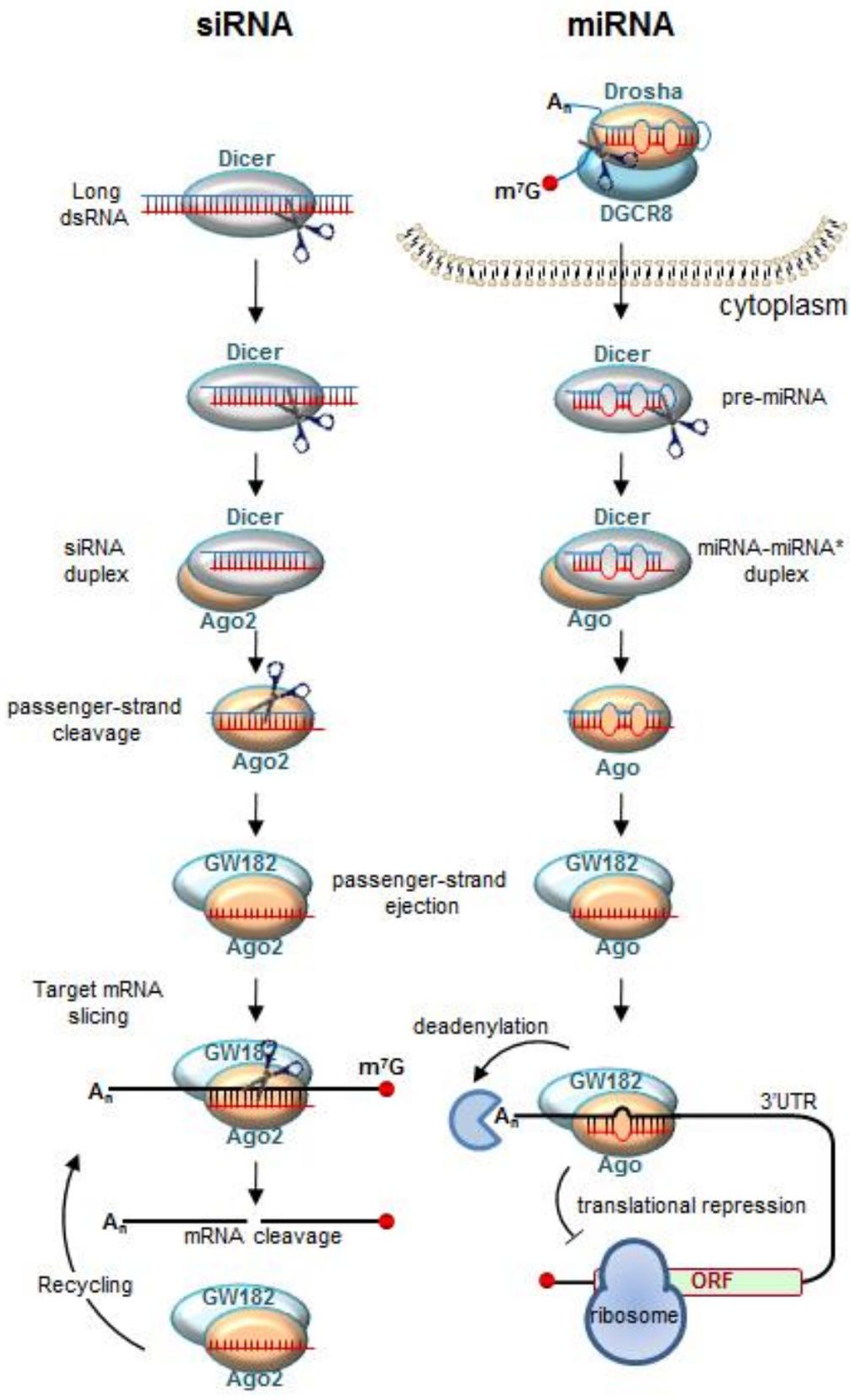


Figure 1-1. GW bodies (GWBs) are heterogeneous structures with some obvious differences in protein composition (7). A) HEp-2 cells costaining with prototype human anti-GWB serum 18033 (green), known to contain antibodies to GW182, hAgo2, and Ge-1, but not Dcp1, and rabbit anti-Dcp1 antibody (often used as a marker for P bodies; red) demonstrate that although there are many costained foci (arrowheads), not all GWBs contain detectable Dcp1 (arrows). B) HEp-2 cells costaining with mouse anti-hAgo2 monoclonal 4F9 (green) and rabbit anti-RCK/p54 (red) polyclonal antibodies demonstrate again that most foci have both (arrowheads) but not all containing both RCK/p54 and hAgo2 (arrows). Nuclei (blue) were counterstained with DAPI. Bar, 10 μ m.

Figure 1-2. Biogenesis and mechanism of the two main classes of small regulatory RNA. Long double stranded RNA (dsRNA) derived from viral infection, transposon or convergent transcription can be recognized and processed by Dicer, an RNAase III endoribonuclease, to become 20-25 bp small interference RNA (siRNA, left panel). The siRNA duplex including both the guiding strand (red) and passenger strand (blue) is transferred from Dicer onto Ago2, the catalytic component of RNA-induced silencing complex (RISC) complex also possessing endonuclease activity on its C-terminal PIWI domain. Core protein components Ago and GW182 are representing the holo-RISC complex. During the loading process, the passenger strand is cleaved and ejected. Ago2 and GW182 together with the guiding strand identify and hybridize perfectly with the target mRNA. Ago2 then catalyzes the cleavage at the middle region of the siRNA-mRNA duplex to slice the mRNA into two halves. The complex may be recycled for multiple rounds of cleavage. In contrast, miRNA is often derived from primary miRNA (pri-miRNA) which is transcribed from a single miRNA gene locus, multiple gene clusters, or processed from introns. Pri-miRNA has a 5'-end cap, a poly-A tail, and a hairpin stem-loop secondary structure and is processed by Drosha-DGCR8 protein complex in the nucleus to become hairpin precursor miRNA (pre-miRNA). Pre-miRNA is translocated into cytoplasm through a nuclear transporter and then bound by Dicer. Similar to the siRNA pathway, Dicer processes the pre-miRNA into matured miRNA-miRNA* duplex (guiding strand and passenger strand (also known as miRNA*)) which is then transferred to Ago. The guiding strand loaded complex forms imperfect complementary with its target mRNA preferentially at the 3'UTR. All the Argonaute family proteins can be involved in miRNA-mediated silencing and GW182 plays a critical role in translation repression, deadenylation, and mRNA decay. Modified from Jinek, M. and Doudna, J.A. (12)



CHAPTER 2 DIVERGENT GW182 FUNCTIONAL DOMAINS IN THE REGULATION OF TRANSLATIONAL SILENCING

Background

MicroRNAs (miRNA) are endogenous 20-25 nt RNAs largely transcribed from independent miRNA genes or gene clusters and play many key roles in a variety of normal and pathological cellular processes (13). MiRNAs are incorporated into the RNA-induced silencing complex (RISC) to effect translational repression or RNA degradation of their target mRNAs (2,7,17,48,49). The Argonaute protein family, a highly conserved key component of the RISC complex, is represented by four proteins (Ago1-Ago4) in mammals that are involved in miRNA-mediated translational silencing (15). Only Ago2 harbors RNase H-type activity in its C-terminal P-element induced wimpy testis (PIWI) domain and is known to function in small interfering RNA (siRNA)-mediated slicing of mRNA targets by endonucleolytic cleavage (16,50,51).

GW182 (Gene name TNRC6A) was first identified and characterized by our laboratories in 2002, as a novel protein recognized by an autoimmune serum from a patient with motor and sensory neuropathy (1). It is an 182 kDa protein characterized by multiple glycine (G) and tryptophan (W) motifs and is an essential component of GW bodies (also known as mammalian processing bodies, or P bodies) (6,7). Two isoforms of GW182, named TNGW1 (long isoform) and GW182 (short isoform) respectively, have been subsequently reported with TNGW1 being identical in sequences with GW182 but has additional N-terminal 253 amino acids (aa) containing trinucleotide glutamine-repeat (TNR Q-repeat) domain (35). In the GW182 family, there are three paralogues of TNRC6 (GW182-related) proteins comprising GW182/TNGW1, TNRC6B (containing 3 isoforms) and TNRC6C in mammal, a single *Drosophila* ortholog

(dGW182, also known as Gawky) and two *C. elegans* ortholog AIN-1 and AIN-2. (2,3,13,40,52). They are known to play a critical role in the silencing and degradation of miRNA-targeted mRNAs across different species (18,19,21-23,25,26,31,35,38,41,52-61). Significant progress has been made in characterizing the 3'UTR sequence element required for efficient targeting and regulation of miRNA (62,63) but the detailed molecular basis of the miRNA-mediated translational silencing and mRNA degradation, especially with respect to their role of human GW182/TNGW1 is not completely understood (2,3,13,40,52). The Argonaute proteins, including Ago1 to Ago4, are the most highly characterized factors in the miRNA-induced silencing complex (miRISC), where they bind miRNA to mediate recognition of target mRNAs (12,64). Argonaute proteins artificially tethered to the mRNA 3'UTR induce translational silencing (47,56,65). However, the Ago-miRNA/mRNA complex requires recruitment of additional protein factors to effect subsequent translational repression (35,45,54). Multiple candidates have been proposed to play an important role in the miRNA-mediated translational silencing. Among these, GW182 is a conserved factor that retains a key role in miRNA-mediated translational repression and mRNA degradation across different species, as evidenced by studying of GW182 proteins in humans (18,19,21,22,25,26,38,56,58), *Drosophila* (23,41,45,53-55,57,59), and *C. elegans* (43,61). An important feature of the GW182 family in this process is its conserved ability to bind with Ago proteins (18,19,21,22,31,38,41,43,54,56,59,60). In addition, the GW182 family is shown to induce translational silencing effect despite the absence of Ago2 (35,41,56,59). Knockdown of individual GW182 related proteins by specific siRNAs only partially rescue the repression indicating the functional redundancy among

those paralogues (22). However, they appear not to have identical roles in repression as TNRC6B and TNRC6C form distinct protein complexes with the four human Argonaute proteins (18).

Significant efforts have been made to map the repression domains of human (18,19,22) and *Drosophila* GW182-related proteins (23,41,53,55). The C-terminal domain including the DUF, M-GW, RRM, and C-GW is commonly identified as the 'silencing domain' in a variety of species. However, it is controversial and remains to be confirmed if the "N-terminal Ago binding domain" spanning the N-GW region possesses full silencing effects (23,53), is partially active (22) or completely inactive (18,19,41), albeit these studies use slightly different deletion construct boundaries and/or different species.

In the current study, mapping of the repression domain(s) of human GW182 was performed by generating a series of deletion constructs covering the full-length GW182 protein. Two non-overlapping domains, a middle GW182 fragment $\Delta 12$ (aa896-1219) and a C-terminal GW182 fragment $\Delta 5$ (aa1670-1962), were shown to trigger translational silencing when tethered to the 3'UTR. We showed that these domains representing the minimum length of middle- and C-terminal deletion constructs caused comparable silencing effects on the reporter compared to two full-length GW182 isoforms, TNGW1 and GW182. The present study defined a novel silencing domain on human GW182 and the role of GW/WG motifs within this domain.

Materials and Methods

Plasmids

The cDNAs of TNGW1, GW182, TNRC6B, $\Delta 1$, $\Delta 10$, $\Delta 12$, $\Delta 7$, $\Delta 8$, $\Delta 5$, Ago2, and PIWI were constructed as described in our previous studies (7,35,56). The N-terminal

construct '1-565' was generated by restriction digestion using enzymes HpaI and SmaI to excise the 3' fragment from the full-length construct pENTR-TNGW1; the construct was completed by T4 DNA ligase reaction to re-circularize the truncated linearized plasmid. Δ 5a, Δ 5b, Δ 8, and Δ 11 were generated by polymerase chain reaction (PCR) using GW182 cDNA as the template. The PCR cloning primers were: Δ 5a, forward 5'-AAAAAGCAGGCTCCTCATCCTTGAACACCACG-3', reverse 5'-AGAAAGCTGGGTTCAGCTGGAACCTGGGGTATATCT-3'; Δ 5b, forward 5'-AAAAAGCAGGCTCCTCATCCTTGAACACCACG-3', reverse 5'-AGAAAGCTGGGTTCAGTCTCCACAGTTAGTTCCCGACA-3'; Δ 8, forward 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATTAGACAGAATGGCAATCC-3', reverse 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTAGGCAACATCAAGGCATAG-3'; Δ 11, forward 5'-AAAAAGCAGGCTTCACTTGGGGAAACAACATA-3', reverse 5'-AGAAAGCTGGGTTCACCTCTGGTGAGTCTCTCGAAAA-3'. Fragments Δ 12a and Δ 12b were directly synthesized by GenScript Corp (Piscataway, NJ). The cDNA of Δ 12a mutant (Δ 12am, all GW or WG changed to AA) was also synthesized by GenScript. The PABPC1 expression vector was purchased from OriGene (Rockville, MD). All of the variants used in current study were subcloned into the Gateway destination vector for GST, GFP, or NHA (35) expression using the Gateway LR recombination reaction (Invitrogen, Carlsbad, CA). The tethering assay plasmids, including pCIneo-NHA vector, NHA-Ago2, Renilla luciferase (RL), and firefly luciferase (FL), with or without the 5BoxB structure in the 3'UTR, were obtained from Dr. Witold Filipowicz, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland (47). The miR20

luciferase reporter RL-20 bulge and RL-20 perfect was obtained from Dr. Phillip Sharp, Massachusetts Institute of Technology (66). All DNA constructs used in this study were confirmed by direct DNA sequencing.

Antibodies

Rabbit polyclonal anti-GST and RCK/p54 were purchased from MBL International (Woburn, MA). Mouse monoclonal anti-HA was purchased from Covance (Emeryville, CA). Mouse monoclonal anti-tubulin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Transfection

HeLa, A549 and HEK293 cells were cultured in DMEM containing 10% fetal bovine serum in a 37°C incubator with 5% CO₂. The plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instruction. The tethering assays were performed in a 24-well plate format. Six hundred nanograms of NHA-GW182 construct were co-transfected with either 10ng RL-5BoxB/100ng FL or 100ng FL-5BoxB/10ng RL in HEK293 cells. Cells were harvested 48 hours after transfection for luciferase assays. For the GST pull-down assays, 2 µg of GST-tagged GW182 construct was co-transfected with 2 µg NHA-tagged construct into HeLa cells. HeLa cells were harvested 24 hours after transfection, lysed by NET/NP40 buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.3% NP40) with Complete Protease Cocktail Inhibitor (Roche Diagnostics, Indianapolis, IN), and then applied to GST pull-down assays.

GST Pull-Down Assays and Western Blot Analysis

Cell lysates were sonicated at 20% amplitude for 10 sec three times on ice and then centrifuged at 13,200 rpm for 5 min. A fraction of soluble lysate was mixed with

Laemmli sample buffer as input for Western blot analysis. Two hundred mL of the soluble fractions were incubated with Glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ) and mixed at 4°C for 2 h for GST pull-downs. After the incubation, the beads were washed with NET/0.3% NP40 buffer four times and the samples were eluted in Laemmli sample buffer. The input and GST pull-down samples were separated on 10% polyacrylamide gels and transferred to nitrocellulose and western blotting performed as described previously (34). The dilutions of primary antibodies were: 1:1000 for anti-GST, and 1:1000 for anti-GFP.

Micrococcal Nuclease Assay

Three parallel transfections in HeLa cells were set up with indicated combinations of GST-PIWI and NHA-tagged constructs. Whole cell lysates were harvested in EDTA-free lysis buffer containing 150mM NaCl and EDTA-free Protease Cocktail Inhibitor (Roche Diagnostics, Indianapolis, IN) 24 hours after transfection. Samples (200ul) were diluted in equal volume of the same buffer without NaCl to adjust to a final concentration of 75mM NaCl. Untreated group was immediately transferred on ice until ready for the pull-down assay. Mock and micrococcal nuclease (MNase) treated groups were added 10µL 0.1M CaCl₂. 0.2U MNase (Sigma, St. Louis, MO) was added only to the MNase treated group. Both Mock and MNase groups were incubated in 37°C for 10 minutes. Twenty µL 0.5M EGTA was added to inactivate MNase. An aliquot of cell lysate from each group were separated for RNA extraction using mirVana total RNA isolation kit (Applied Biosystems, Foster City, CA). All three groups were subjected to GST pull-down assay protocol described above.

Tethering Assay and miRNA Interference Assay Using a Dual Luciferase Assay

HEK293 cells were harvested 48 hours after transfection with tethering constructs and dual luciferase reporters. The FL and RL activities were measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) following the manufacturer's protocol. Relative luciferase activities were calculated as the ratio of targeted luciferase activities over control luciferase activities (47). The repression levels of experimental groups were calculated by the percentage reduction of relative luciferase activities compared with that in the NHA control group (35). The relative repression effects of each construct were normalized to that of NHA, which was standardized as 1. Data for each construct were collected in 6 to 18 replicates. HeLa and A549 adenocarcinoma human alveolar basal epithelial cells were also used to address any cell specific effects. For miRNA interference assays, RL-20 bulge and RL-20 perfect, containing 7 copies of miR-20 target sites at the 3'UTR which form imperfect or perfect matches with endogenous miR-20 were co-transfected into HEK293, HeLa, and A549 cells with FL internal control and tested constructs. Luciferase activities were measured as described above. Cell lysates from representative luciferase assays were mixed directly with Laemmli sample buffer and separated in 4-20% HCl-Tris Ready Gels (BioRad, Hercules, CA) to quantify the expression levels of different NHA-tagged GW182 constructs. Samples were then transferred to nitrocellulose membranes and analyzed by Western blot using anti-HA tag antibody. To avoid the relatively narrow dynamic range of traditional film systems, bands visualized by an enhanced chemiluminescence assay were captured with a Geliance 600 (PerkinElmer, Waltham, MA) to obtain optimal images. The results were then analyzed by GeneTools software

(PerkinElmer) or Image J (<http://rsbweb.nih.gov/ij/>) to quantify the amount of protein expressed in individual assays.

siRNA and qRT-PCR

HeLa cells were seeded at a concentration of 5×10^4 cells/well into 24 well plates in 0.5 mL culture medium. siGENOME SMARTpool siRNA for GFP, GW182 (NM_014494), and TNRC6B (NM_015088) or RCK/p54 (NM_004397) were purchased from Dharmacon, Inc (Lafayette, CO). The final concentration used for transfection was 100 nM. Four different duplexes of GW182 siGENOME siRNA were purchased separately from Dharmacon (Cat No. D-014107-01 ~ D-014107-04). Since duplex 1 targets sequences residing in $\Delta 5$, only duplexes 2-4 were used to knockdown endogenous GW182 in experiments when the co-expression of $\Delta 12$ or $\Delta 5$ was required. To monitor the efficiency of the siRNA knockdown, parallel experimental groups were set up. Total RNA samples were harvested using mirVana total RNA isolation kit (Applied Biosystems) 48 hours after siRNA transfection following the manufacturer's instructions. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The relative mRNA levels of target genes were measured in duplicate using TaqMan Fast Universal Master Mix (Applied Biosystems) with the corresponding TaqMan Gene Expression Assay (Applied Biosystems). Quantification of mRNA degradation using SYBR-Green quantitative real time polymerase chain reaction (qRT-PCR) was described (35).

Results

Non-Overlapping GW182 Fragments Δ 12 and Δ 5 Harbored Comparable Repression Effects to Full-Length GW182/TNGW1

As shown in Figure 2-1A, human GW182 and its longer isoform TNGW1 (35) contain three glycine/tryptophan (GW)-rich regions in their N-terminal, middle, and C-terminal domains (N-GW, M-GW, and C-GW, respectively), as well as an RNA recognition motif (RRM, aa1780-1853, cd00590). Another interesting domain is a short sequence element termed the “Argonaute hook” (Ago hook, aa1076-1144, pfam10427) that binds to PIWI domains of Ago proteins (60). There is also a short stretch of glutamine repeats in the N-terminal domain of TNGW1 (trinucleotide repeat (TNR) Q-repeat, aa93-127) (35) and a glutamine/asparagine-rich region (Q/N-rich, aa1264-1553) between the N-GW and M-GW regions (67). A conserved ubiquitin-associated domain (UBA) (59) and another domain of unknown function (DUF, aa1604-1641) (22,25) are also shown in recent reviews (3,40). Right after reporting our findings that human GW182 induced silencing independent of Ago2 (35), the current study was initiated with multiple truncated constructs of GW182 spanning the full-length protein (Figure 2-1) to narrow down the region responsible for the repression effect in the tethering assay. These constructs were adapted to the tethering assay (35,47) and examined for their repression effects accordingly. As shown in the right panel, the relative repression effects observed for different GW182-truncated constructs was sorted into three categories: 1) no repression effect including 1-565, TNR, Δ 1, and QN; 2) high repression effect comparable to full length protein including Δ 10, Δ 12, Δ 8, Δ 7, and Δ 5; 3) Δ 11, which had a low to moderate repression effect. Interestingly, the results revealed that there were more than one non-overlapping region able to induce a repression effect

when tethered to the 3'UTR of the reporter mRNA. GW182 fragment $\Delta 12$ and $\Delta 5$ were the smallest representative, non-overlapping constructs that retained the most repression effect of the full-length protein without being expressed significantly higher than other truncated constructs (Figure 2-2). Both FL-5Boxb and RL-5Boxb reporters were used in these tethering assays and similar effects were observed from using either reporter. In subsequent studies, primarily the RL-5Boxb reporter was utilized. Since $\Delta 12$ and $\Delta 5$ were reported to bind to Ago proteins (56), an initial interpretation was that their repression activities were related to their binding of Ago proteins. Contradicting this hypothesis, $\Delta 1$, an N-terminal truncated construct of GW182 that strongly binds to all four human Ago proteins (56) did not show repression in the tethering assay. This finding is consistent with previous reports that Ago proteins were not the direct effectors of repression (19,41). In summary, a novel domain $\Delta 12$ was identified in human GW182/TNGW1 with a comparable silencing activity to the established silencing domain $\Delta 5$ and full-length GW182/TNGW1.

Contribution of Defined Domains Within $\Delta 12$ and $\Delta 5$ In Reporter Silencing and Ago2 Binding

An intriguing finding was that the two identified GW182 domains with repression activities had different defined domains: the Ago hook domain in $\Delta 12$ (Figure 2-3A) and the RRM domain in $\Delta 5$ (Figure 2-3B). The Ago hook has been shown to bind to Ago2 independently in vitro but it is not conserved in all GW182-related proteins (3,41,60). The RRM is linked to RNA binding activity (1) and is highly conserved in the GW182 family. Initial experiments were designed to examine whether the Ago hook or RRM was important for the repression effect of $\Delta 12$ or $\Delta 5$ in human GW182 respectively. To better evaluate the silencing roles of these two domains, truncated constructs were

generated (Figures 2-3A and 2-3B). When $\Delta 12$ was expressed as $\Delta 12a$ and $\Delta 12b$, which contain about equal distribution of GW/WG motifs, both truncated constructs had a significant reduction in repression activity compared to $\Delta 12$. Compared to the N-terminal half $\Delta 12a$, which lacked the Ago hook but retained most of the repression activity of $\Delta 12$, the C-terminal half $\Delta 12b$ containing the Ago hook significantly lost the repression activity (Figure 2-3A). Thus, these results showed that the Ago hook was not critical for $\Delta 12$ -induced repression in the tethering assay.

To examine the importance of the RRM domain, two C-terminal deletion constructs of $\Delta 5$ were generated (Figure 2-3B). Fragment $\Delta 5b$, which contained the RRM domain, M-GW region and part of the C-GW region, retained repression activity when compared to $\Delta 5$ or GW182/TNGW1. The repression capacity of $\Delta 5a$, which contained M-GW but not the RRM domain, was significantly reduced compared to full-length $\Delta 5$ but still retained about 50% of repression compared to NHA control (Figure 2-3B). Our data with human GW182 were consistent with recent reports on human TNRC6C (22) and dGW182 (3,40,41,55) showing that the RRM and its flanking sequences enriched in GW/WG motifs (M-GW and C-GW) were pivotal for the repression, whereas the Ago hook contributed little to the repression induced by $\Delta 12$.

To determine whether the observed repression activity was correlated with the Ago binding function, $\Delta 12$ or its deletion constructs were analyzed for Ago2 binding activity using a GST pull-down assay (Figure 2-3C). The results showed that NHA- $\Delta 12$, NHA- $\Delta 12a$, NHA- $\Delta 12b$, and NHA- $\Delta 5$ were all pulled-down by GST-PIWI albeit at different efficiency. For a semi-quantitative analysis, when normalized to the pulled-down GST-PIWI levels and compared to NHA- $\Delta 12$, only 1.1% and 20% of the

expressed NHA- Δ 12a and NHA- Δ 12b, respectively, were pulled down,(Figure 2-3C). This estimation was consistent with the results showing that the Ago hook alone was insufficient to achieve maximum binding to Ago2 (19,21). Intriguingly, the integrity of Δ 12 was important as it bound to the Ago2 PIWI domain substantially stronger than the Δ 12a or Δ 12b fragments alone, implying the full-length Δ 12 formed a higher order structure to perform both Ago2-binding and silencing of the bound mRNA. The positive control NHA- Δ 10 containing all three defined Ago-binding sites reported by Takimoto et al. (21) efficiently bound to GST-PIWI as evidenced by up to 85% pull-down of the input protein (Figure 2-3C). NHA-TNR, previously shown not to bind to GST-PIWI, served as a negative control in this experiment (56). When NHA- Δ 5 was compared to NHA- Δ 12, only 4.2% versus 18% bound to GST-PIWI representing 23% relative efficiency. The very weak binding of this C-terminal GW182 fragment Δ 5 to Ago2 might explain why this interaction was not reported in previous studies (3). In order to further characterize whether the interaction between GST-PIWI and NHA- Δ 12 or NHA- Δ 5 was RNA dependent, whole cell lysates were harvested 24 hours post transfection and treated with or without MNase prior to the pull-down assay. RNA degradation was monitored by 18S and lamin A/C RNA levels using qRT-PCR (Figure 2-4A). The interactions of NHA- Δ 12 and - Δ 5 with GST-PIWI were shown to be RNA independent (Figure 2-4B). Taken together, these data showed that the Ago hook domain was neither critical for Δ 12-induced repression in the tethering assay nor important for optimal binding to Ago2. Its full function may rely on the tertiary structure and its relationship to adjacent Ago binding sites. The RRM in Δ 5 contributed to the full repression activity and Δ 5 interacted with Ago2 relatively weakly when compared with Δ 12.

Reducing Endogenous Repressor Levels Did Not Affect Reporter Repression Induced by $\Delta 12$ and $\Delta 5$

Although it was demonstrated earlier that Ago2 protein was not required for tethered GW182-mediated repression in the tethering function assay (35,56,59), it was still possible the repression mediated by $\Delta 12$ or $\Delta 5$ relied on recruitment of other important factors of the miRNA pathway machinery, including endogenous GW182 paralogues. This possibility was tested in a series of tethering assays in HeLa cells where siRNA knockdown was used as an approach to evaluating the roles of GW182 and/or TNRC6B in the repression mediated by $\Delta 12$ or $\Delta 5$. To knockdown endogenous GW182, a pool of three siRNAs targeted to GW182 mRNA, but not $\Delta 12$ and $\Delta 5$, was used as described in Methods. The other two factors examined were RCK/p54 and TNRC6B, both of which are reported to be important for miRNA function. For example, knockdown of RCK/p54 impaired miRNA function (68) and tethered RCK/p54 (69,70) or TNRC6B (18,19,22) induced translational repression effects. Since HeLa cells expressed both GW182 and TNRC6B but only very low levels of TNRC6C compared to A549 or HEK293 cells (Figure 2-5), knockdown of both GW182 and TNRC6B together was also performed to create a “GW182-free” background in HeLa cells. The luciferase activity in each knockdown experiment for NHA- $\Delta 12$ or NHA- $\Delta 5$ tethering was normalized to the same siRNA transfected control NHA group. With about 70% reduction of endogenous GW182 or TNRC6B mRNA levels compared to mock transfected or siGFP transfected controls (Figure 2-6B, C, D), the repression effects of tethered $\Delta 12$ and $\Delta 5$ were not altered significantly (Figure 2-6A; t-test p value >0.05 between siGFP and other siRNA knockdown in both NHA- $\Delta 12$ and NHA- $\Delta 5$ groups). Efficient knockdown of GW182, TNRC6B and RCK/p54 was monitored by either qRT-

PCR or Western blot (Figure 2-6B-6E). An additional control experiment was performed to monitor the effects of siGW182 and siGW182/siTNRC6B knockdown in the tethering assay using NHA-TNGW1, NHA- Δ 12, or NHA- Δ 5. Figure 2-7 shows the siRNA transfection significantly affected the reporter silencing induced by NHA-TNGW1, but not NHA- Δ 12, or NHA- Δ 5, as would be expected since by design the siRNAs targeted endogenous GW182/TNGW1/TNRC6B and the NHA-TNGW1 construct but not NHA- Δ 12 or NHA- Δ 5. The apparently reduced repression effects in both Δ 12 and Δ 5 tethering assay compared to data shown in Figures 2-1 and 2-3 might be resulted from the introduction of siRNA transfection. In summary, knockdown of endogenous repressors GW182, TNRC6B, and RCK/p54 did not significantly affect the repression activities of Δ 12 and Δ 5 in tethering assay. Our findings are in agreement with those previously described in *Drosophila* (23) and further emphasizes the independence of GW182 in inducing translation silencing in human cells.

Δ 5, Not Δ 12, Enhanced Ago2-Mediated Repression in Tethering Function Assay

Since both Δ 5 and Δ 12 have comparable silencing activity when tethered to the luciferase reporter 3'UTR (Figure 2-1 and 2-3) and none of the known endogenous factors tested were required for their repression function, it remained unclear whether their mechanism of inducing repression was similar. Therefore, experiments were designed to determine whether the expression of Δ 5 and Δ 12 as a GST-tag fusion proteins could interfere with NHA-Ago2 tethered assays, as shown in Figure 2-8. As expected, the positive control NHA-TNGW1 typically showed 85-90% repression expressed as a reduction of luciferase activity compared to the NHA control when tethered to the RL-5boxB reporter (35). There was also the typical ~60% repression

compared to the NHA control when NHA-Ago2 was tethered to the RL-5boxB reporter (35,47). Interestingly, there was significantly (33%) enhanced repression with the co-expression of GST- Δ 5 (Figure 2-8A, *, *t*-test, $p < 0.01$, $n = 3$). In contrast, significant differences were not observed for co-expression of GST- Δ 12, -Ago2, or -QN (Figure 2-8A). Furthermore, neither GST- Δ 5 nor $-\Delta$ 12 co-expression affected repression in the tethering assay utilizing NHA-GW182 or NHA-TNGW1 (data not shown). Since both NHA-GW182 and NHA-TNGW1 consistently showed the highest levels of repression, it was not likely that any further enhancement could be observed for GST- Δ 5 or GST- Δ 12. Similar significantly enhanced repression for NHA-Ago2 was observed by co-expression of GFP- Δ 5 but not GFP- Δ 12 (Figure 2-8B) indicating the effect is not related to the GST tag. The Δ 5-mediated enhancement in repression by NHA-Ago2 implied that this could be due to binding to other translational machinery or RNA decay factors that remain to be determined. Although Δ 12 strongly bind to Ago2, it did not affect repression by tethered Ago2.

Δ 12, Not Δ 5, Interfered with Endogenous miRNA Repression

In our previous study, it was shown that multiple GW182 regions (Δ 1, Δ 5, and Δ 12) were able to bind Ago proteins (56). Thus, the next experiment was to examine whether these three Ago-binding regions would interfere with endogenous miRNA function in a dominant-negative manner. The RL reporter for miR-20 (RL-20 bulge), which contains seven miR-20 target sites and forms bulge structures with miR-20 (66), was used to monitor the cellular miR-20 functional status. In normal conditions, the expression of RL-20 bulge is inhibited by the endogenous basal miR-20 (data not shown). To our surprise, when GST-TNR, $-\Delta$ 1, $-\Delta$ 12, or $-\Delta$ 5 were co-transfected with the RL-20 bulge reporter using the dual luciferase system, only GST- Δ 12 increased the

expression of RL-20 bulge in all three conditions when increasing amounts of GST plasmids were used (Figure 2-9A). The results showed that although $\Delta 1$, $\Delta 12$, and $\Delta 5$ were able to bind Ago protein, only $\Delta 12$ impaired miR20-induced repression with >3-fold increase in luciferase activity ($p < 0.0001$) and $\Delta 1$ showed mild release of RL-20 bulge reporter silencing ($p < 0.01$).

As already shown in Figure 2-3, the Ago hook in $\Delta 12$ was neither critical for $\Delta 12$ -induced repression in a tethering assay nor responsible for maximum binding with Ago2. Therefore, the next question was whether the Ago hook domain could be responsible for the miRNA interference effect or de-repression observed for $\Delta 12$. To also avoid any potential tag-dependent effects when only GST-tag was used, NHA-tagged constructs including $\Delta 12a$ and $\Delta 12b$ were employed to repeat the experiment. Meanwhile, another RL reporter with miR-20 target sites forming perfect match with endogenous miR-20, thus acting as reporter for the siRNA pathway, was utilized to determine whether this interference also applies to the siRNA pathway. The results clearly showed that $\Delta 12a$, which lacked the Ago hook, retained almost the same capability to interfere with 20 bulge reporter miRNA function (Figure 2-9B, left panel, $p < 0.001$). However, $\Delta 12b$, which contained the Ago hook domain, mildly altered the miR20-induced repression (Figure 2-9B, left panel). This interference was only observed with the 20 bulge but not the 20 perfect reporter, thus demonstrating overexpression of $\Delta 12$ and its deletion constructs impaired reporter silencing in a miRNA-specific manner. Compared to the 20 bulge reporter, the 20 perfect reporter showed enhanced repression effects from efficient mRNA degradation induced by the siRNA machinery and it therefore served as a useful functional control. Note that $\Delta 12b$

bound to Ago2 more efficiently than $\Delta 12a$ (Figure 2-3C) and yet its miRNA interference effect was weaker, indicating there were additional factors involving in interfering this miRNA-mediated silencing. This led us to further examine the role of GW/WG motifs residing in this fragment by generating mutations of those motifs.

Substitution of GW/WG Motifs with Alanines in $\Delta 12a$ Hindered Its Tethering Assay Activity, as Well as Its Interference with Endogenous miRNA Repression

Since the results showed that the Ago hook was not critical for function but $\Delta 12a$ retained almost the same capability to interfere with RL-20 bulge reporter, it was postulated that the GW/WG motifs in $\Delta 12a$ might be important for functions besides binding to Ago2 (Figure 2-10A). A $\Delta 12a$ mutant ($\Delta 12am$) was then generated to replace all GW/WG residues in $\Delta 12a$ with alanine-alanine (AA) residues. In a tethering assay using RL-5BoxB, NHA- $\Delta 12am$ was devoid of translation silencing when compared to NHA- $\Delta 12a$ and other relevant controls (Figure 2-10B). When $\Delta 12am$ was analyzed in the interference of RL-20 bulge reporter repression, the ability of $\Delta 12a$ to release miR-20 activity was abolished in $\Delta 12am$ (Figure 2-10C). These data suggested that the GW/WG motifs in $\Delta 12a$ were important for the silencing in the tethering assay and interference in miR-20 bulge reporter function. Since it was shown that $\Delta 12a$ did not bind efficiently to Ago2 (Figure 2-3C), the GW/WG motifs in $\Delta 12a$ might be responsible for both mediating strong repression and impairing miRNA-mediated silencing. It appeared that the GW/WG motifs in different regions of GW182 have different functional roles. For example, $\Delta 1$ also possessed multiple GW/WG motifs and a defined Ago binding site but it was not efficient in both tethering and 20 bulge interference assays compared to $\Delta 12$. Further, mutation of some of the GW motifs on $\Delta 1$ did not abolish the Ago binding (56). However, it is acknowledged that these mutations may influence the

global folding of the fragment and more detailed mutagenesis is needed in future studies to further define the roles of these GW/WG motifs. Collectively, GW/WG motifs in $\Delta 12a$ region showed significant effects in silencing tethered mRNA and impaired miRNA-induced repression. GW/WG motifs located in different regions of GW182 might have different functional preferences or formed a particular three-dimensional structure that requires further investigation.

$\Delta 12$ and $\Delta 5$ Bound to PABPC1 but Only Mildly Affected miRNA Degradation

In the eukaryotic cap-dependent translation initiation step, mRNAs usually form circularized structures facilitated by the binding between the cap binding complex eIF4E/4G and Poly-A binding protein PABPC1 to favor association with the 40S ribosome (71). Thus, PABPC1 has been shown to play a critical role in mRNA degradation (25). To determine whether the two GW182 silencing domains $\Delta 12$ and $\Delta 5$ play differential roles in mRNA degradation, GST pull-down experiments were designed to investigate the interaction between GW182 fragments with PABPC1. The positive control $\Delta 7$ harboring the C-terminal half of TNWG1, including the PAM2 (DUF) domain, bound strongly to GST-PABPC1 (Figure 2-11A). This data is consistent with the results observed with TNRC6C that the PAM2 domain is the major binding site to PABPC1. $\Delta 5$ showed weak binding (arrow) for PABPC1 and this is consistent with a recent report (26) describing weak binding activity in the C-terminal region of GW182. It is noteworthy that $\Delta 12$ showed intermediate binding activity to PABPC1 compared to $\Delta 7$ and $\Delta 5$. In order to further characterize whether the interaction between GST-PABPC1 and NHA- $\Delta 12$ or NHA- $\Delta 5$ was RNA dependent, whole cell lysates were harvested 24 hours post transfection and treated with or without MNase prior to the pull-down assay as described in Figure 2-4. The interactions of NHA- $\Delta 12$ and - $\Delta 5$ with GST-PABPC1

were shown to be RNA independent (Figure 2-12). To further fine mapping the PABPC1 binding sites on two GW182 repression domain, additional pull down assays have been set up to examine the detailed interaction. PABPC1 interacted with NHA- Δ 12b and NHA- Δ 5b its interaction with GW182 repression domains (Figure 2-13). Note that the interaction of Δ 12 and Δ 5 with endogenous PABPC1 was not observed potentially reflecting that these are weak interactions (data not shown).

A qRT-PCR was utilized to measure the level of degradation of the RL-5Boxb reporter mRNA in tethering assay normalized to the FL mRNA level (35). As shown in Figure 2-11B, both NHA- Δ 12 and - Δ 5 induced comparable, mild mRNA degradation when tethered to the reporter comparable to the activities of NHA-GW182 and -TNGW1, respectively. The observation that NHA- Δ 12 induced mild reporter mRNA degradation is consistent with the recent report that a Δ 12-comparable dGW182 silencing domain (aa205-490, cf. dGW182) also induced mRNA degradation in tethering assay (53). This difference in the extent in mRNA degradation may be explained by variations in methodology used to detect degradation (i.e. qRT-PCR in this study versus Northern blot (53)), as well as differences in the activity of human GW182 versus the Drosophila orthologs.

Discussion

Δ 12 and Δ 5 Mapped as Two Non-Overlapping Domains Mediating Repression

Since its discovery in 2002 (1), increasing and compelling evidence from different laboratories has shown that GW182 and its paralogues have important functions in miRNA-mediated translational repression, as well as mRNA degradation (18,19,21-23,25,26,31,35,38,41,53-60). A summary of the functional characteristics of different domains of GW182 in this study is shown in Figure 2-14, where their differential Ago2

binding, repression in tethering assays, enhancing Ago2-mediated repression, and releasing endogenous miR-20 repression are compared and contrasted. Our previous data showed translational repression of tethered Ago2 to reporter 3'UTR required GW182 and that tethered GW182 exerted a stronger repression than tethered Ago2 (35). As exemplified in the present study, these observations led to further mapping of the functional repression domain(s) of human GW182 by generating a series of deletion constructs using tethering assays. Recent reports have shown that the C-terminal domain of human GW182/TNGW1, TNRC6B, and TNRC6C (18,19,22) and *Drosophila* GW182 (23,41,53,55) exert strong translational repression when tethered to the 3'UTR of the reporter mRNA. Our current data from human GW182 truncated constructs also support the conclusion that GW182 C-terminal fragments $\Delta 7$, $\Delta 8$, and $\Delta 5$ indeed inhibit luciferase activity of the 5BoxB reporter when tethered to its 3'UTR. Among those, $\Delta 5$ (aa1670-1962) was the minimum domain that retained the full activity. It was well established by Izaurre and colleagues that the M-GW and C-GW domains in the C-terminus of *Drosophila* GW182 act as a bipartite silencing domain and the RRM contributed to, but was not required for, silencing in tethering and complementation assays (3,40,41,55). In a study of TNRC6C by Filipowicz and co-workers, C-terminal mutation and deletion constructs were generated to elucidate the importance of the integrity of M-GW and C-GW in silencing domains; only RRM mutations mildly affected the repression tethered to 3'UTR (22). Our data of human GW182 supported these observations by showing the repression activity of $\Delta 5a$ with the RRM and C-GW deletion was impaired but still maintained ~50% repression through the M-GW. $\Delta 5b$

with the C-terminal $\Delta 5$ deletion but containing intact M-GW, RRM and C-GW retained full repression activity.

In contrast to reports to date, the current study demonstrated that another repression domain $\Delta 12$ (aa896-1219), in the middle region of human GW182 appears to be crucial for this repression function. This is the first time that the functional repression domain $\Delta 12$ is identified within the described “N-terminal Ago binding domain” (Figure 2-14) of human GW182. However, it should be noted that some TNRC6C fragments such as aa1-405 or aa1-1304 have been shown to be partially active with about 50% repression activity in tethering assays (22). Our data ruled out the Ago hook as a critical region for repression activity. Since the Ago hook domain was defined in binding the Ago PIWI domain both *in vitro* and *in vivo* (60), a number of recent studies showed that additional Ago hook-independent GW182 fragments could efficiently bind Ago protein in human GW182, TNRC6B, and TNRC6C as well as dGW182 (18,19,21,41,56). For example, the report of Takimoto et al. (21) identified three defined Ago2-binding domains that corresponded to aa697-739, aa969-1031, and aa1059-1163 (Figure 2-14). It has also been shown that deletion of the Ago hook in GW182 and TNRC6C did not totally abolish the binding to Ago proteins (19). Chekulaeva et al. observed that three dGW182 regions were responsible for the repression in *Drosophila* (23). The first repression region was the C-terminal domain of dGW182; the second repression region was the N-terminal domain aa1-605 (cf. dGW182); the third region (aa605-830, cf. dGW182), including the QN rich region, also triggered repression. We note that comparable activity for the corresponding region in human GW182 was not observed in our present study, an inconsistency that could be due to paralogue- or

species- specific effects. During the preparation of this manuscript, Chekulaeva et al. published a report showing the functional significance of GW/WG repeats on dGW182 repression domain (aa205-490) that induced reporter silencing (53). These investigators showed alignment of their repression domain with other GW182 homologs and found that mutation of certain conserved amino acid residues abolished repression induced by these dGW182 fragments. Interestingly, the alignment showed the second dGW182 repression domain corresponded closely to $\Delta 10$ (aa655-1343). In agreement with this finding, $\Delta 10$ induced repression in our tethering assay was clearly observed (Figure 2-1). In addition, the $\Delta 12$ repression domain defined in our study represents a new core repression region with somewhat higher repression than in $\Delta 10$ (Figure 2-1, $\Delta 12$ versus $\Delta 10$).

In order to investigate whether the repression effects caused by these fragments were direct or indirect, knockdown-tethering experiments utilizing endogenous GW182, TNRC6B, or RCK/p54 did not dramatically impair the 5BoxB reporter repression tethered by $\Delta 12$ or $\Delta 5$. Thus, our study shows that there are two functionally independent repression domains in human GW182 with differences discussed further in the next paragraph. It is possible that $\Delta 5$ has a more important and more direct repression function than $\Delta 12$ because of the apparent higher tethering activity (Figure 2-1, and Figure 2-3A) and its ability to enhance Ago2-mediated repression in a tethering assay (Figure 2-8). However, since it is still unclear how these domains bind *in vivo*, it can be argued that $\Delta 12$ can play an equally important role.

GW182 has been shown to bind multiple Ago-miRNA complexes (19,21,41,56). Studies have showed that closely spaced miRNA target sites often act synergistically

and result in stronger repression than those separated by greater distances (62). The demonstration that there is more than one repression domain in each molecule of GW182 as described in this study and described by others in *Drosophila* (23,53), suggests that re-examination is in order to elucidate how enhanced repression may be triggered when miRNA sites are approximated to each other in 3'UTRs. The functional advantages of the dual-repression domain GW182 with $\Delta 12$ and $\Delta 5$ having their own functional bias requires elucidation. It is conceivable that GW182, and perhaps its paralogues, function best to regulate mRNA with multiple miRNA binding sites. Future studies will need to address the molecular mechanism how mRNAs with multiple putative miRNA binding sites will benefit the efficiency of regulation in concerted manners.

GW182 Mediated Translational Repression Distinguished from miRNA Deadenylation and Decay

MicroRNAs bind primarily to the 3'UTR of their target mRNA, and mediate translational repression and/or mRNA decay, although the detailed molecular mechanism is still not completely understood. Several models have been proposed indicating that translation repression is achieved by inhibition of translational initiation, elongation, or mRNA deadenylation (2,13). A recent report utilizing *in vitro* translation extracts from mouse Krebs-2 ascites cells showed miRNA-mediated deadenylation occurred 1-2 hours after initial translational inhibition (25). These investigators and others also showed that the C-terminal domain of GW182 bound to deadenylases through the DUF conserved domain located between the QN-rich domain and the RRM domain (25,26) (also known as PAM2 domains (40), Figure 2-14) and recruited deadenylases to the target mRNA. This deadenylation process requires binding of

Ago2 and GW182 paralogues and this binding appears to be RNA independent (25). Similar results have been observed in mouse 3T3 cells where it was shown that when mRNA is bound by miRNA, a two-step deadenylation takes place followed by decapping (58). In *Drosophila* S2 cells, the DUF domain of dGW182 binds to PABPC1 and thus competes for eIF4G binding and leading to the disruption of the circularized mRNA structure that would normally favor translation (57). It is proposed that PAM2, M2 (between PAM2 and RRM), and C-terminal region on dGW182 together define a binding region to PABPC1 (40,57) (Figure 2-14). In contrast, PAM2 on TNRC6C appears to be the major binding site for its interaction with PABPC1 (25,40). Another weak PABPC1 binding site was subsequently identified on the TNRC6C C-terminal domain downstream from the RRM (26). These recent data indicate that there are differences in GW182-PABPC1 complex formation when different GW182-related proteins are compared. Of relevance to the present study, both $\Delta 5$ and $\Delta 12$ domains lack the known PABPC1 binding domain DUF (Figure 2-14) but still caused remarkable repression in a tethering assay and exhibited enhanced Ago2-mediated repression when compared to $\Delta 7$ or $\Delta 8$ that contains the DUF domain. GST pull-down assays indicated $\Delta 7$ strongly bound to PABPC1 whereas both $\Delta 12$ and $\Delta 5$ bind weakly to PABPC1 (Figure 2-11); nevertheless, these reactivities with PABPC1 appeared specific as several other controls including $\Delta 1$, which binds Ago2, were negative. Thus, it is interesting that the two defined repression domains, lacking DUF, somehow still associated with PABPC1. It can be speculated that when mRNA is bound by specific miRNA-Ago complex, translational inhibition occurs relatively rapidly and is mediated by one or the other repression domains of GW182 (or from its paralogues), but the

deadenylation step can be delayed. This process may be reversible within a narrow time frame and the repressed mRNA still may be released for further translation. Whether $\Delta 12$ and $\Delta 5$ play a role in this control will need further study. Our study demonstrated that human GW182 repression domains can be clearly separated from the putative PABPC1 binding domain DUF.

Distinct Characteristics of $\Delta 12$ and $\Delta 5$ Implying Differential Functions for GW182?

It has been shown by us (31,56) and others (19,21,41) that the N-terminal and middle region of the GW182 family and dGW182 possess multiple Ago2 binding sites. Our co-IP experiments showed that $\Delta 12$ bound strongly to Ago2, while $\Delta 5$ had a substantially lower affinity for Ago2. Co-expression of $\Delta 12$, but not other GW182 fragments such as $\Delta 5$, $\Delta 1$, $\Delta 11$, or TNR, significantly inhibited miRNA rather than siRNA activity, as determined by the difference between activity of RL-20 bulge and RL-20 perfect miRNA reporters, although $\Delta 1$, $\Delta 5$, and $\Delta 11$ still bind to Ago2. The impairment of miRNA function following overexpression of dGW182 or human GW182 paralogue fragments that bind Ago has been reported. For example, overexpression of the N-terminal half of Drosophila GW182, which bind Ago1 protein, impaired miRNA function and the impairment was rescued by overexpression of Ago protein (54). Other data showed that synthetic peptides or recombinant proteins corresponding to the Ago hook domain impaired translational efficiency in an in vitro translation assay (60) or impaired miRNA-mediated deadenylation (25). In particular, let-7-mediated translational repression is inhibited by a GW182 fragment containing multiple Ago2-binding sites in vitro (21). As summarized in Figure 2-14, clearly there are distinct properties of $\Delta 12$ and $\Delta 5$.

Why did $\Delta 5$ enhance Ago2 repression effects in the tethering assay but have no apparent effect on miR-20 repression? Why did $\Delta 12$ abolish miR-20 repression, but not the siRNA-like repression by miR-20, or affect Ago2 repression in tethering assay? Clearly, more studies are needed to address these questions. Speculations on the potential implication of these questions will undoubtedly stimulate discussion, debate and future research directions. GW182 was identified as a marker for cytoplasmic foci GW/P bodies (1) and it is likely that there is a certain amount of “soluble” cytoplasmic pool versus “GW/P body-bound insoluble” pool of this protein. The distribution between the two putative pools has not been studied extensively as there are many factors that may influence this dynamic process. For example, transfection of siRNA into culture cells (34) or lipopolysaccharide stimulated monocytes (72) cause an increase in number and size of these foci and probably switch GW182 from the soluble to the insoluble pool. In contrast, induced cell quiescence (6) or blocking the biogenesis of miRNA (33) lead to disassembly of GW/P bodies and increases in the soluble pool of GW182. A technical consideration is that, the separation of soluble versus insoluble pool also likely depends on the composition of the lysis buffer including whether commonly used detergents are used. The significance of the two distinct repression domains in GW182 and their relationship to two or more cytoplasmic pools also deserves consideration. Whether certain GW182 can exert inhibition of repression as demonstrated by transfected $\Delta 12$ in miR-20 repression will also need to be explored.

In conclusion, this study identified two distinct repression domains in GW182 using tethering assays and showed their characteristics in different functional assays. Observations that GW182 is characterized by having multiple Ago binding sites with

different binding affinities, as well as two distinct repression domains, is highly suggestive of its role in stabilizing multiple “repressed” Ago-miRNA-mRNA complexes or in aggregating Ago-miRNA-mRNA complexes to establish an efficient repressed state. Alternatively, our data also suggest that GW182 may regulate the fate of repressed mRNA and potentially direct the repressed complex to decay or reversal to a translational state.

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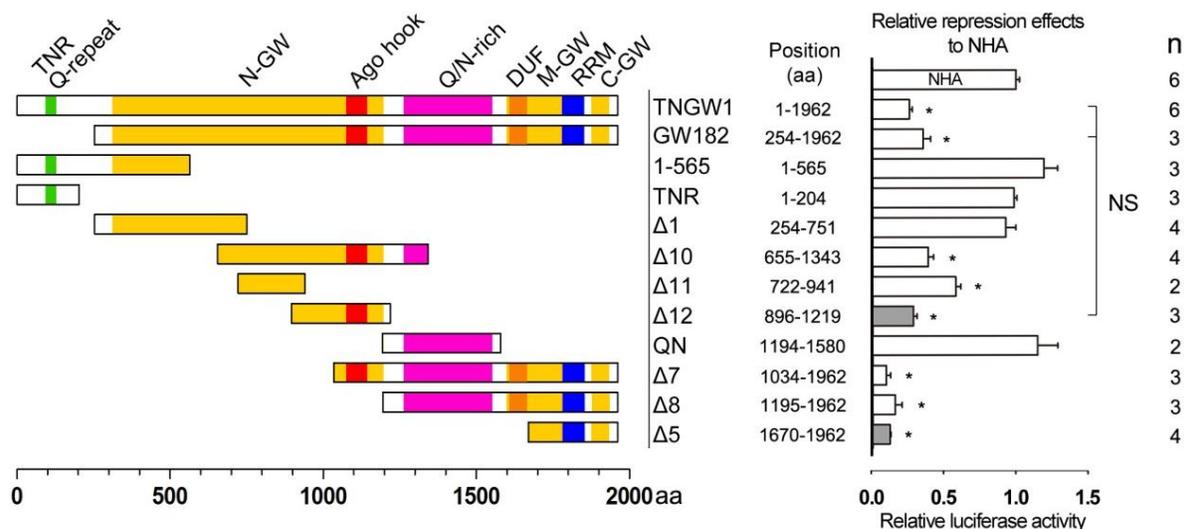


Figure 2-1. $\Delta 12$ and $\Delta 5$ are two non-overlapping GW182 domains harboring repression in tethering function assay. The left panel shows GW182/TNGW1 and their series of truncation constructs. Amino acid residues of GW182 constructs are referenced to TNGW1, the longer isoform of GW182 (GenBank Accession NM_014494.2). TNR Q-repeat (green), glutamine repeat at the N-terminal domain of TNGW1; N-GW, M-GW, and C-GW (yellow), three glycine/tryptophan-rich regions; Ago hook (red), a region reported to bind Ago protein; Q/N-rich (purple), glutamine/asparagine-rich region; DUF, domain of unknown function (orange); RRM (blue), RNA recognition motif. The right panel shows relative repression effects on reporter (either FL-5BoxB or RL-5BoxB; data combined as no difference was observed between reporters) by tethering the corresponding construct to the 3' UTR of luciferase mRNA. Their repression effects were normalized to NHA control, which was assigned as 1. Bar graphs show averages with standard errors (error bars); n, numbers of repeated experiments; * represents significant difference from NHA in *t*-test, $p < 0.01$; NS, no statistical significance.

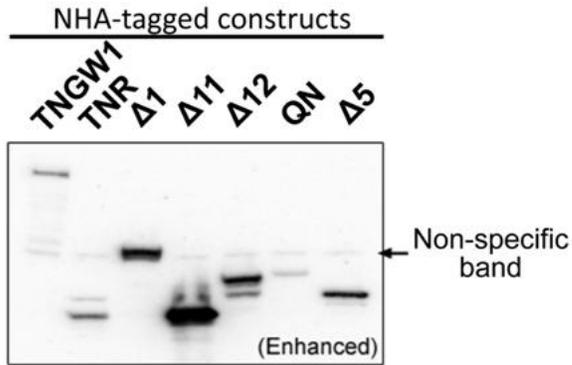


Figure 2-2. The expression of selected NHA-constructs from representative tethering experiments were monitored by western blot using anti-HA monoclonal antibody. Signals were enhanced by increasing contrast to better visualize the low level of the NHA-TNGW1 band.

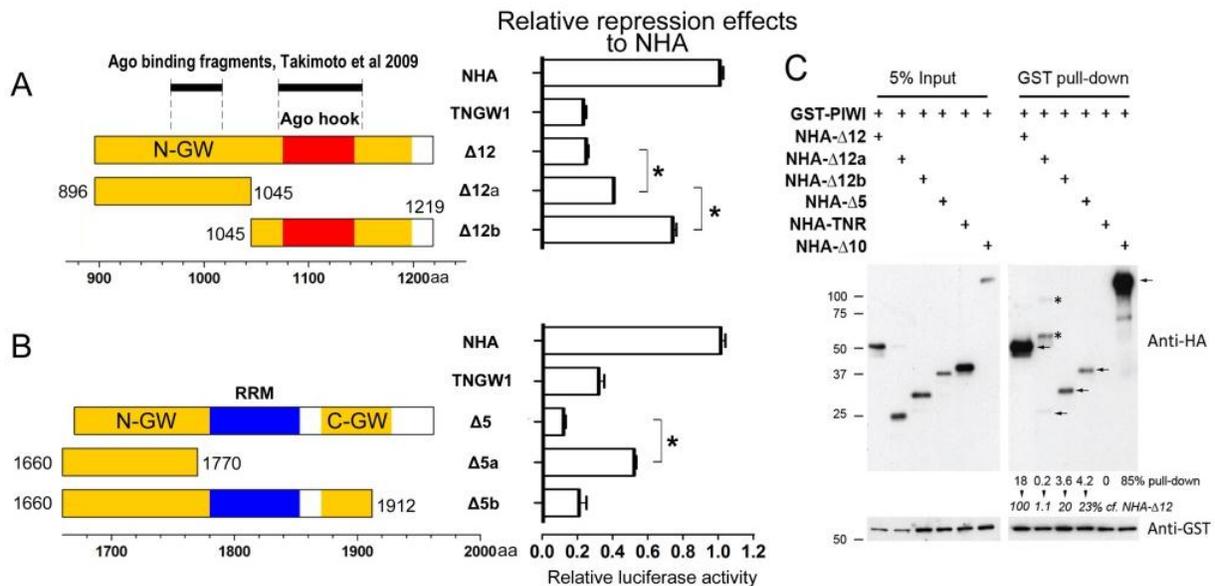


Figure 2-3. Roles of Ago hook and RRM domains for repression in tethering function assay and binding to Ago2. A) The Ago hook in Δ12 was not critical for its repression effect. Compared to Δ12a which still retained 60% repression compared to NHA, Δ12b had only 27% repression. Results are expressed as mean ± standard error from three independent experiments. * represents significant difference between Δ12a to Δ12 and Δ12a to Δ12b using *t*-test, $p < 0.01$. B) RRM domain and its flanking sequences in Δ5 were required for maximal repression effect. The repression activity of Δ5a was significantly reduced to only 55% of Δ5 (* represents significantly different compared to Δ5 in *t*-test, $p < 0.01$). The repression effect of Δ5b was not significantly different from that of Δ5 ($p = 0.12$). Results are expressed as mean ± standard error from three independent experiments. C) Semi-quantitative Western blot analysis showed differential binding of NHA-GW182 domains to GST-PIWI in GST pull-down assay. Bands (arrows) in blotting data were quantified and normalized to the total input (left panel) to obtain percent pull-down as shown for each lane at the bottom of the anti-HA panel. NHA-Δ12a, NHA-Δ12b, and NHA-Δ5 showed only 0.2%, 3.6%, 4.2% pull-down, respectively, whereas NHA-Δ12 showed 18%. Thus NHA-Δ12a, NHA-Δ12b, and NHA-Δ5 showed only 1.1%, 20%, 23% binding, respectively, to GST-PIWI compared to NHA-Δ12 as 100%. NHA-TNR and NHA-Δ10 served as negative and positive controls respectively. *, nonspecific bands.

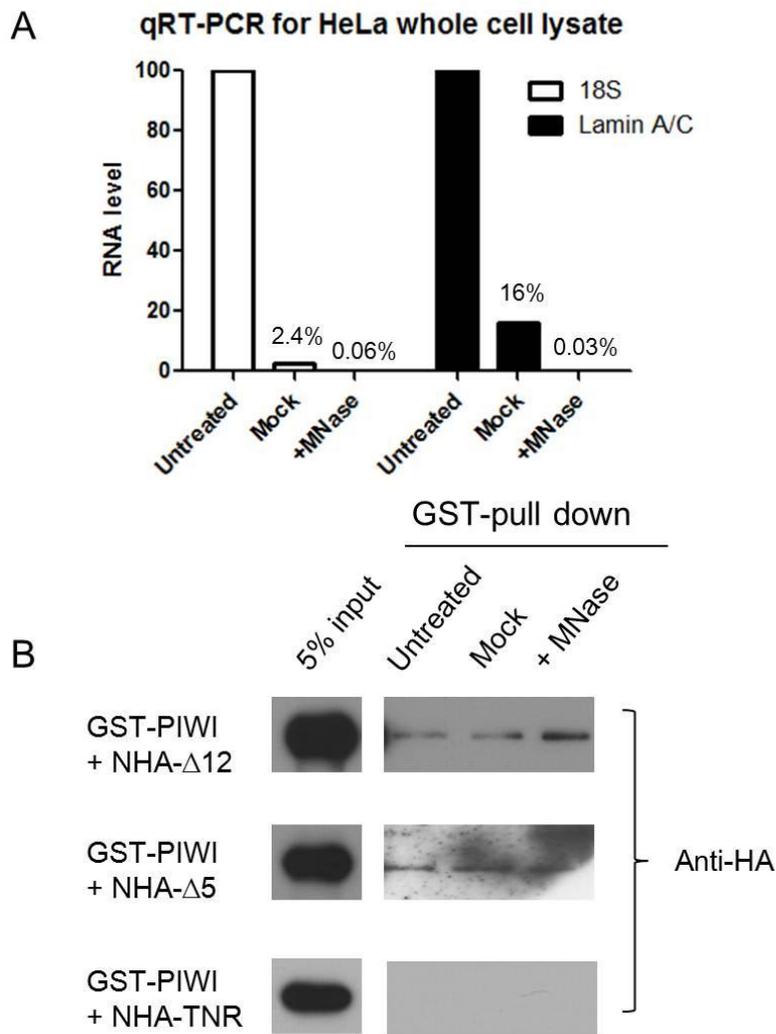


Figure 2-4. The interactions of GW182 repression domains and Ago2 PIWI domain was independent of RNA. A) The conditions for MNase digestion effectively reduced the RNA level in HeLa whole cell lysates. Note that the incubation conditions for MNase (Mock) already demonstrated significant degradation of RNA. B) Interactions of Δ 12 and Δ 5 with PIWI were independent of RNA. NHA-TNR served as negative control.

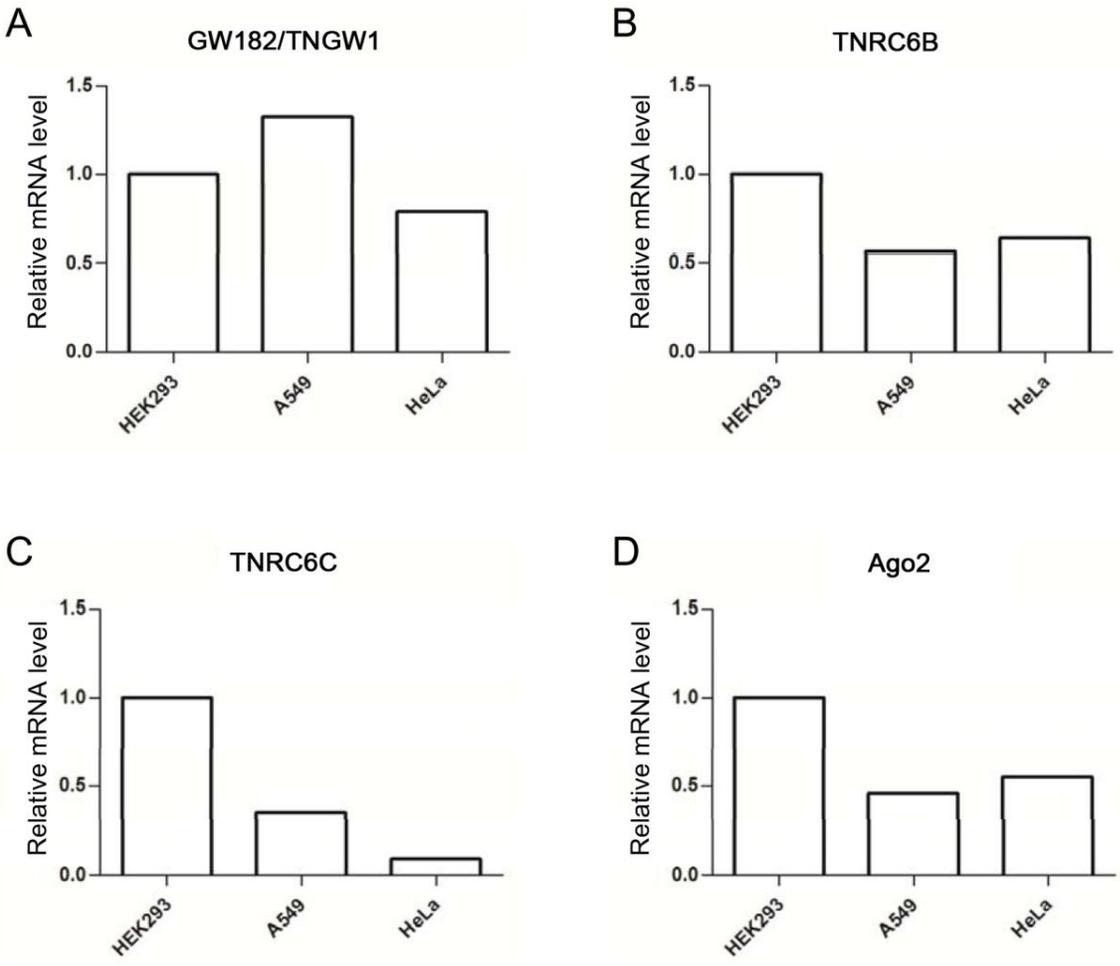


Figure 2-5. TNRC6C expression was very low in HeLa cells compared to A549 and HEK293 cells. To evaluate the relative expression levels of GW182-related protein and Ago2 in different cell lines that were used to perform tethering and RNA interference assay, RNA from each cell line was extracted and examined by qPCR. For comparison, mRNA levels in HEK293 were normalized to 1.

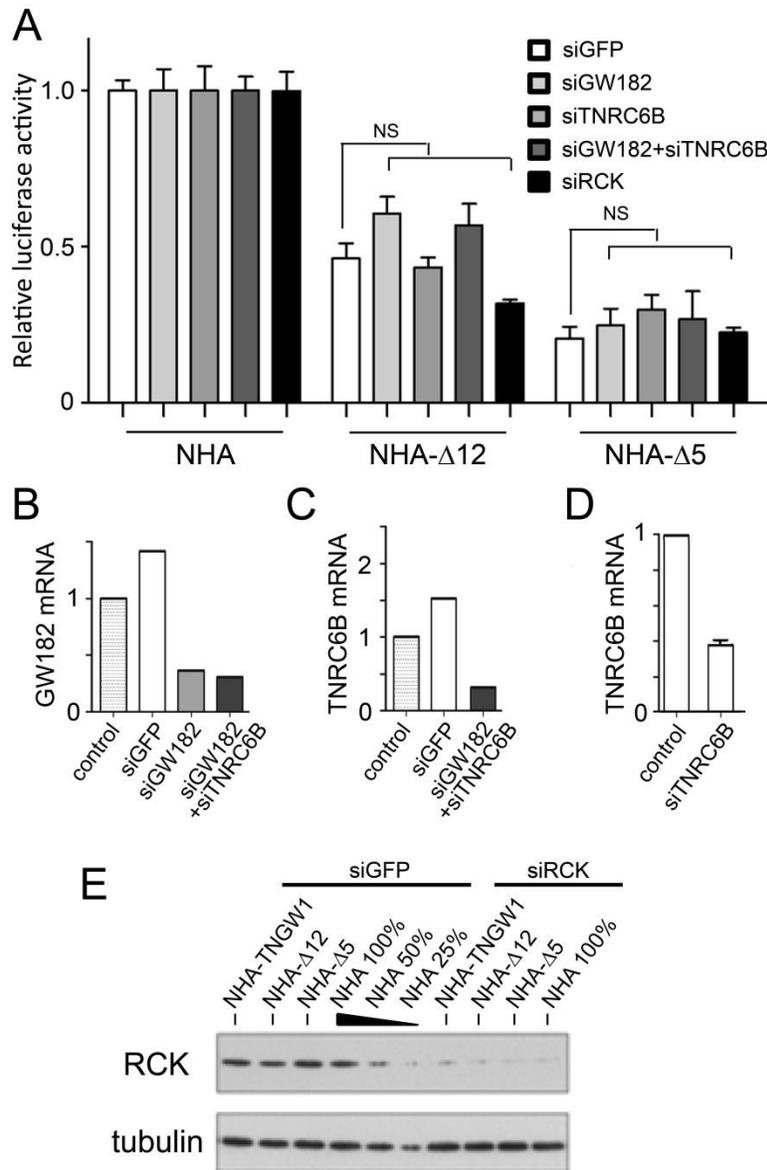


Figure 2-6. Knockdown of endogenous repressors did not affect repression activity of the two defined domains $\Delta 12$ and $\Delta 5$ in the tethering assay. A) Repression by $\Delta 12$ and $\Delta 5$ were not significantly altered when GW182, TNRC6B, GW182/TNRC6B, or RCK/p54 was knocked down by respective siRNAs. Results are expressed as mean \pm standard error from three independent experiments. There is no statistical significance difference comparing each knockdown to siGFP within NHA- $\Delta 12$ or - $\Delta 5$ group (NS, *t*-test). Efficiency of siRNA knockdown was monitored in each individual experiment using qRT-PCR for B) GW182, C-D) TNRC6B compared to the untreated HeLa cell control, or E) Western blot analysis for RCK/p54 compared to siGFP transfected controls. The extract from cells transfected with NHA and siGFP was loaded at three concentrations (100%, 50%, and 25%) to demonstrate the semi-quantitative detection of RCK/p54.

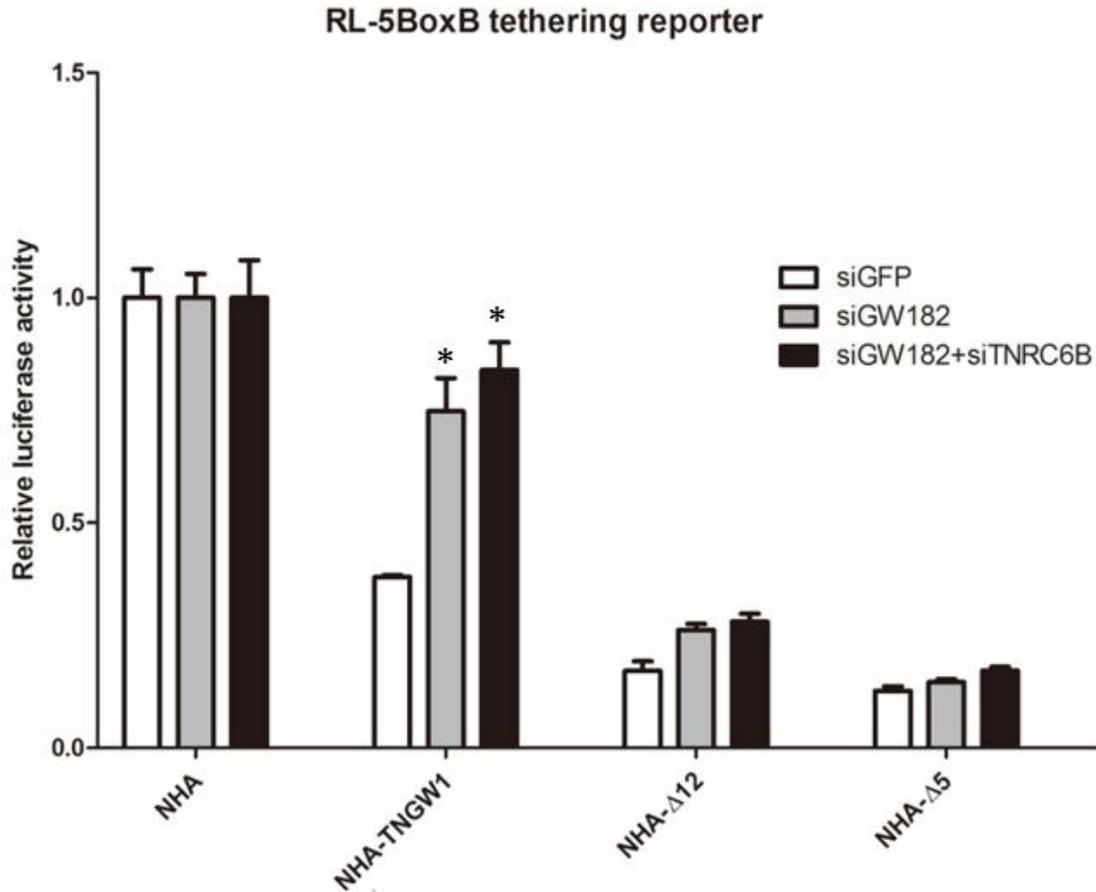


Figure 2-7. Tethered NHA-TNGW1 was employed as an additional control to monitor the GW182 siRNA effects. Repression by $\Delta 5$ and $\Delta 12$ in tethering assay were independent of endogenous GW182/TNGW1. SiGW182 knockdown impaired NHA-TNGW1 induced tethering repression as expected. In comparison with control siGFP, both siGW182 and the combination of siGW182+siTNRC6B severely impaired the tethered reporter repression indicating the full length TNGW1 was efficiently knockdown. Results are expressed as mean \pm standard error from three independent experiments. * represents significant difference in *t*-test compared with siGFP, $p < 0.01$.

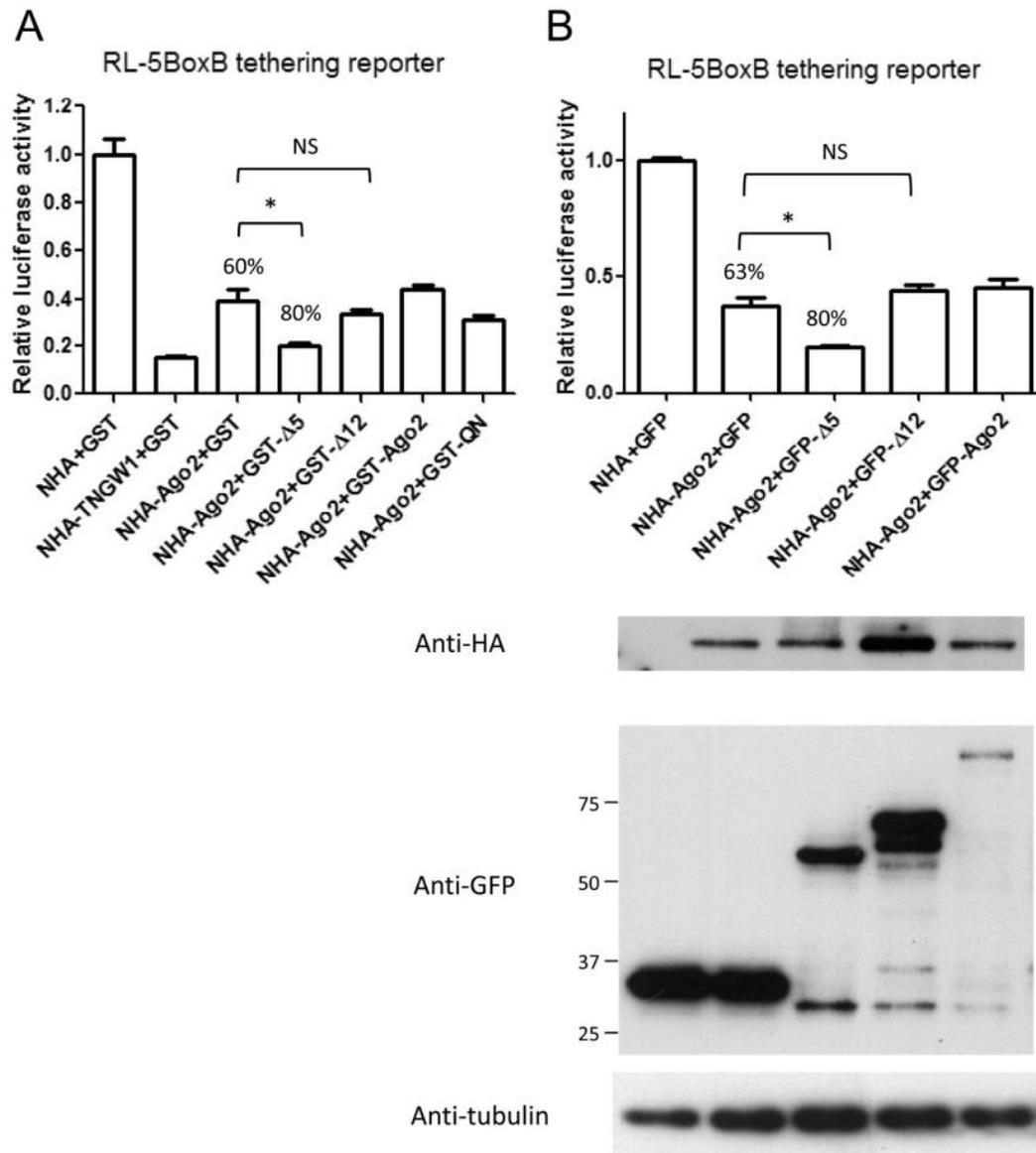


Figure 2-8. $\Delta 5$, but not $\Delta 12$, enhanced Ago2-mediated repression in the tethering function assay. A) Co-expressing GST- $\Delta 5$ with NHA-Ago2 that was tethered to the RL-5boxB reporter enhanced the NHA-Ago2-mediating repression from 60% to 80% (33% enhanced repression). In contrast, co-expression of GST- $\Delta 12$, -QN, or -Ago2 did not show significant change. Results are expressed as mean \pm standard error from three independent experiments. * represents *t*-test compared NHA-Ago2+GST- $\Delta 5$ with NHA-Ago2 alone, $p < 0.01$. B) Identical experiments as in panel A except GFP-tagged proteins were used in place of GST fusion proteins. Results are expressed as mean \pm standard error from three independent experiments. Representative cell lysates were analyzed by Western blot to demonstrate expression of NHA-Ago2 and GFP fusion proteins with tubulin expression shown as loading controls.

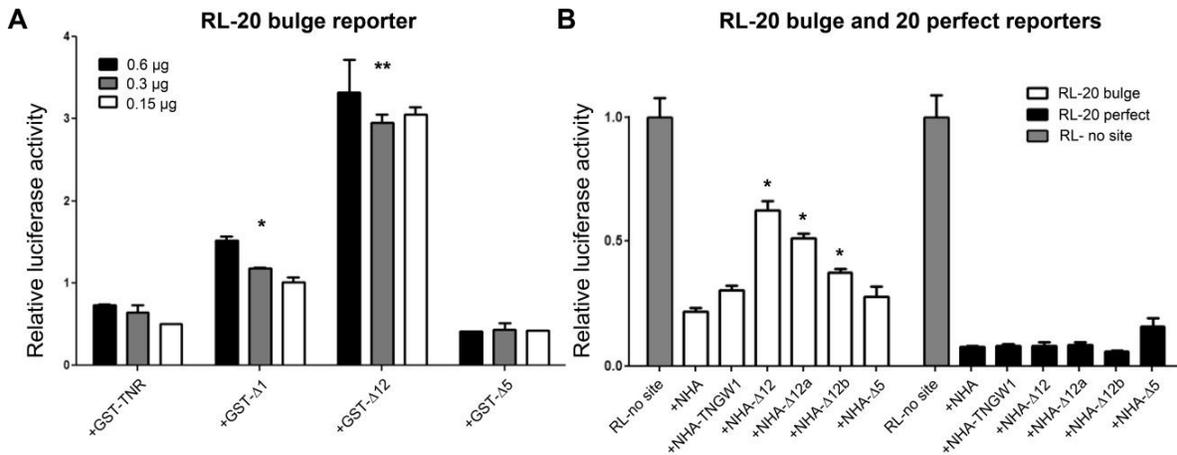


Figure 2-9. Δ 12 significantly interfered with endogenous miRNA repression. A) GST- Δ 12, but not GST- Δ 5, interfered with miR-20-mediated repression. Three different amounts (0.6, 0.3, 0.15 μ g) of GST-TNR, - Δ 1, - Δ 12, and - Δ 5 plasmids were co-transfected with the RL-20 bulge reporter into HEK293 cells. The RL-20 bulge expression was significantly increased when cells co-expressed Δ 12. Overexpression of Δ 1 also mildly interfered with miRNA function. * represents significant difference in *t*-test compared with +GST-TNR, $p < 0.01$, $n = 3$; ** represents highly significant difference in *t*-test compared with +GST-TNR, $p < 0.0001$, $n = 3$. B) Δ 12 and its deletion constructs only interfered with miRNA but not siRNA mediated repression. NHA tag, NHA-TNGW1, - Δ 12, - Δ 12a, - Δ 12b, and - Δ 5 were co-transfected with reporters RL-20 bulge/FL or RL-20 perfect/FL in HEK293 cells. Compared to the NHA control, the relative activity of RL-20 bulge was significantly increased in cells expressing NHA- Δ 12 and - Δ 12a/b but not in cells expressing -TNGW1 or - Δ 5. NHA- Δ 12 and its deletion constructs did not interfere with RL-20 perfect reporter that repressed by siRNA pathway. Results are expressed as mean \pm standard error from three independent experiments. * represents significant difference in *t*-test compared with +NHA, $p < 0.01$.

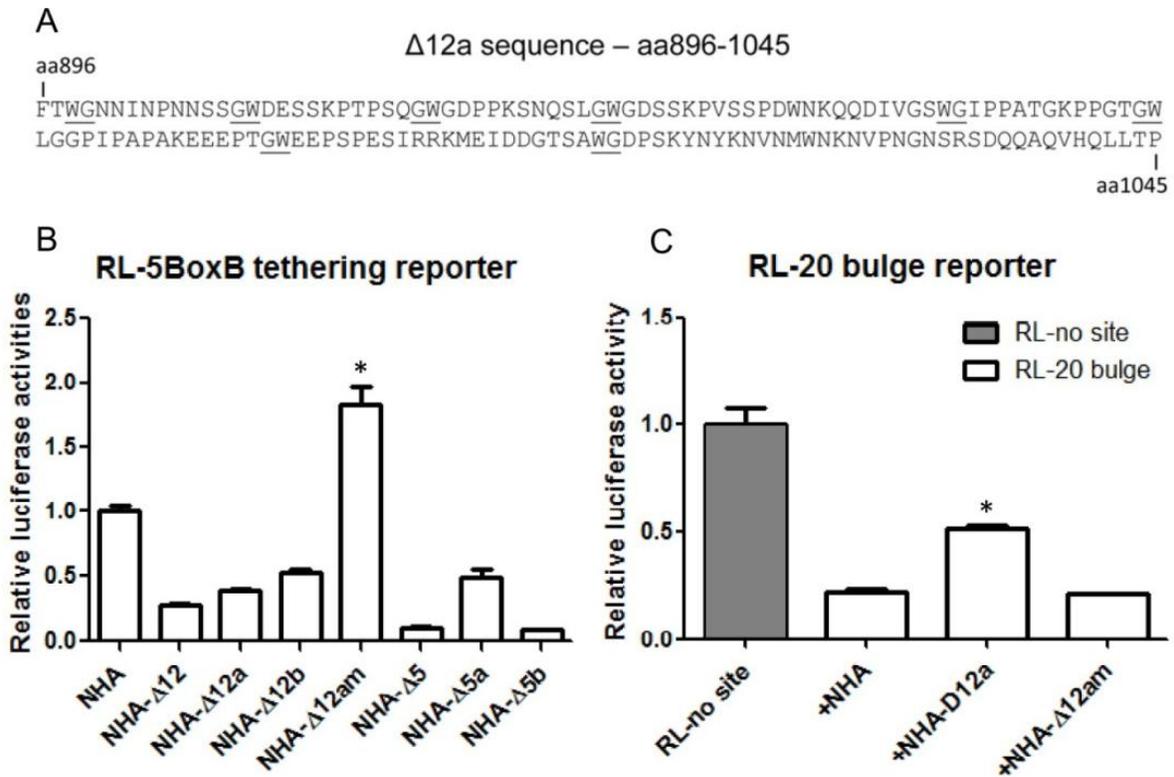


Figure 2-10. Substitution of glycine (G) and tryptophan (W) residues with alanine (A) in GW1Δ12a interfered with its repression on reporter and interference in miRNA repression activity. A) Amino acid sequence of Δ12a shown with GW and WG residues are underlined; these residues are substituted with AA to generate a mutant Δ12am. B) Δ12am had no repression activity in RL-5BoxB tethering assay. NHA-Δ5 and its deletion constructs served as positive controls. * represents significant different in *t*-test compared with NHA-Δ12, $p < 0.01$, $n = 3$. C) GW/GW mutated Δ12a (Δ12am) no longer affected miRNA mediated repression. Experiment was performed as described in Figure 2-9B.

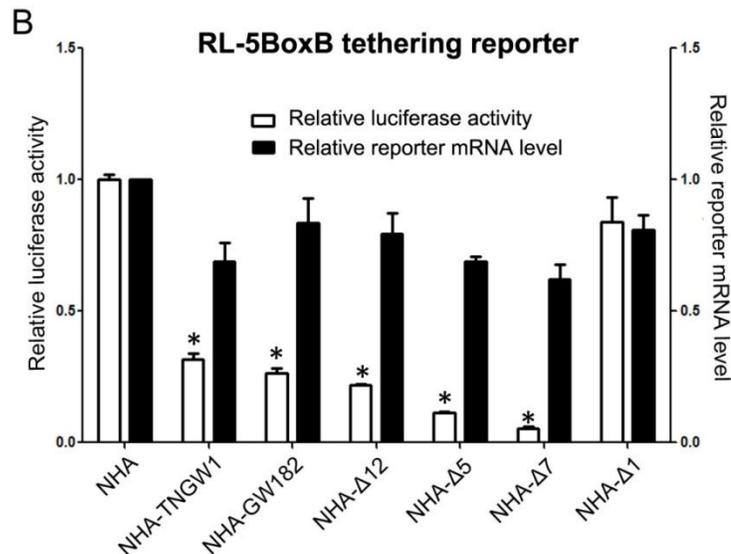
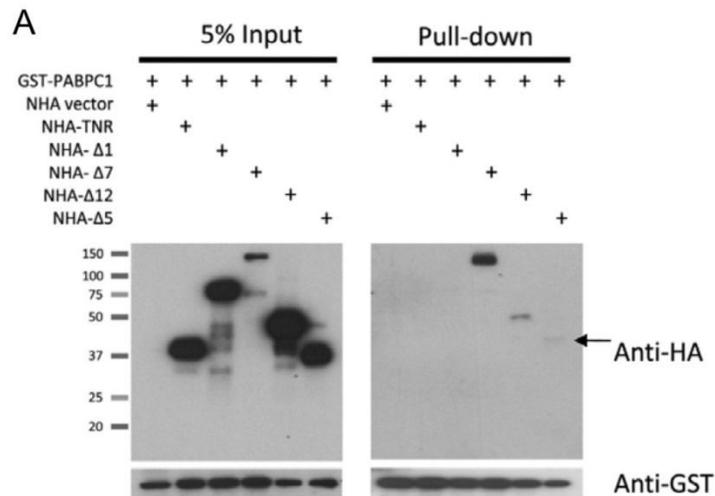


Figure 2-11. Binding of $\Delta 12$ and $\Delta 5$ to PABPC1 did not significantly affect reporter mRNA degradation. A) Differential binding of GW182 fragments $\Delta 12$, $\Delta 7$, and $\Delta 5$ to PABPC1. GST-PABPC1 was co-transfected with different NHA-tagged constructs into HeLa cells as shown above the panels for the designed GST pull-down assay. After 24 hours, cell lysates were harvested and analyzed by GST pull-down followed by Western blot analysis. GST-PABPC1 strongly pulled down NHA- $\Delta 7$ compared to NHA- $\Delta 12$ and - $\Delta 5$ (arrow). B) Both tethered $\Delta 12$ and $\Delta 5$ induced primarily translational repression with only moderate reporter mRNA degradation. To determine the mRNA level of the reporter in tethering assay, a pair of each RL and FL primers was utilized in SYBR-Green qRT-PCR. The RL mRNA level was normalized to FL mRNA. All results are expressed as mean \pm S.D. from three independent experiments. * represents significant difference in *t*-test compared with NHA, $p < 0.01$. No significant difference observed in mRNA degradation for any of the constructs compared with NHA.

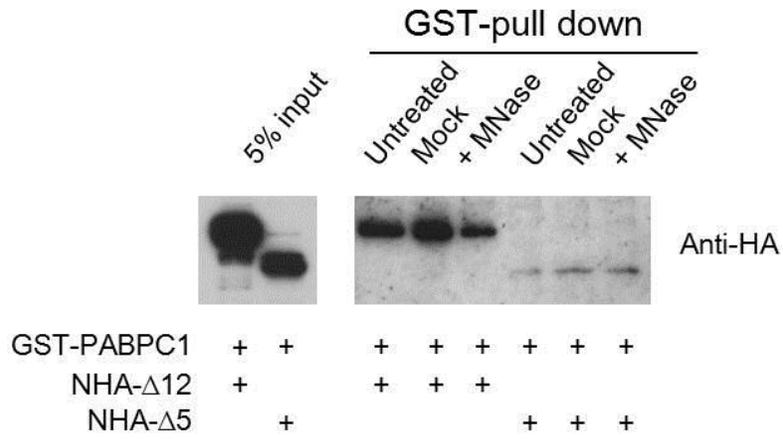


Figure 2-12. The interactions of GW182 repression domains and PABPC1 was independent of RNA. MNase digestion effect has been demonstrated in Figure 2-4.

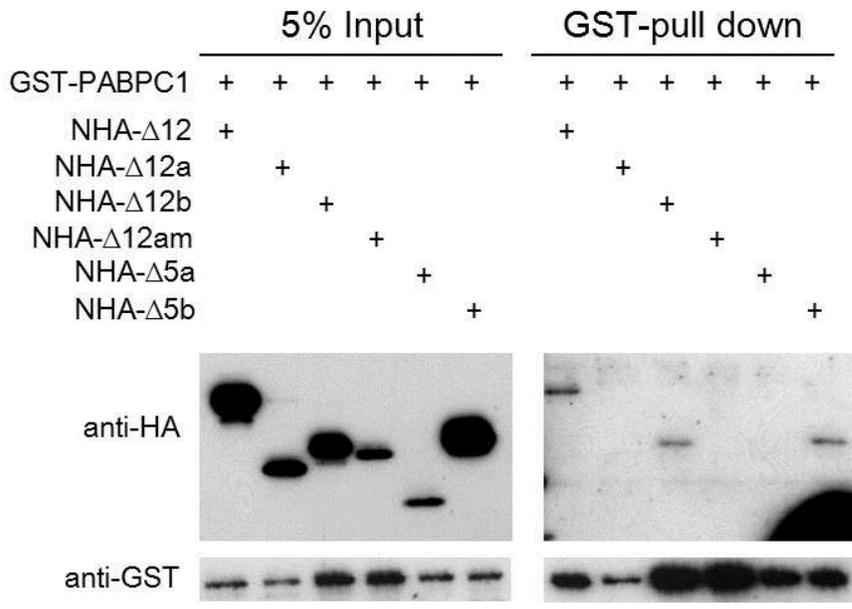


Figure 2-13. NHA-Δ12b and NHA- Δ5b bind to GST-PABPC1. GST-PABPC1 was co-transfected with different NHA-tagged constructs into HeLa cells as shown above the panels for the designed GST pull-down assay. After 24 hours, cell lysates were harvested and analyzed by GST pull-down followed by Western blot analysis. NHA-Δ12 served as a positive control as shown in Figure 2-11.

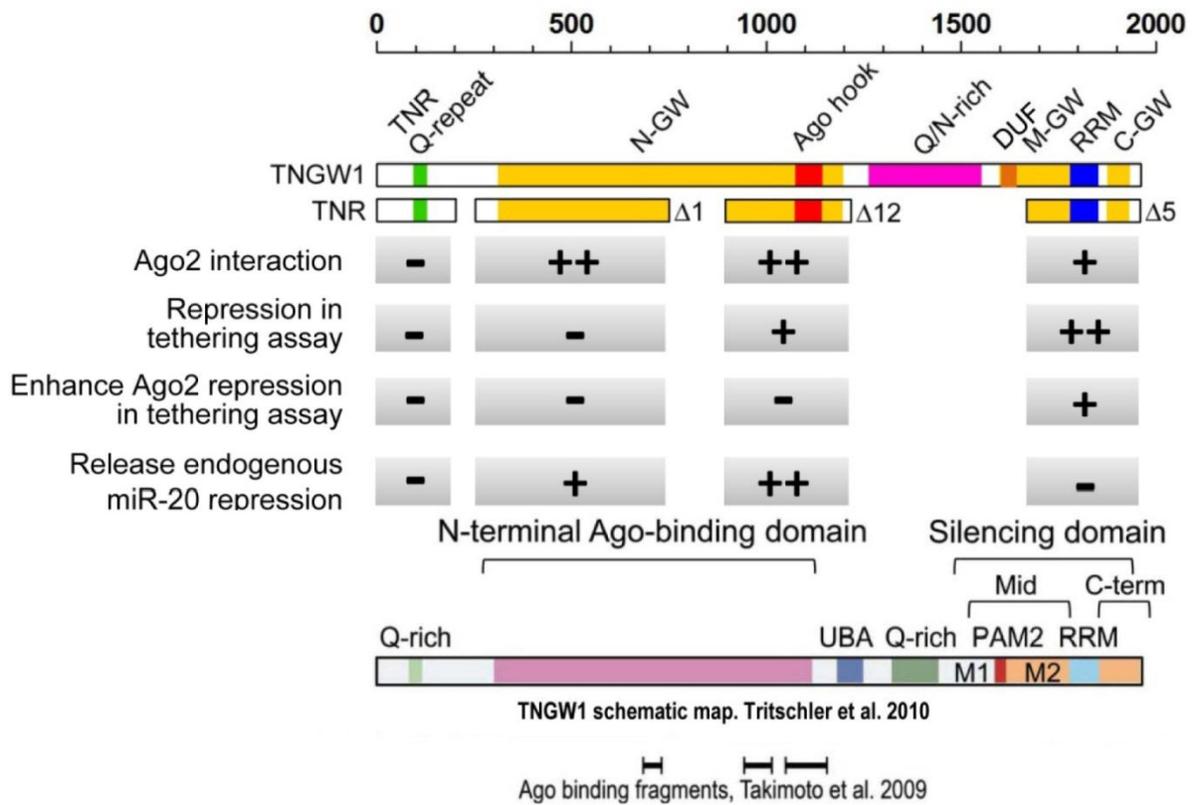


Figure 2-14. Summary of GW182 domain functional characteristics. DUF, sequence identified to be important for PABPC1 binding. A reference schematic map of TNGW1 in one recent review (40) is included for comparison. UBA, Ubiquitin-associated domain; PAM2, PABP-interacting motif 2. Regions M1 and M2 together with PAM2 formed the Mid region. Middle region and C-terminal region but not RRM defined the bipartite silencing domain.

CHAPTER 3
DEFINING A NOVEL ROLE OF GW182 IN MAINTAINING MICRORNA STABILITY

Background

MicroRNAs (miRNAs), a family of 20~25 nt single-stranded non-coding RNAs, has become a major focus in many biological processes due to their potent ability to moderate target mRNA function (17). To date, 50% or more mRNAs are regulated by miRNAs involved in almost all known cellular pathways (5,13). miRNAs are commonly transcribed from endogenous genomic loci and their biogenesis comprises multiple steps, including the processing of primary miRNAs (pri-miRNAs) by Drosha in the nucleus and precursor miRNAs (pre-miRNAs) by Dicer in cytoplasm to become mature miRNAs. The guiding strands then directly associate with Argonaute (AGO) proteins, core components of the RNA-induced silencing complex (RISC), serving as address labels to guide RISC to downregulate their target mRNAs by imperfect sequence matches (2,4).

Maintaining proper levels of cellular miRNA is important for normal cellular activities. Aberrant expression of certain miRNAs has been described in many diseases, including cancer (73). Although different mechanisms have been reported to regulate each step of the miRNA biogenesis process, the understanding of mature miRNA turnover is lagging behind (4,74). Several recent studies begin to shed light on this issue by demonstrating that particular mature miRNAs show dynamic and distinct turnover rates during the cell cycle (75,76) or from cells that are grown at different densities (77), even though those miRNAs are closely related to or are from the same gene clusters. Others show that miRNA decay in retina neurons is much faster than non-neuronal cells and this may contribute to neuronal activity (78). In contrast, global

transcriptional arrest reveals the majority of miRNA remain stable, with a few exceptions, suggesting a protective mechanism against decay under quiescent condition (79). Argonaute proteins directly associate with miRNAs and may protect miRNA from being degraded from ribonuclease. In fact, Argonaute proteins have been shown to positively correlate with mature miRNA levels (80). A very recent report directly shows that Argonaute protein increased miRNA abundance due to enhanced mature miRNA stability (81).

Recent observations that miRNA can be exchanged between cells via secretory exosomes, multivesicular endosome-derived vesicles ranging from 50 to 100 nm, that are actively secreted through an exocytosis pathway for intercellular cross-talk (82). This introduces the possibility that cells can regulate their intracellular miRNA level through this process (83-86). Alternatively, mature miRNA has been proposed to be degraded by endogenous ribonuclease. Significant effort has been made to identify the ribonuclease that is responsible for this process in different species (79,87,88) and has been specifically termed “microRNase” (89). However, a critical question how microRNase accesses the RISC-protected miRNA remains.

It has been ten years since GW182 and GW bodies (also known as mammalian P-bodies) were initially discovered by our immunostaining and immunoprecipitation with an autoimmune disease patient serum (1). The unique subcellular localization of GW182 and unusual high glycine-tryptophan repeat (GW) distribution had immediately drawn our attention. The function of GW182, however, remained elusive until Argonaute proteins were shown by us and others to co-localize and directly bind to GW182 in humans (31,38,90), *Drosophila* (44,46), *C. elegans* (43) and *Arabidopsis* (91), linking

GW182 to the miRNA silencing pathway. GW182 binds to Argonaute proteins through its unique GW/WG repeats (21,41,60,92) and this interaction is crucial for miRNA-mediated translational repression, as well as mRNA destabilization (35,54,58,61). GW182 and its longer isoform TNGW1 serve as downstream repressors of Argonaute proteins for gene silencing (35,41,46). Extensive mapping of AGO-interacting and repression domains on GW182 generated a consensus that the GW182 N-terminal GW-rich domain primarily binds to AGO on multiple sites, whereas the C-terminal domain possesses an intrinsic silencing ability (18-22). However, in addition to that, the N-terminal effective domain, which actually covers much of the middle region of GW182, has also been identified to demonstrate silencing activity in both *Drosophila* (53,93) and humans (22,24). It is still unclear how miRNA hampers mRNA translation, but important progress has recently been made to illustrate the mRNA decay mechanism induced by miRNA (5). The conserved GW182 Pam2 motif [Poly-A binding protein- (PABP-) interacting motif 2] functions as a major docking site to interact with PABP (25,26). Three very recent studies simultaneously report GW182 can directly interact and recruit the CCR4-NOT deadenylase complex through C-terminal conserved W motifs independent of PABP interaction (27-29). Therefore, GW182 functions as a coordinative platform that disrupts circularized mRNA by interacting with PABP to initiate translational interference. The proposed mechanism includes displacement of PABP from the Poly-A tail and deadenylation by recruitment and activation of deadenylase (27-29). Since GW182 binds to Argonaute proteins to play a central role in miRNA-mediated gene silencing, an interesting question is whether the AGO-GW182

interaction also plays a role in protecting AGO-associated mature miRNA on top of their silencing function.

In the present study, we have identified a novel function of GW182 in protecting mature miRNA from being degraded by interacting with Argonaute proteins. Beside its silencing activities, this study suggests an additional role of GW182, that of maintaining AGO-bound miRNA stability.

Materials and Methods

Plasmids, siRNA, and miRNA-mimic

The cDNAs of TNGW1, GW182, TNRC6B, TNRC6C, TNR, $\Delta 1$, $\Delta 12$, $\Delta 12a$, $\Delta 12b$, and Ago2 were constructed as described in previous studies (20,24,35). All cDNAs were cloned in described tethering assay plasmid pCIneo-NHA vector (35). GFP-GW182, -TNRC6B, and -TNRC6C were cloned using the Gateway (Invitrogen) clone system. NHA-GW182 and -TNGW1 with 5 mutations (5568 T to C, 5571 T to C, 5577 C to G, 5578 C to A, 5580 C to A) that were resistant to shRNA knockdown without affecting protein sequences (silent mutation) were generated by a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). pSuper-pre-miR-146a and pCDNA-pre-miR-155 were generously provided, respectively, by Dr. Nan Shen at the Shanghai Institute for Biomedical Sciences (94) and Dr. Rolf Renne at the University of Florida (95). Ago2 mutant constructs F2V2 and F2W2 were kindly provided by Dr. Zissimos Mourelatos at the University of Pennsylvania. siRNAs against XRN1, XRN2, and RRP41 were synthesized as described in other studies (96,97) by Dharmacon (Thermo Fisher Scientific, Lafayette, CO). miRNA-mimics were purchased from Applied Biosystems (Carlsbad, CA).

Lentiviral Constructs

ShRNA sequence against GW182/TNGW1 common region (5567-5585bp on TNGW1 mRNA) is shown in Figure 3-1A, upper panel. shRNA sequence against TNRC6B and TNRC6C was selected from a previous report (22). shRNA sequence mutant (shGW182-mut) was cloned based on the same strategy serving as a negative control (Figure 3-1A, upper panel). All shRNA sequences were fused with miR-30 stem loop backbone structure (Open Biosystems, Huntsville, AL) to achieve better processing efficiency. Primer sequences for shRNA containing BamHI/MluI restriction enzyme cutting sites, miR-30 stem loop, and RNA Polymerase III stop codon are shown in Table 3-1. Forward and reverse primers were then directly annealed and cloned into lentiviral vector pTYF-EF carrying GFP and puromycin-resistant markers. ShRNA expression was driven by Pol III-specific H1 promoter, a transcriptionally weaker promoter to avoid major cytotoxic effects from lentiviral transduction (98). GFP was used to monitor the transduction efficiency and identify transduced cells, and puromycin-resistant marker for establishing long-term stable cells. Viruses were produced as described (99). HEK293 cells were transduced with lentivirus that contained shRNA and GFP marker at a multiplicity of infection (MOI) of 20–40 in the presence of 5 µg/mL polybrene. Forty-eight hours post-transduction, live cells were observed under fluorescent microscope for GFP expression. Cells were then transferred into puromycin-2.5 µg/mL medium for culture.

Cell Culture and Transfection

HEK293 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin in a 37° C incubator with 5% CO₂. Plasmid coding pre-miRNA, siRNA, and miRNA-mimic were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. In brief, 5x10⁵

wildtype or GW182-KD 293 cells were seeded into 12-well plates one day prior transfection. The next day, 2 µg plasmids, 100 nM siRNA, or 40 nM miRNA-mimic were transfected into cells for various time points as indicated. Cells were washed once with PBS and subjected to total RNA extraction or whole cell lysate preparation.

Nucleofection

Cells were transfected by electroporation using the Nucleofector system as per manufacturer's instructions (Lonza, Walkersville, MD). In brief, 5×10^5 cells were seeded into T-25 flask two days prior to nucleofection and maintained in a 37° C incubator with 5% CO₂. On the day of nucleofection, cells were trypsinized and counted. One million cells were mixed with 1-5 µg of plasmid DNA together with 100 µL Cell Line Nucleofector Solution V from Amaxa Cell Line Nucleofector Kit V specific for HEK293 cells (Lonza Cologne AG, Cologne, Germany). The cell-plasmid mixture was immediately transferred into a certified cuvette and inserted into the Nucleofector II device (Lonza Cologne AG) and transfection was initiated with program Q-001. After nucleofection, ~500 µL of the culture medium was immediately added and gently transferred into prepared 6-well plates for continuous culture. Viewed under a fluorescent microscope, GFP expression was visualized within 12 hours with more than 80% transfection efficiency.

RNA Extraction, Reverse Transcription and Quantitative PCR (qPCR)

Total RNA samples from cultured cells were harvested using a mirVana total RNA isolation kit (Applied Biosystems) following the manufacturer's instructions. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit or miRNA Reverse Transcription Kit (Applied Biosystems). The relative mRNA or miRNA levels of target genes were measured in duplicate using TaqMan Fast Universal Master

Mix (Applied Biosystems) with the corresponding TaqMan Gene Expression or miRNA expression Assay (Applied Biosystems). Primer with the sequence specific to the unique TNGW1 region was used to distinguish TNGW1 and GW182 isoforms. Ribosomal 18s and small RNA RNU44 served as normalizers for mRNA or miRNA levels, respectively.

***In Vitro* Assay for GW182-Dependent Differential miRNA Sensitivity to RNase**

Mouse monoclonal anti-Ago2 (4F9) was used to immunoprecipitate endogenous Ago2 as described (100). Rabbit polyclonal anti-Ago2 (C34C6, Cell Signaling, Danvers, MA) was used for Ago2 Western blot. Rabbit polyclonal anti-GW182 (5182) was previously described and used for Western blot (35). To determine the role of GW182 in affecting miRNA half-lives, 40 mM miR-146a-mimic was transfected into both wildtype and GW182-KD cells. Cells were harvested 6 hours after transfection, lysed using NET/NP40 buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.3% NP40) with Complete Protease Cocktail Inhibitor (Roche Diagnostics, Indianapolis, IN), and then immunoprecipitated by mouse anti-Ago2 monoclonal antibody 4F9 (100). Cell lysates were sonicated for 10 sec three times on ice and then centrifuged at 13,200 rpm for 5 min. Supernatant was mixed with 200 μ L 4F9 and 50 μ l Protein L beads at 4° C for 1 hour. After incubation, the beads were washed a total of four times with NET/NP40 buffer containing 500 mM NaCl. Beads were aliquoted evenly and treated with different concentration of RNase at 37° C for 30 min. Total RNA samples were harvested using mirVana total RNA isolation kit (Applied Biosystems) and the levels of miRNA determined by a Taqman qRT-PCR (Applied Biosystems). To monitor the levels of GW182, parallel cell lysates and IP samples were analyzed by Western blot.

Secretory Exosomes Purification

Exosomes purification was performed as described (101). HEK293 cell culture medium was subjected to a series of differential centrifugations. In brief, raw cell culture media was processed by centrifuging at 1000 g for 5 min to remove live cells, 3000 g for 10 min to remove dead cells, and 10000 g for 30 min to remove cell debris, then passed through a 0.22 μm filter to remove large secretory vesicles and residual debris. The exosomes enriched medium was then either directly exposed to RNA extraction or further processed at 100,000 g ultracentrifugation for 1 hour to collect the exosome pellet. The exosome pellet was washed once with cold PBS and centrifuged for an additional hour at 100,000 g. The final exosome pellet was resuspended in 30 μL of PBS for RNA analysis or Western blot. Mouse monoclonal anti-CD63 (BD Pharmingen, San Diego, CA) and rabbit polyclonal anti-Rab5b (Santa Cruz Biotechnology, Santa Cruz, CA) were used as exosomal markers.

Calculation of miRNA Secretion Ratio

The calculation of the miRNA secretion ratio was based on described methods (85) with some modifications. The fold changes of intracellular and extracellular miRNAs were determined by qRT-PCR. The overall intracellular miRNA fold changes were calculated by PCR fold change per ng x total RNA harvested. The overall extracellular miRNA fold changes were calculated by PCR fold change per media volume x total volume. Thereby the ratio of extracellular/intracellular miRNA was calculated.

Results

Knockdown of GW182 and Its Paralog TNRC6B Impaired miRNA Stability

A lentiviral-based shRNA strategy was used to generate stable knockdown cells (Materials and Methods in this Chapter). The shRNA sequence against GW182 and its

mutated negative control are shown in Figure 3-1A, upper panel. This negative control was tested throughout the entire study to ensure that none of the observations were related to false positive from the lentiviral transduction. Nearly 100% of lentiviral transduction efficiency was obtained after 48 hours, as demonstrated by the GFP reporter expression, and cells were switched into a 2.5 $\mu\text{g}/\text{mL}$ puromycin-containing medium for long-term maintenance (referred as GW182-KD and shGW182-mut cells). Lentiviral GW182-KD showed a specific and effective knockdown of 79.1% of GW182 mRNA without affecting its paralogs, TNRC6B and TNRC6C, or its interaction partner, Ago2, and pre-miRNA processor Dicer mRNA (Figure 3-1A, lower left panel). In contrast, shGW182-mut did not significantly affect expression. Western blot was carried out to measure the GW182 and Ago2 protein level in all three cell lines (Figure 3-1A, lower right panel). Cell lysates were loaded in three different concentrations to obtain a better quantification in Western blot. Compared to wildtype 293, GW182-KD and shGW182-mut cells showed elevated Ago2 protein levels (compare lanes loaded with 12.5 μg total protein); this increase in Ago2 levels as a result of elevated levels of siRNA was reported in another study (102). In contrast, both GW182 and TNGW1 proteins were effectively knocked down in GW182-KD, but not in shGW182-mut cells.

To observe the GW182 knockdown effect, we went on to examine transfected miRNA levels in GW182-KD cells and their wildtype controls. Two plasmids expressing miR-146a and miR-155 were transfected in wildtype and GW182-KD cells. Interestingly, both transfected miR-146a and miR-155 level were significantly lower (50.7%, $p < 0.01$, and 64.2%, $p < 0.01$, respectively) in GW182-KD cells compared to wildtype that was normalized to 1 (Figure 3-1B). Renilla and Firefly luciferase reporters were transfected

in both wildtype and GW182-KD cells in separate experiments, which demonstrated that they have comparable transfection efficiency in GW182-KD cells (data not shown). This interesting observation led us to hypothesize the role of GW182 in regulating miRNA stability. In independent experiments to directly evaluate the mature miRNA levels, 40 nM of miR-146a-mimic and miR-132-mimic were transfected into wildtype, GW182-KD, and shGW182-mut cells seeded in equal cell number. Cells were harvested at 6, 24, and 48 hours to analyze for miRNA levels by qPCR. At all three time points, both transfections exerted significantly low miRNA levels, specifically in GW182-KD cells compared to wildtype that was normalized to 1, and shGW182-mut controls (Figure 3-1C). For instance, miR-146a levels in GW182-KD cells were 42.7% ($p < 0.01$) at 6 hours compared to the level in wildtype and dropped to 25.5% ($p < 0.001$) at 48 hours. Similarly, miR-132 levels in GW182-KD cells were 57.4% ($p < 0.05$) at 6 hours and 44.7% ($p < 0.001$) at 48 hours compared to wildtype. Based on this data, the half-life of miR-146a was determined to be 15.3 and 9.0 hours in wildtype and GW182-KD cells, respectively. Similarly, the half-life of miR-132 was 11.9 hours in wildtype and 8.8 hours in GW182-KD cells. Thus the half-lives of miR-146a and miR-132 were reduced by 41.1% and 26.0%, respectively, in GW182-KD cells compared to wildtype.

Synthetic mature miRNA-mimics were therefore primarily used in a subsequent study to evaluate the impact of GW182 knockdown on mature miRNA levels. miRNA-mimics have been used in similar studies to monitor the dynamic changes of miRNA during different cellular activity and proved to be faithfully recapitulating endogenous miRNA activity (75,77). Transfected miRNA-mimics offered a boosted signal for easy detection, as well as potentially saturating compensatory GW182 paralogs. miR-146a-

mimic transfection was also performed in concentrations of 5, 10, and 40 nM for 24 hours, and significant lower levels from each concentration (41.4%, 31.8%, and 24.4%, respectively; $p < 0.01$) were observed in GW182-KD cells compared to wildtype in all three concentrations tested (Figure 3-1D). Transfection of 40 nM miRNA-mimic was primarily used throughout the rest of the study. Taken together, these data demonstrated that GW182 knockdown significantly reduced mature miRNA-mimic levels.

In mammals, there are three paralogs of GW182 with gene names TNRC6A, B, and C (5). These paralogs shared broad functional similarities in terms of AGO interaction and miRNA-mediated mRNA repression in tethering functional assays (18,19,22). Transient knockdown of individual paralog caused partially de-repression of reporter activity, also implying their functional redundancy (22). However, clear functional difference of these paralogs has not been described. To investigate whether TNRC6B and TNRC6C also play a role in regulating miRNA stability, lentiviral shRNA-based stable TNRC6B knockdown (6B-KD) and TNRC6C knockdown (6C-KD) cells were also established. MiR-146a-mimic was again transfected into wildtype, GW182-KD, 6B-KD, and 6C-KD cells for 6 and 24 hours. Intriguingly, TNRC6B, but not TNRC6C, was also required for miRNA stability in these two time points (Figure 3-1E). The lentiviral shRNA knockdown efficiency and specificity is shown in Figure 3-2. Collectively, knockdown of GW182 and its paralog TNRC6B reduced mature miRNA stability. Beside its miRNA silencing activity, the present study shows for the first time a novel function of GW182 in regulating miRNA stability.

Replenishment of GW182 and Its Paralogs Restored the Shortened miRNA Stability

Since knockdown of GW182 and TNRC6B reduced miRNA stability, it was important to perform complementation experiments to examine the role of GW182 in regulating miRNA stability. To this end, we tested the wild type and a series of deleted GW182 constructs (Figure 3-3A). Transfections of 5 μ g NHA vector, NHA-Ago2, and NHA-GW182 were introduced into wildtype cells by nucleofection for 16 hours, followed by transfection of an miR-146a-mimic. Total RNA was harvested 6 hours later for miR-146a qRT-PCR analysis (Figure 3-3B, open bars). miR-146a levels in wildtype cells transfected with vector alone were normalized to 1. miR-146a level increased 42.2% ($p < 0.05$) when co-transfected with NHA-Ago2, consistent with previous observation that elevated Ago2-enhanced miRNA level (80). NHA-GW182 transfection only mildly increased the transfected miR-146a level and was not statistically significant compared to the NHA vector control in wildtype 293 cells. The transfected miR-146a level in GW182-KD cells without plasmid transfection (no vector) showed reduced level (53.9%) compared to wildtype, consistent with data already shown in Figure 3-1C. The transfected miR-146a level in vector alone transfection did not show any significant difference compared to no vector control in GW182-KD cells (Figure 3-3B). In contrast, the transfected miR-146a level in GW182-KD cells was restored to comparable levels as in wildtype cells with the expression of a GW182 construct that was specifically mutated to be resistant to the lentiviral shRNA in GW182-KD cells, in a dose-dependent manner (Figure 3-3B, 1 μ g, 74.0%, $p < 0.05$; 2 μ g, 74.1%, $p < 0.05$; and 5 μ g, 91.4%, $p < 0.001$ compared to 56.9% for vector alone). Interestingly, transfection of the previously defined repression domain Δ 12 also strongly restored transfected miR-146a

levels comparable to transfected full-length GW182 (1 μ g, 87.4%, $p < 0.001$; 5 μ g, 106%, $p < 0.001$ vs 56.9% for vector alone). Another GW182 fragment $\Delta 1$ showed mild restoration of miR-146a level (66.7% vs 56.9%, $p < 0.05$). Transfection of Ago2 in GW182-KD cells also showed mild restoration (70.8% vs 56.9%, $p < 0.05$) and this might imply GW182 was a limiting factor in this reaction. All transfected constructs were monitored in Western blot, ruling out the effects caused solely by differential protein expression (Figure 3-3B).

Since $\Delta 12$ was able to significantly restore the stability of the transfected miRNA, two $\Delta 12$ truncations, $\Delta 12a$ and $\Delta 12b$, were nucleofected in GW182-KD cells for a direct comparison with $\Delta 12$ (Figure 3-3C). Interestingly, neither $\Delta 12a$ nor $\Delta 12b$ exerted comparable complementary effects as $\Delta 12$, indicating the importance of integrity for the whole $\Delta 12$ fragment. This was consistent with the previous study that neither $\Delta 12a$ nor $\Delta 12b$ recapitulated full-length $\Delta 12$ function in interaction with Ago2 and tethering repression (24). Expression of transfected constructs was monitored in Western blot (Figure 3-3C).

Re-expression of TNGW1 in GW182-KD cells by transfection of a shRNA-resistant GFP-TNGW1 construct in increasing amounts (1, 2, and 5 μ g) also restored transfected miR-146a levels, peaking at the 2 μ g level (Figure 3-3D). Units of 5 μ g of GFP-TNRC6B or GFP-TNRC6C transfected by nucleofection into GW182-KD cells for cross-complementation analysis each restored the stability of transfected miR-146a (Figure 3-3D). The expression levels of TNGW1, TNRC6B, and TNRC6C were monitored by qPCR (Figure 3-3E). These data indicated the functional redundancy of the GW182

family in regulating miRNA stability, probably by their comparable affinity to Argonaute proteins (19).

Collectively, re-expression of GW182 and its paralogs TNRC6B and TNRC6C, as well as one of its middle repression domains, $\Delta 12$, could significantly restore the stability of transfected miRNA levels in GW182-KD cells. These data strongly support the hypothesis that GW182 regulates miRNA levels by binding to Ago2.

GW182 Knockdown Decreased Exosomal Secretion of miRNA

In GW182-KD cells, there were two possible pathways for reduction of the transfected miRNA, either secreted into extracellular space via exosomes or degraded intracellularly by cytoplasmic ribonucleases. To address these two possibilities, extracellular secreted miRNA levels were investigated first. Recent studies suggest that the extracellular miRNAs are primarily protected by exosomes, the secretory vesicles shed from almost all cell types, and that GW182 may be involved in this process (83,84). Either upregulation of endogenous or exogenous miRNA results in elevated miRNA secretion via exosomes that can be taken up by recipient cells and has been demonstrated to silence reporter mRNA (85,86). However, it is disadvantageous to use a miRNA mimic to evaluate miRNA secretion rates in GW182-KD cells due to their persistence in the media and interference with secreted miRNA measurements (85). To overcome this issue, plasmids expressing miR-146a and miR-155 were transfected into wildtype and GW182-KD cells to examine the export of these miRNAs. In agreement with a previous study (85), both miR-146a and miR-155 secretions by exosomes increased in the culture medium (Figure 3-4A). To further confirm miRNA was secreted via exosomes, an established differential ultra-centrifugation protocol (101) was applied to transfected 293 cells media for exosomes purification. Two exosomes markers, CD63

and Rab5b (103), were used to confirm the successful purification by Western blot (Figure 3-4B). Upregulation of either miR-146a or miR-155 did not appear to increase exosomal CD63 or Rab5b suggesting the transfection of miRNA expression plasmids did not increase non-specific exosomal export in these experiments. RNA was extracted from both the exosome fraction and the supernatant depleted of exosomes. Our data showed the majority of extracellular miRNA (was co-purified with exosomes (Figure 3-5B, miR-146a: 92.7% vs 7.3%; miR-155: 78.7% vs 21.3%). Both wildtype 293 and GW182-KD cells were transfected with miR-146a and miR-155 plasmids, as shown in Figure 3-5C. Relative fold change of both intracellular and extracellular miR-146a and miR-155 compared to GW182-KD and wildtype cells was determined by qPCR and the ratios of extracellular to intracellular miRNA were calculated based on a previous report (85) with modifications (Materials and Methods in this Chapter). In both plasmid transfections, GW182-KD cells showed a reduced ratio compared to wildtype, demonstrating that the knockdown of GW182 decreased miRNA secretion via exosomes (Figure 3-5C). Note that the percentage of miRNA in the exosomal fraction was low (0.1% for miR-146a and 1% for miR-155) and this was consistent with data from a report evaluating the miRNA level in secretory microvesicles compared to their cellular content in THP-1 monocytes (86).

In summary, elevated intracellular miRNA was detected in exosomes and GW182 knockdown reduced both miR-146a (37.5%, $p < 0.05$) and miR-155 (48.2%, $p < 0.05$) secretion. The fraction of miRNA exported was low and thus ruling out secretion as a major miRNA turnover pathway when GW182 was knocked down. These results

demonstrated that the reduced miRNA level in GW182-KD cells was an intracellular event and not due to elevated secretion.

GW182 Protected Argonaute-Bound miRNA from Degradation by Direct Interaction

By excluding the possibility that the reduced stability of transfected miRNA in GW182-KD cells was due to enhanced secretion into extracellular space, the alternative possibility was that the knockdown of GW182 caused AGO-bound miRNA to be more readily accessed and degraded by cytoplasmic ribonuclease. Previous studies identified different exoribonuclease responsible for miRNA degradation in *Arabidopsis* (87), *C. elegans* (88), and humans (79) described as “microRNase (89).” In order to identify the ribonuclease that plays a role in GW182-KD cells, the effect on siRNA knockdown of the three major candidate ribonucleases (5'-3' exoribonuclease XRN1, XRN2, and 3'-5' exoribonuclease complex component RRP41) were examined in GW182-KD cells. First siRNA for XRN1, XRN2, and RRP41 were transfected into wildtype and GW182-KD cells. After 48 hours, miR-146a-mimic was subsequently transfected, and RNA was harvested after another 6 hours for qPCR analysis. Interestingly, the miR-146a levels remained unchanged in wildtype 293 cells with all three individual nuclease knockdowns compared to levels in cells transfected with two siRNAs controls (siLamin A/C, Figure 3-6A, left panel, and siGFP, data not shown). Remarkably, the miR-146a level in GW182-KD cells transfected with siRRP41 was 51.4% higher than the same levels in siLamin A/C, siXRN1, and siXRN2 (Figure 3-6A, right panel, $p < 0.01$). These data demonstrated that the miRNA was protected from degradation by the AGO-GW182 complex in wildtype cells and therefore knockdown of these nucleases did not show any effect. However, in GW182-KD cells, AGO-bound miRNAs were more sensitive to degradation,

primarily by the RRP41-dependent 3'-5' exoribonuclease complex, which is consistent with a previous report showing the role of this exoribonuclease as miRNase in mammalian cells (79). Furthermore, mRNA qPCR analysis revealed only RRP41, but not XRN1 or XRN2, mRNA was significantly elevated by 52.6% in GW182-KD cells; this apparent compensatory effect might further implicate a role of RRP41 in this process (Figure 3-7).

To further confirm that GW182 protected AGO-bound miRNA from ribonuclease degradation, immunoprecipitation of Ago2 was performed with monoclonal anti-Ago2 4F9 using lysates from wildtype and GW182-KD cells transfected with miR-146a-mimic for 6 hours. After three washings, the beads were aliquoted equally into five tubes. One set of aliquoted samples was saved for Western blot analysis to demonstrate the efficient knockdown of both TNGW1 and GW182 in GW182-KD cells (Figure 3-6B, right panel). The four remaining tubes were subjected either to no treatment or to three different concentrations of RNase A treatments incubated in 37° C for 30 min. Total RNA was extracted from the beads and qRT-PCR was performed to evaluate the transfected miR-146a and endogenous miR-16 levels (Figure 3-6B, left two panels). miRNA levels in untreated groups of wildtype and GW182-KD were individually normalized to 1. Both miR-146a and miR-16 precipitated from GW182-KD cell lysates showed increased sensitivity to RNase A treatments compared to those from wildtype lysates. miR-146a level from GW182-KD cell lysate was 25.3% lower than that from wildtype treated with 10 µg/mL RNase A. In contrast, compared to the wildtype, the endogenous miR-16 level from GW182-KD lysate was reduced 49.5% using only a 1 µg/mL RNase A treatment. The difference in sensitivity was likely due to the high

number of copies of overexpressed miR-146a compared to endogenous miR-16. Thus these data demonstrated similar sensitivity to RNase A for both miRNA-mimic and endogenous miRNA in GW182-KD cells and the role of GW182 in protecting miRNA by binding to Ago2.

In order to provide additional evidence that the AGO-GW182 interaction is critical for AGO-bound miRNAs, a set of Ago2 constructs with differential GW182 binding abilities was employed in the next experiment. Two Ago2 constructs harboring mutations on two conserved phenylalanines (Phe470 and Phe505) in the MID domain to valines (F2V2) or tryptophans (F2W2) were first studied for their mRNA cap-binding ability (104). It was demonstrated that F2V2 severely impaired binding to GW182 and mature miRNA loading, whereas F2W2 partially retained its ability to interact with GW182 and *Drosophila* miRNAs (41,46,58). NHA-tagged Ago2 wildtype and mutated plasmids were then transfected into wildtype and GW182-KD cells. Twenty-four hours post-transfections, the miR-146a-mimic was transfected for an additional 6 hours. Cell lysates were harvested for HA immunoprecipitation and associated RNAs were extracted for miRNA analysis. F2V2 showed significantly lower miRNA association from both wildtype and GW182-KD cells in agreement with previous studies that this construct was defective in miRNA loading (41,46). Ago2 wildtype and F2W2 showed comparable miRNA loading (Figure 3-6C, left panel). When comparing miRNA associated with Ago2, F2V2, and F2W2 in Ago2 IP, F2V2 showed a significantly lower level (35% in wildtype Ago2 pulldown and 41.3% in GW182-KD pulldown) compared to Ago2 wildtype of miRNA association in both wildtype and GW182-KD cells, consistent with previous observations. Importantly, however, both Ago2 wildtype and F2W2-bound

miRNA from GW182-KD cell lysate showed significantly lower levels compared to that from wildtype lysate (Figure 3-6C, left panel, 41.7% and 40.4% reduction, respectively; $p < 0.05$). No significant difference was observed for F2V2-bound miRNA from wildtype and GW182-KD cells. Since previous studies suggested AGO loading is dispensable to GW182 (41), our data suggested that GW182 protected loaded-miRNAs only in Ago2 constructs with normal miRNA loading capacity. Western blot confirmed the precipitated Ago2 constructs and their putative interactions with endogenous GW182 (Figure 3-6C, right panel).

Collectively, our data strongly supported that GW182 binds to Argonaute proteins in RISC complex and play an important role in maintaining AGO-bound miRNA stability.

Discussion

How GW182 Maintains miRNA Stability by Binding to Argonaute Proteins

In the past ten years since GW182 was identified (1), great progress has been made to establish the role of this unique protein as a central player in miRNA-silencing pathways (2,5,13). It is now becoming clearer that this multi-domain protein anchors to the miRNA-mRNA complex by binding to Argonaute proteins and facilitates translational repression, as well as mRNA deadenylation (27-29). Argonaute proteins directly associate with miRNA (15,105) and require GW182 interaction for their functions in translational inhibition and mRNA decay (35,54,58,61). Argonaute proteins have been proposed to be limiting factors in forming miRISC complexes as overexpression of each individual AGO protein enhanced mature miRNA abundance (80). In fact, our data was consistent with a previous study that concluded that the overexpression of Ago2 in wildtype cells enhanced transfected miRNA levels. However, reduced miRNA level in GW182-KD cells could not be simply explained by a reduction in Ago2 levels. In fact,

GW182-KD and shGW182 mutant cells showed that elevated Ago2 protein levels likely correlated with the elevated level of shRNA (102). This data suggested a direct role of GW182 in maintaining miRNA stability.

Transient knockdown of GW182 and its paralogs individually causes partial alleviation of repression, indicating they are functionally redundant (22). In our study, stable knockdown of TNRC6B also caused reduction of miRNA but this was not observed when TNRC6C was knocked down. The TNRC6B shRNA appeared to be less potent than TNRC6C as we could achieve only a 33.4% knockdown for TNRC6B mRNA, but a 53.6% knockdown for TNRC6C mRNA. This may be due to the different efficacies of shRNAs. The functional differences among GW182 and its paralogs have not been observed so far in different functional assays (19,22), but TNRC6C seems to be dispensable and tissue-specific as HeLa cells have less than 10% of TNRC6C mRNA levels comparing to HEK293 cells (24). Interestingly, re-expression of any GW182-family protein could restore the stability of transfected miRNA in GW182-KD cells, once again indicating their functional redundancy. A previous study suggests that the three GW182 paralogs and four AGO paralogs can form discrete complexes of 12 in total (18) raising the possibility for differential binding of these complexes to different miRNA subsets. However, recent PAR-CLIP experiments demonstrate that AGO and the GW182 family of proteins are associated with similar subsets of miRNA (106). Conserved domains or amino acids, especially tryptophan on GW182 proteins, usually play a critical functional role by interacting with AGO proteins and affecting their silencing activity in tethering assays (24,27-29). Further mapping of the domains that can rescue miRNA stability in GW182-KD cells identified a previously defined silencing

domain $\Delta 12$ with repression activity in a tethering assay on the middle region of GW182 (24). The $\Delta 12$ activity may exert its ability to bind to Ago2, as it possesses two pre-defined Ago2 binding region (21). The consensus is that the major GW182 silencing domain represented by the C-terminal domain $\Delta 5$ was less effective (or mildly effective) in restoring stability of the transfected miR-146a in GW812-KD cells (data not shown). Note that although $\Delta 5$ has a strong silencing function, it has low or no AGO binding ability (107). Notably, the conserved tryptophan on the GW182 sequence has proved to be very important for interaction with AGO (21,41,60,92) and the deadenylation machinery (27-29). Future experiments should be carried out to further define conserved amino acid residues for their role in protecting miRNA stability.

Two sets of immunoprecipitation experiments were designed to directly evaluate the requirement of GW182 in the AGO-miRNA complex for miRNA stability. Endogenous Ago2 IP from wildtype and GW182-KD cells showed a distinct sensitivity to RNase A treatments with miRNA being more stable in the presence of GW182. Transfected Ago2 wildtype and two conserved phenylalanines mutated to tryptophans (F2W2) that retained miRNA association showed a significant difference in miRNA pulled down from wildtype cells or GW182-KD cells. In comparison, miRNAs recovered from F2V2 that impaired miRNA loading did not show a significant difference between wildtype and GW182-KD. These data implied the presence of GW182 in the AGO-miRNA complex is critical for loaded-miRNA stability, although GW182 is not required for the miRNA loading process (41,46). Previous studies have shown that Argonaute proteins can bind to miRNA through its MID and PAZ domains (15,105). In fact, several reports have investigated the interplay between AGO-miRNA and AGO-GW182 in the

RISC complex. For example, Eulalio et al. (41) and Miyoshi et al. (46) have systematically examined the different *Drosophila* Ago1 mutations/deletions for their ability to load miRNA and subsequent GW182 interaction. Their data indicated that GW182 and miRNA binding sites on AGO proteins are uncoupled, defining miRNA loading and AGO-GW182 interaction as two separate events. Our results confirmed previous results that F2V2, but not Ago2 or F2W2, showed an impaired miRNA loading. Since the knockdown of GW182 did not affect miRNA loaded onto AGO proteins, the reduced Ago2 and F2W2-bound miRNA from GW182-KD cell lysate IP may be due to a lack of GW182 protection from endogenous miRNase. Eulalio et al. also found that some *Drosophila* Ago1 mutations, such as F777A, showed no GW182 interaction in immunoprecipitation, but could still pull down miRNA Bantam. However, the investigators stated that, although these mutations did not interact with GW182 in IP, they exhibited a residual binding ability in vivo. Taken together, our data, along with previous reports, suggest that the absence of GW182 did not alter the miRNA loading onto AGO proteins or the ability of AGO to function in direct association with miRNAs, but may make them more accessible and vulnerable to ribonuclease.

Reduced miRNA in GW182-KD Cells Was Not Due To Enhanced Secretion into Extracellular Space

It has been noticed that many body fluids, including plasma, urine, milk, and saliva, contain miRNAs (108). These miRNAs are apparently resistant to nucleases, suggesting the existence of a protective mechanism (108). Exosomes were initially studied as a vehicle to exchange macromolecules, such as MHC molecules between cells in the immune system (82). Recent exosomes profilings reveal a broad spectrum of genetic materials including mRNA and miRNA that can be taken up by and become

functional in recipient cells (83). Secretory exosomes derive from endo-lysosomal compartments termed multivesicular bodies. Blocking multivesicular body formation compromises miRNA-mediated gene silencing linking miRNA activity with endosomal trafficking (84,109). In particular, GW182, but not Ago2, was found to enrich in exosomes, suggesting a possible alternative miRNA turnover pathway (84). Our data from cells with elevated endogenous miRNA confirmed the secretion of miRNA mainly via exosomes. These exosomal miRNAs play an important role in cell-to-cell communication by regulating recipient cellular activity (86). However, our data using miRNA expression plasmids showed a relatively low ratio between extracellular and intracellular miRNA. Therefore, the results suggest that the miRNA secretion via exosomes may not be a major miRNA turnover channel. The decreased stability of transfected miRNA in GW182-KD cells was not due to elevated secretion into extracellular space, but rather due to rapid endogenous degradation. Reduced secretion in GW182-KD cells is consistent with the notion that GW182 may play a role in miRNA secretion via exosomes. However, GW182 was not detected in the purified exosomes in our experiments to date. Further experiments are certainly required to elucidate whether there is a role for GW182, as well as other RISC components, in miRNA secretion.

Identification of Critical microRNase Affecting miRNA Stability in GW182-KD Cells

A few studies have reported variations in the stability of miRNAs in different cell types or conditions. For example, cardiac-specific miR-208 is highly stable (110), whereas miRNAs in retinal neurons decay much faster than in non-neuronal cells (78), likely caused by their tissue-specific activities. Cell cycle stage-specific miRNA expression patterns have also been investigated. miR-29b was generally unstable in all cell cycle stages but specifically accumulated during mitosis (75). In contrast, the miR-

16 family is accumulated when cells are arrested in G₀, but decreased rapidly during cell cycle re-entry (76). Some miRNAs, such as miR-141, but not miR-200c, that are transcribed in the same gene cluster, are very sensitive to cell density and cell-cell contact (77). Consensus sequences have been identified for mature miRNA degradation, implying some sequence-specific recognition (76,77). In our experiments, the stability of plasmid-derived miRNA and synthetic miRNA appeared universally reduced, implying a common mechanism of degradation pathway. However, additional regulation of the degradation of specific miRNA controlled by GW182 remained a viable question and identifying putative co-factors may help understand this process.

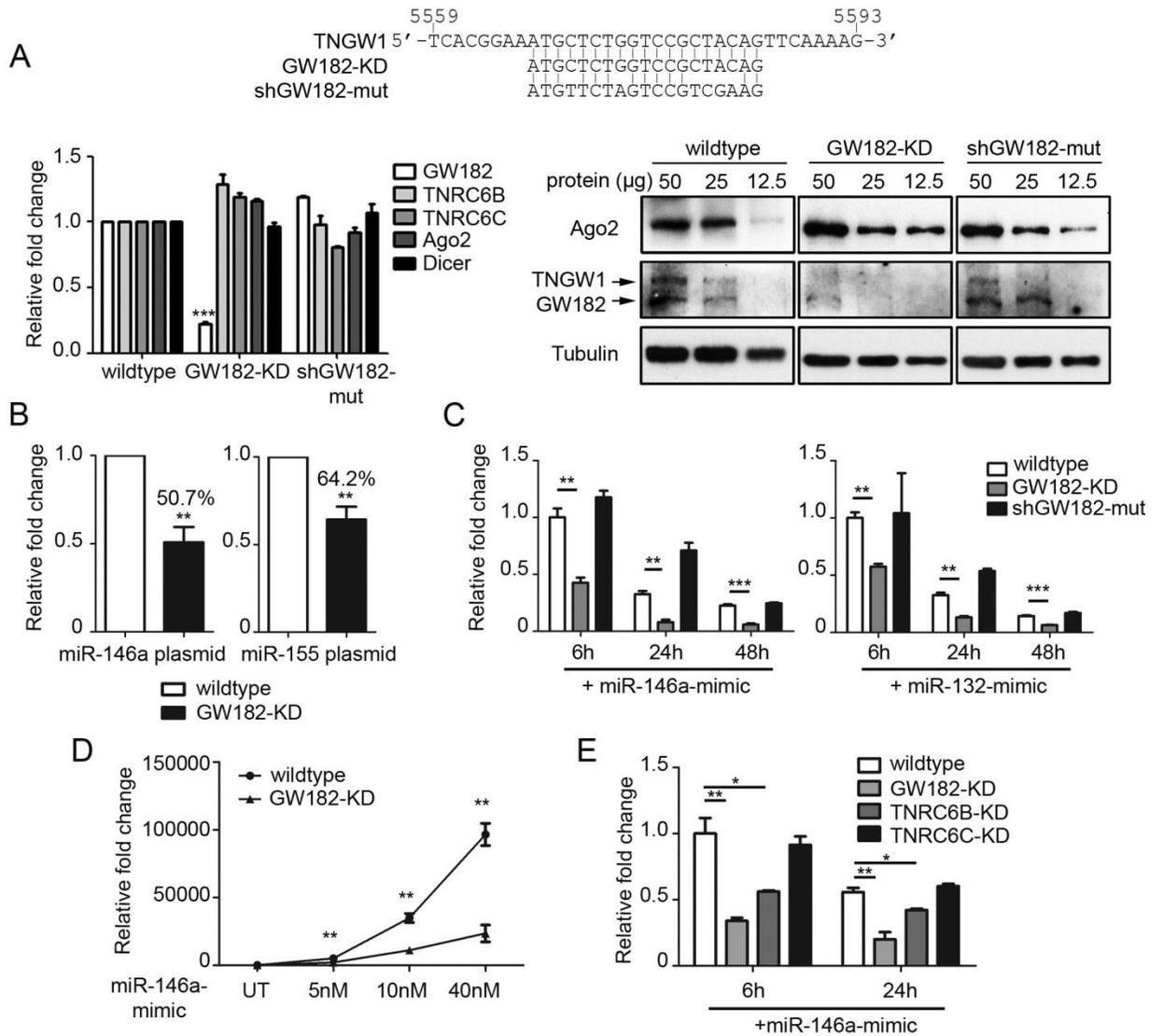
Important steps have been made to identify the ribonucleases that are responsible for miRNA degradation. A family of small RNA degrading nucleases in Arabidopsis can efficiently degrade mature miRNA in vitro as 3'-5' exonuclease (87). A screen of a nuclease panel has identified XRN-2 as the 5'-3' exonuclease that degrades mature miRNAs in *C. elegans* both in vitro and in vivo (88). Global transcription blocking has identified a few miRNA targets, such as miR-382, but they are not stable in HEK293 cells. Knocking down RRP41 results in significant and specific stabilization of miR-382 (79). Our data supported this observation. In wildtype 293 cells, neither knockdown of XRN1, XRN2, or RRP41 showed any significant elevation of transfected miR-146a levels. Remarkably, knockdown of RRP41, but not XRN1 or XRN2, in GW182-KD cells showed a significant elevation of the synthetic miR-146a level, suggesting that the RRP41-dependent 3'-5' exonuclease complex was involved in this process. Accordingly, we found that RRP41, but not XRN1 or XRN2, mRNA levels readily increased in GW182-KD cells compared to wildtype cells, implicating an associated

functional role. However, how the RRP41/exosome complex is recruited on site remains to be determined. Knockdown of RRP41 was only effective when GW182 was knocked down, indicating the role of GW182 in protecting AGO-bound miRNA. Interestingly, the levels of GW182 and GW/P bodies fluctuated during the cell cycle, suggesting the delicate control of GW182 level in a specific cellular state (6). In addition, the size and number of GW/P bodies increased when functional siRNAs were transfected (34). Although the changing levels of GW182 have not been examined in great detail, it is clear that the expression in GW182 is relatively low compared to other genes, such as Ago2. Thus a subtle change of GW182 may have a significant impact on miRNA stability.

In summary, our data suggest a model where GW182 interacts with Ago2 through its N-terminal AGO-binding domain and protects the 3' end of AGO-loaded miRNA from being degraded by a 3'-5' exoribonuclease complex (Figure 3-8).

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Figure 3-1. Knockdown of GW182 and TNRC6B impaired miRNA stability. A) Lentiviral shRNA effectively and specifically knockdown GW182. ShRNA for GW182/TNGW1 (5567-5585nt) cloned into a lentiviral expression vector for stable knockdown in HEK293 cells (GW182-KD). A negative control lentiviral construct was also generated with mismatched mutations (shGW182-mut) based on the same strategy. Lower left panel, GW182-KD effectively and specifically knockdown GW182 without significantly affecting its paralogs TNRC6B, TNRC6C, its interaction partner Ago2, and Dicer mRNA as determined by qRT-PCR. The two stable HEK293 cell lines were therefore referred as GW182-KD and shGW182-mut cells. Lower right panel, total cell lysate from wildtype HEK293 and two stable cell lines were loaded at 50, 25 and 12.5 μ g total protein. Western blot showed GW182-KD, but not shGW182-mut, was specifically depleted of both GW182 and TNGW1 proteins. Ago2 protein levels were elevated in both stable cells comparing to parental HEK293 cells. B) Level of transfected miR-146a and miR-155 reduced in GW182-KD. Plasmid encoding pre-miR-146a and pre-miR-155 were transfected in both wildtype HEK293 and GW182-KD cells. qPCR demonstrated that mature miR-146a and miR-155 level were reduced to 35.9% and 74.8%, respectively, in GW182-KD cells compared to wildtype which was normalized to 1. C) Transfected miRNA levels reduced in GW182-KD. Synthetic miR-146a- and miR-132-mimic were transfected into wildtype, GW182-KD, and shGW182-mut cells for 6, 24, and 48 h. The miRNA levels in wildtype with 6 hour mimic transfection were normalized to 1. D) Transfected miR-146a after 24 hours showed more rapid elimination in 5nM, 10nM and 40nM transfection concentrations in GW182-KD cells compared to wildtype. E) Stability of transfected miRNA was affected most in GW182-KD and TNRC6B-KD, but not in TNRC6C-KD cells. Lentiviral shRNA against TNRC6B and TNRC6C were transduced into 293 cells to generate TNRC6B and 6C stable knockdown cells (6B- and 6C- KD). miR-146a-mimic was transfected into wildtype, GW182-, 6B- and 6C-KD cells. Knockdown of TNRC6B also affected miR-146a stability after 6 hours and 24 hours transfection although the effect was weaker than in GW182-KD cells. TNRC6C knockdown did not affect transfected miR-146a stability. The miRNA levels in wildtype with 6 hour mimic transfection were normalized to 1. miRNA results are expressed as mean \pm S.D. from three independent experiments. *t*-test was used for statistics. *, $p < 0.05$; **, $0.01 < p < 0.05$; ***, $p < 0.01$.



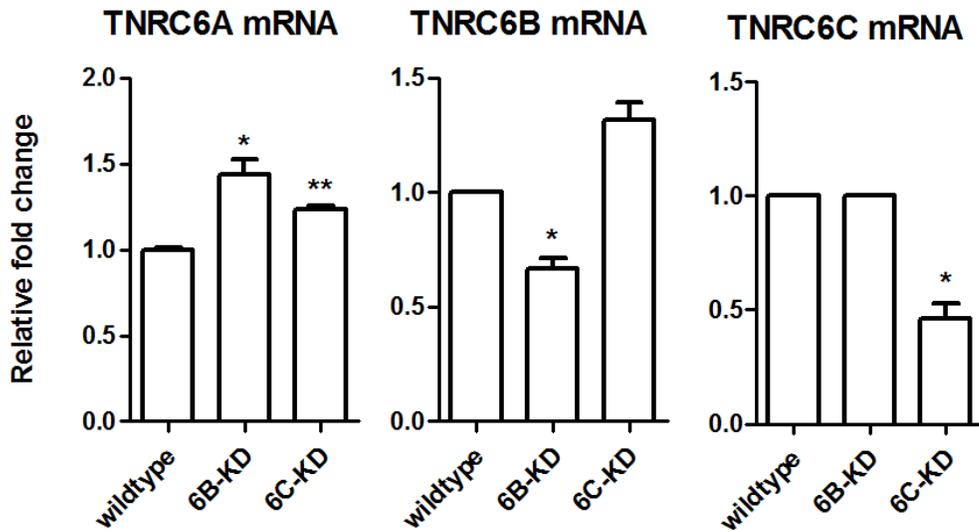
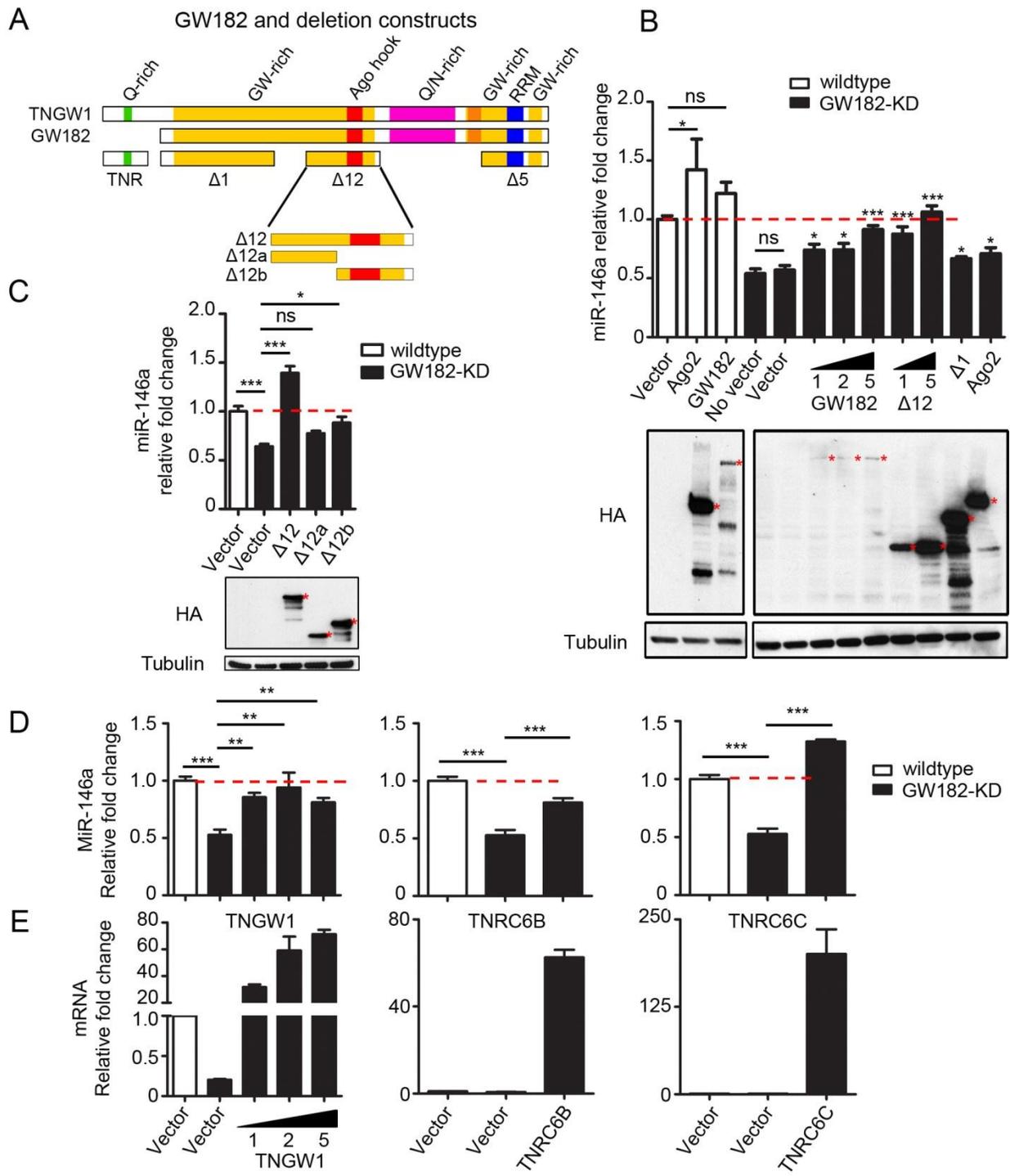
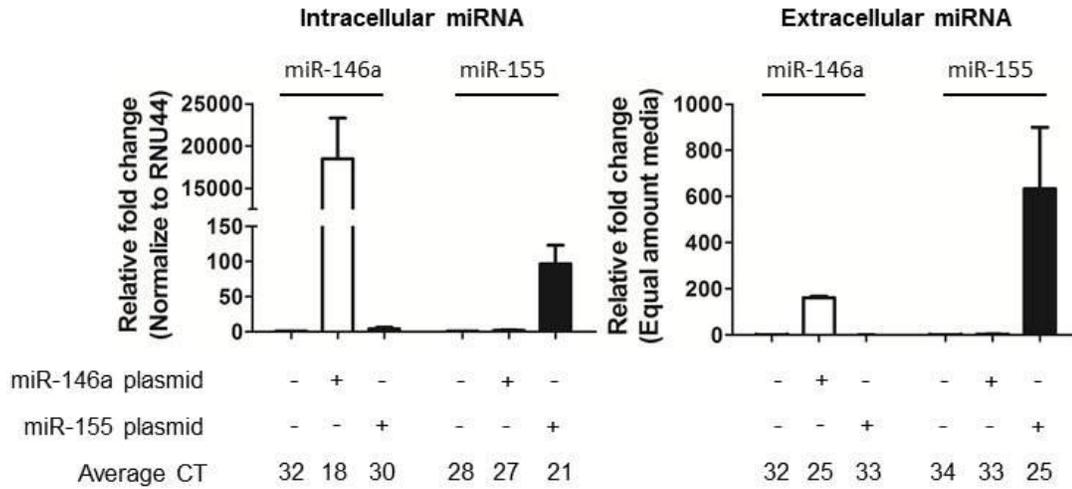


Figure 3-2. TNRC6B and TNRC6C specifically knocked down by lentiviral shRNA. TNRC6B and TNRC6C were knocked down 33.4% and 53.6% in 6B-KD and 6C-KD cells, respectively. Interestingly, TNRC6A/GW182 mRNA levels were significantly elevated in both 6B- and 6C-KD cells (43.7%, $p < 0.05$ and 23.5%, $p < 0.01$, respectively).

Figure 3-3. Replenishment of GW182 and its paralogs restored the shortened miRNA stability. A) Schematic map showed TNGW1, GW182 and their truncated domains. B) Re-expression of GW182 and its silencing domain $\Delta 12$ in GW182-KD cells significantly restored miRNA level. Wildtype 293 cells were nucleofected with NHA vector alone, NHA-tagged Ago2 or GW182. NHA-GW182 full length mutations on shGW182 binding sites (GW182) was nucleofected into shGW182-KD cells in three titrations (1, 2, and 5 μg). NHA- $\Delta 12$ ($\Delta 12$) was transfected in two titrations (1 and 5 μg). Other constructs including $\Delta 1$ and Ago2 were also nucleofected in 5 μg . miR-146a-mimic was subsequently transfected 16 hours post nucleofection and cells were harvested 6 hours later. The synthetic miR-146a level in GW182-KD cells was significantly restored to comparable to that in wildtype cells (open bar) with re-expression of GW182 and $\Delta 12$ in dose dependent manner. Another GW182 fragment $\Delta 1$ and NHA-Ago2 only had mild effects in GW182-KD cells compared to vector alone, which did not show significant effects and served as negative control. Expression of transfected proteins were monitored by blotted with anti-HA antibody. miR-146a level from wildtype cells with vector transfection was normalized as 1. C) Fragment of $\Delta 12$ rendered less effective in complementation assay. D) Re-expression of GW182 longer isoform TNGW1, TNRC6B, or TNRC6C in GW182-KD cells significantly restored miRNA level. Wildtype 293 cells were nucleofected with NHA vector alone. GW182-KD cell were nucleofected with NHA- TNGW1 with mutations on shGW182 binding sites in three concentrations (1,2 and 5 μg), or GFP-tagged TNRC6B, TNRC6C in 5 μg concentration. miR-146a-mimic was subsequently transfected 16 hours post nucleofection and cells were harvested 6 hours later. miR-146a level from wildtype cells with vector transfection was normalized as 1. E) mRNA of transfected TNGW1, TNRC6B AND TNRC6C were measured by qRT-PCR.



A



B

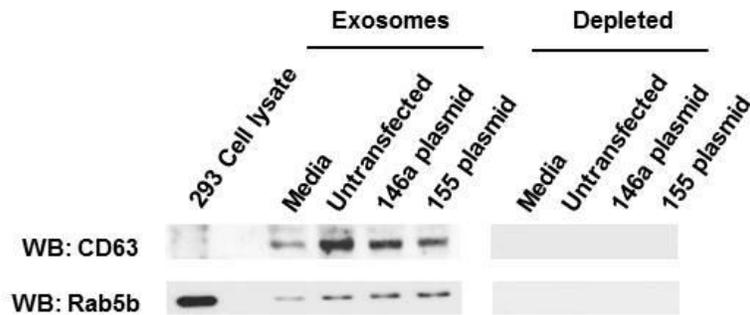


Figure 3-4. miRNAs were secreted into extracellular space via exosomes. A) miRNA secreted into culture media in 293 cells. Plasmids encoding miR-146a and miR-155 were transfected in wildtype 293 cells for 24 hours. Cells were pelleted and culture media undergone a series of differential centrifugation to remove cell debris. Both intracellular and extracellular miRNAs fold changes were measured by qPCR relative to untransfected controls. Average CT values are shown for intracellular miRNA in 25 ng total RNA or equivalent of extracellular miRNA in 10 μ l culture media. B) Purified exosomes from 293 cell culture media with or without plasmid transfection was lysed and blotted for two exosomes marker proteins CD63 and Rab5b to confirm a successful enrichment of exosomes. Whole cell lysate of 293 cells was loaded as positive control.

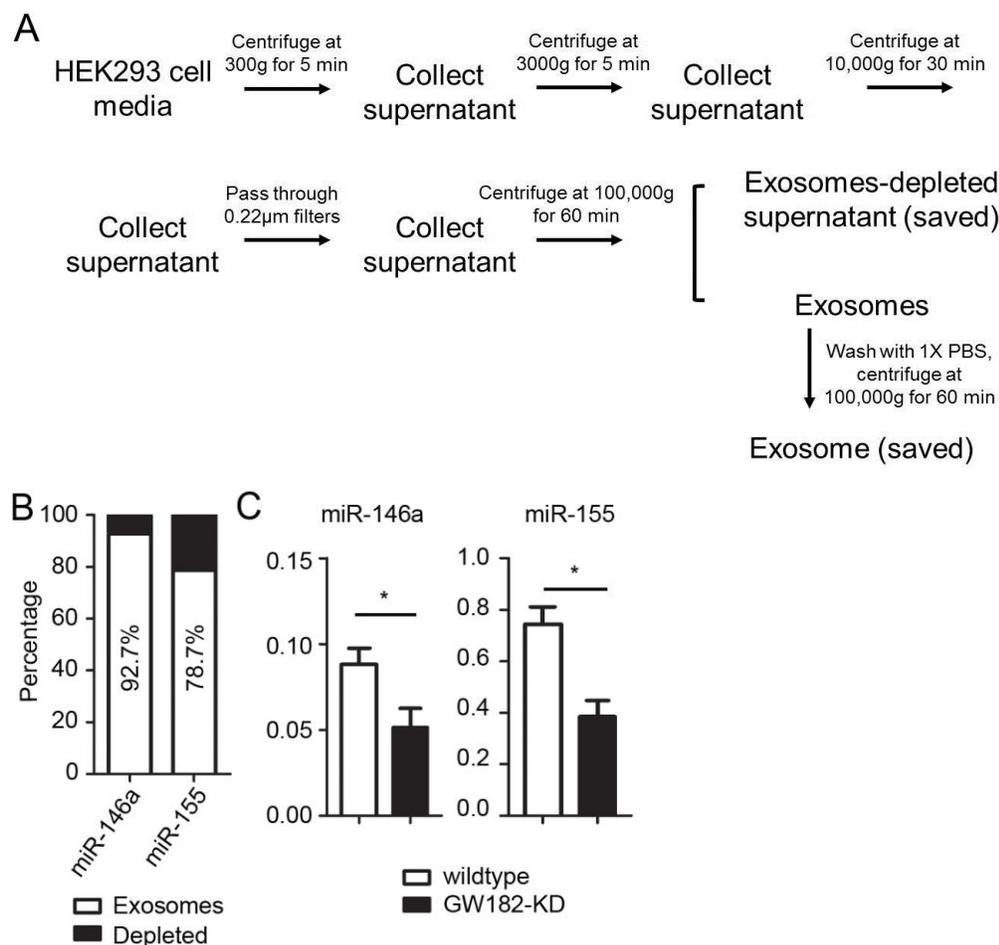
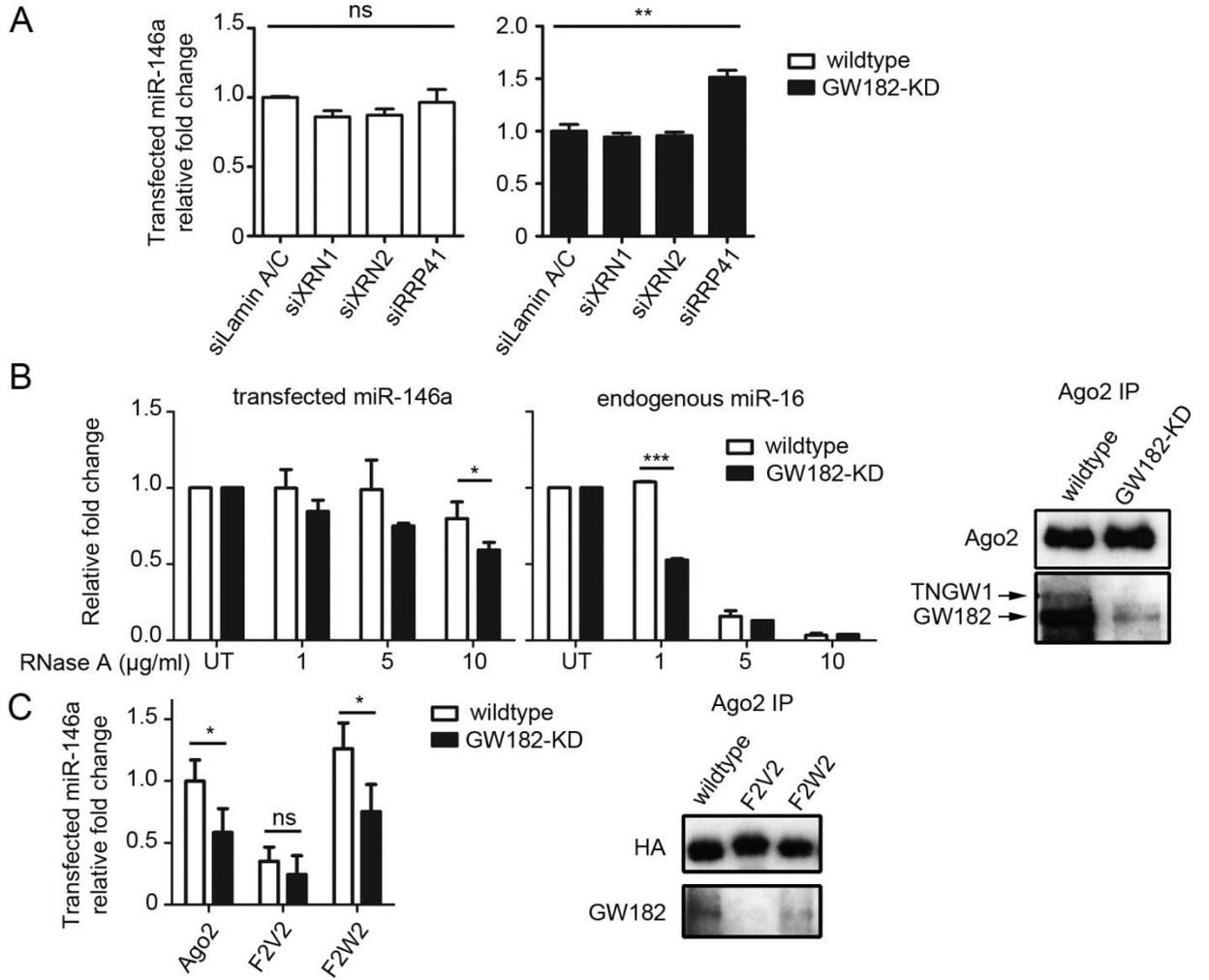


Figure 3-5. Knockdown of GW182 reduced miRNA secretion via exosomes. A) Exosomes purification strategy. B) Majority of secreted miR-146a and miR-155 was co-purified with secretory exosomes. Exosomes were purified by ultracentrifugation from cell culture media. Total RNA was extracted from exosomes fraction and supernatant that was depleted of exosomes. C) Knockdown of GW182 reduced miRNA secretion. Wildtype and GW182-KD cells were transfected with miR-146a and miR-155 expression plasmids. Both whole cells and culture media were harvested 24 hours post-transfection and subjected to total RNA extraction and qPCR. Knockdown of GW182 reduced miR-146a and miR-155 ratio outside/inside cells suggesting an intrinsic mature miRNA degradation mechanism. Two or five microgram of plasmids were transfected in both cells independently for accurate measurements. Three independent data sets were acquired and pooled for statistical analysis.

Figure 3-6. GW182 protected loaded miRNA decay by interacting with Argonaute proteins. A) Exoribonuclease complex was responsible for miRNA degradation in GW182-KD cells. siRNA against 5'-3' exoribonuclease XRN1, XRN2 and 3'-5' exoribonuclease complex component RRP41 was transfected into wildtype and GW182-KD cells. Forty-eight hours after transfection, miR-146a-mimic was transfected to evaluate its stability. MiR-146a level showed no significant difference in wildtype cells when any of the three nucleases was knocked down compared to control siRNA for lamin A/C. However, miR-146a level was significantly extended to 51.4% more in GW182-KD cells only when RRP41 was knockdown. B) miRNA prone to degradation without GW182 in Ago-RISC complex *in vitro*. Ago2 immunoprecipitation was performed using lysates from both wildtype and GW182-KD cell transfected with miR-146a-mimic. After three washing, the beads were aliquoted equally into five tubes. Four aliquots were subjected to either untreated (UT), or incubated 1, 5, or 10 $\mu\text{g/mL}$ RNase A at 37 °C for 30 min. All four beads associated total RNA was extracted and qRT-PCR was performed to evaluate the residual transfected miR-146a and endogenous miR-16 level. The remaining aliquot analyzed by Western blot for GW182 and Ago2 levels. C) Argonaute constructs showed decrease miRNA association in the absence of GW182. miRNA associated with wildtype NHA-Ago2 and two conserved phenylalanines (Phe470 and Phe505) mutation constructs to valines (F2V2, poor miRNA loading GW182 binding) and tryptophans (F2W2, retained miRNA loading and GW182 binding) were compared in HA-pull down assays using both wildtype and GW182-KD cell lysate. RNAs from pull down assays were subjected to qRT-PCR. miRNA levels recovered from HA pull down using both wildtype and GW182-KD cell lysates showed strong dependence on GW182 interaction. Ago2 wildtype, F2V2 and F2W2 pull down were blotted by HA and GW182 antibodies.



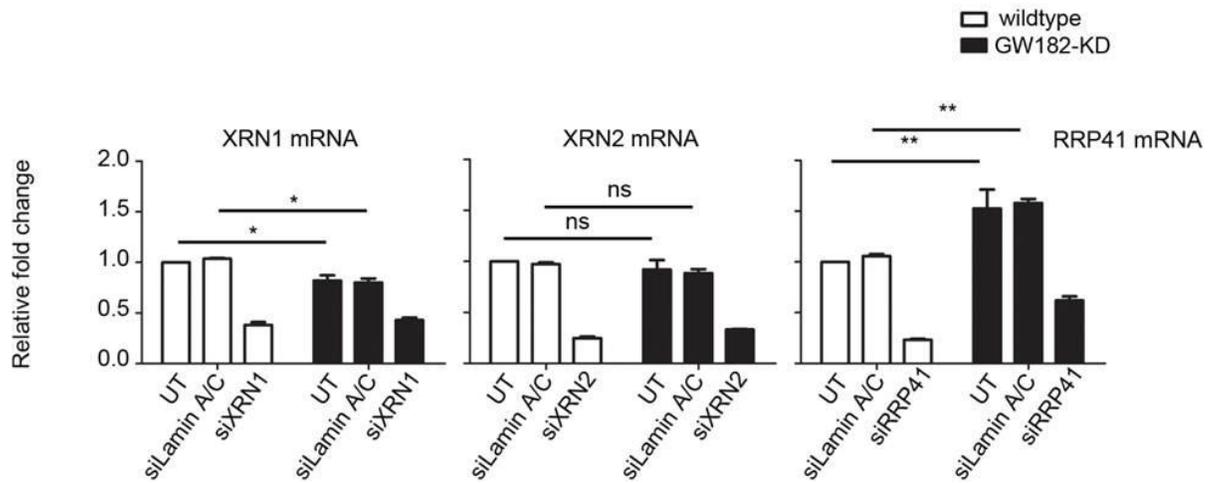


Figure 3-7. siRNA effectively knocked down exoribonucleases. 100nM of each siRNA against XRN1, XRN2 or RRP41 were transfected into wildtype and GW182-KD cells side-by-side with control siRNA to lamin A/C. Forty-eight hours after transfections, cells were harvested and subjected to RNA extraction and qRT-PCR. XRN1, XRN2 and RRP41 mRNA was reduced to 38%, 24%, and 23% in wildtype cells, respectively. The comparison of mRNA level between wildtype and GW182-KD cells revealed that only RRP41 mRNA in GW182-KD cells significantly increased by 52.6% compared to wildtype.

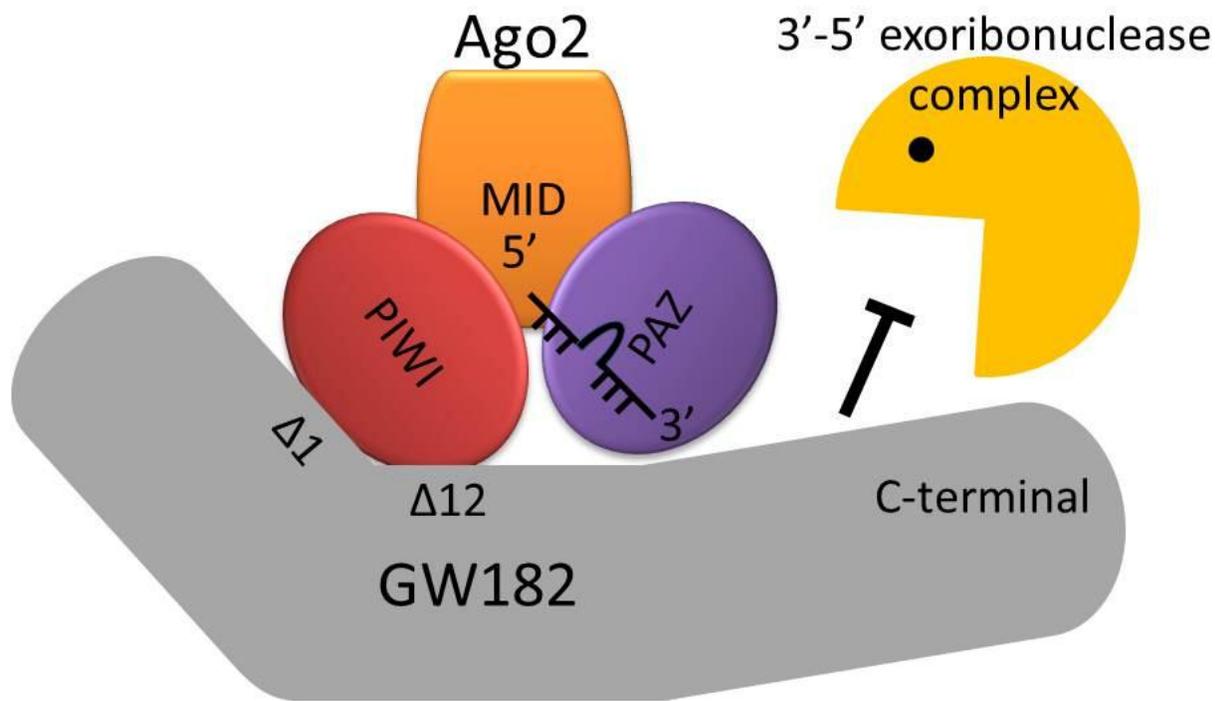


Figure 3-8. A model of GW182 binding to AGO and protected miRNA from being degraded by 3'-5' exoribonuclease. GW182 binds to AGO primarily through its N-terminal region including $\Delta 1$ and $\Delta 12$. This interaction may protect miRNA from being degraded by 3'-5' exoribonuclease complex. (Proteins are not drawn in scale).

Table 3-1. List of primers for lentiviral shRNA knockdown

Targets	Sequence	Length
GW182 (TNRC6A)	Forward: 5'-3' GATCCCGTGAGCGATGCTCTGGTCCGCTACAGTAGTGA AGCCACAGATGTACTGTAGCGGACCAGAGCATTGCCTAT TTTTTGGAAA	87nt
	Reverse: 5'-3' CGCGTTTCCAAAAAATAGGCAATGCTCTGGTCCGCTACA GTACATCTGTGGCTTCACTACTGTAGCGGACCAGAGCAT CGCTCACGG	87nt
GW182 mutant (Negative Control)	Forward: 5'-3' GATCCCGTGAGCGATGTTCTAGTCCGTCGTAGTAGTGAA GCCACAGATGTACTACGACGGACTAGAACATTGCCTATT TTTTTGGAAA	87nt
	Reverse: 5'-3' CGCGTTTCCAAAAAATAGGCAATGTTCTAGTCCGTCGTA GTACATCTGTGGCTTCACTACTACGACGGACTAGAACAT CGCTCACGG	87nt
TNRC6B	Forward: 5'-3' GATCCCGTGAGCGGGAGTGCCATGGAAAGGTATAGTGA AGCCACAGATGTATACCTTTCCATGGCACTCCTGCCTAT TTTTTGGAAA	87nt
	Reverse: 5'-3' CGCGTTTCCAAAAAATAGGCAGGAGTGCCATGGAAAGG TATACATCTGTGGCTTCACTATACCTTTCCATGGCACTCC CGCTCACGG	87nt
TNRC6C	Forward: 5'-3' GATCCCGTGAGCGGCATTAAGTGCTAAACAAATAGTGAA GCCACAGATGTATTTGTTTAGCACTTAATGCTGCCTATTT TTTGGAAA	87nt
	Reverse: 5'-3' CGCGTTTCCAAAAAATAGGCAGCATTAAAGTGCTAAACAA ATACATCTGTGGCTTCACTATTTGTTTAGCACTTAATGCC GCTCACGG	87nt

CHAPTER 4
SUMMARY AND PROSPECTIVE

GW182 Plays a Key Role in miRNA Gene Silencing

Role of AGO and GW182 in miRNA Pathway

The first miRNA was described in *C. elegans* only two decades ago (39) but now miRNA have quickly become one of the major post-transcriptional gene regulation pathway (17). Several viable models have been proposed to illustrate the miRNA mediated silencing mechanism, namely interfering with various steps in translation and triggering mRNA decay (2,5). Either via translational repression or mRNA decay, miRNA-associated protein complex (or RNA-induced silencing complex, RISC) played a central role. Numerous efforts have been put to identify individual proteins in RISC, among which two most important protein families emerge – Argonaute proteins (AGOs) and GW182 proteins. They form tight complex by directly interacting with each other through GW/WG motifs on GW182 to the C-terminal PIWI domain on AGOs (21,41,60,92) and this interaction turns out to be critical in miRNA silencing function (20,35,54,58,61). Crystal structure of *T. thermophiles* AGOs (105,111) and human Ago2 (112) confirms AGO proteins directly associate with miRNAs through its MID and PAZ domains. All four mammalian AGO proteins are thought to play an equal role in miRNA-mediated gene silencing due to their association with similar miRNA pool (106), although only Ago2 possessed endonucleolytic activity in its C-terminal PIWI domain in siRNA pathway to directly cut target mRNA (16). In addition, AGO protein can also interact with Dicer, in a GW182 mutual exclusive manner (46). The Dicer processed mature miRNA duplex could then undergo unwinding step, and only guiding strand could be loaded onto AGO proteins with the help of Dicer complex (113-115). Other

studies show the miRNA:miRNA* duplex could be loaded onto AGO proteins, and the separation of the two strand occurs within AGO proteins (116). The Dicer-AGO complex is also termed as miRNA loading complex (miRLC). In some rare cases, such as miR-451, its maturation is independent of Dicer but requires Ago2 cleavage activity (117,118). Nonetheless, the miRLC dissociated after miRNA unwinding and loading, and AGO then bound to GW182 to form the mature functional miRNA-RISC complex (miRISC). Tethering functional assays offered an unique opportunity to individually examine the silencing potential of each RISC components (47), and GW182 is clearly highlighted as key repressors downstream of AGO proteins (35,41,46). Therefore, it is now well established that AGO proteins play important roles in miRNA maturation, unwinding and loading, whereas GW182 proteins are the actually silencer, in collaboration with other co-factors, triggered translational repression and mRNA degradation (discussed in next section). In fact, deplete both Ago2 and GW182 has been demonstrated to cause severe consequences. Ago2 knockout mice showed embryonic lethality and display several developmental abnormalities (16). Disruption of GW182 in mouse yolk sac led to growth arrest and apoptosis (119). These data confirm the critical role of miRNA and its functional RISC in maintaining normal cellular activities.

GW182 has been shown to bind multiple AGO-miRNA complexes (19-21,41). Studies have shown that closely spaced miRNA target sites often act synergistically and result in stronger repression than those separated by greater distances (62). These observations raise a possibility that multiple AGOs-miRNA complexes anchor to their target mRNA and share one GW182 as their repressor. This may lock GW182 more

tightly onto mRNA with multiple AGO-miRNA-mRNA bindings to further recruit its co-factors for silencing. However, one study showed that GW182 was not permanently associated with mRNA in their pull down experiments (57). Instead, they showed GW182 was actively removed from mRNA by NOT1 complex. Depletion of NOT1 resulted in the retention of GW182 on mRNA (57). This observation led these investigators to conclude that GW182 was required to initiate, but not to maintain, silencing and GW182 dissociated from silenced mRNAs after deadenylation (57). Clearly, more work needs to be done to further confirm the details in this process. For example, in vitro experiments may be set up to determine the hierarchical assembly of RISC components and their dynamics in silencing miRNA targets. Alternatively, live cell imaging can be applied to track the subcellular localization and movement of GW182 and GWBs. In fact, our preliminary data suggest the involvement of cytoskeleton such as actin in this process.

Dual GW182 Silencing Domains Independently Silence miRNA Targets

Although it is now a general consensus that GW182 is the key repressor, some discrepant results remain from various laboratories in establishing its silencing domains. The C-terminal domain of GW182 is widely accepted in possessing autonomous silencing activities defined by bipartite domains flanking the RNA Recognition Motif (RRM, Figure 2-14) (18,19,22,24,41). Our data support this notion since GW182 C-terminal $\Delta 5$ showed strong silencing activity in tethering assays. The silencing activity on the N-terminal and the middle region of GW182 was under debate. Our data from tethering assays together with other groups clearly supported the GW182 middle domain $\Delta 12$ are effective in translational repression or mRNA deadenylation (22,24,53,93). An immediate question therefore needs to be addressed is the need for

GW182 to have multiple silencing domains and how these domains function together in the full-length GW182/TNGW1.

Both transfected $\Delta 12$ and $\Delta 5$ have higher expression levels compared to full-length GW182/TNGW1 in side-by-side transfections yet their repression abilities in tethering assays are comparable. These observations suggest the integrity of the full-length GW182 evolves into such form to achieve maximum silencing effects. Interestingly, when $\Delta 12$ is expressed as fragments $\Delta 12a$ and $\Delta 12b$, which contain about equal distribution of GW/WG motifs, both truncated constructs have significant reductions in repression activity compared to $\Delta 12$. A very recent study systemically examined the role of tryptophans on GW182 N-terminal and C-terminal domains for silencing activity. Their data revealed tryptophans dispersed across both domains and act in an additive manner by recruiting deadenylase complex to trigger both translation repression and mRNA degradation (28). In our immunoprecipitation assay, both $\Delta 12$ and $\Delta 5$ can pull down Poly-A binding protein (PABPC1), suggesting the functional redundancy of different GW182 silencing domains. On the other hand, distinctive functionalities were also clearly observed in between $\Delta 12$ and $\Delta 5$ and summarized in Figure 2-14, including their differential ability for AGO interaction, dominant-negative effects, and even subcellular localization. These differences may define diverse GW182 functions as discussed below. Collectively, GW182 silencing domains worked independently for silencing mRNA targets. The GW182 wildtype protein possesses maximum silencing activity, likely conveyed by its unique tryptophan motifs. My dissertation work for the very first time identified two non-overlapping silencing domains on human GW182 with distinct characters. My research, together with others, leads to

the conclusion that multiple GW182 domains can recruit components of deadenylase by conserved tryptophan residues to inhibit translation and cause mRNA destabilization (28).

The Current Knowledge of GW182 in miRNA-Mediated Translational Repression and miRNA Degradation

miRNAs bind primarily to the 3'UTR of their target mRNA, and mediate translational repression and/or mRNA decay, although the detailed molecular mechanism for translational repression is still not completely understood. Several models have been proposed indicating that translation repression is achieved by either inhibition of translational initiation, elongation, termination or mRNA deadenylation (2,13). Contradiction remains that some investigators believe the translation repression only occurs on the initiation stage or elongation stage but others think it might occur on multiple steps simultaneously.

As discussed in Chapter 2, a recent report utilizing in vitro translation extracts from mouse Krebs-2 ascites cells showed miRNA-mediated deadenylation occurred 1-2 hours after initial translational inhibition (25). These investigators and others also showed that the C-terminal domain of GW182 bound to deadenylases through the DUF (domain of unknown function) conserved domain located between the QN-rich domain and the RRM domain (25,26) (now known as PAM2 domains (40), Figure 2-14) and recruited deadenylases to the target mRNA. It is well established that the mRNA 5' cap and 3' poly-A tail can be brought into close proximity by the interaction between cap binding protein eIF4G to the PABPC1. This looped structure is in favor to recruit ribosomes for active translation (5). One model argues that the newly identified GW182-PABP interaction could competitively disrupt PABP-eIF4G interaction, loosen the

circularized mRNA structure to impair translation. In addition, GW182-PABP interaction may facilitate the dissociation of PABP from poly-A tail to directly expose unprotected poly-A tail from the deadenylase (5,40). In fact, three recent studies simultaneously report GW182 can directly interact and recruit the CCR4-NOT deadenylase complex through its C-terminal domain containing conserved tryptophan in adjacent to glycine/serine/threonine-rich region; they also show that the formation of the GW182-deadenylase complex is independent of PABP (27-29). This model, however, has been subsequently challenged by Fukaya and Tomari et al. (93). These authors utilized in vitro *Drosophila* embryo system to demonstrate neither PABP function nor GW182-PABP interaction was prerequisite for shortening of poly-A tail and pure translational repression (93). The absolute requirement of PABP in miRNA-mediated gene silencing especially deadenylation process waits to be determined.

Another important unanswered question is the temporal hierarchy and dynamics between translational repression versus mRNA deadenylation and decay. In some occasions, pure translation repression is observed with little effect on mRNA stability (24,120). On the other hand, global miRNA-induced mRNA decay is proposed to be predominant for miRNA silencing effects (121). Recent progress has been made to dissect these events using zebrafish (122,123) and *Drosophila* S2 cells (124). These publications reach an agreement that translational repression and mRNA decay are independent events. Translational repression usually occurs in the early stage of repression before complete deadenylation while mRNA decay takes place in the late stage. These observations are consistent with previous in vitro experiments that translation repression occurs in the early stage before complete deadenylation in mouse

Krebs-2 ascites cells (25). Interestingly, Mishima et al. further suggested that translational repression in zebrafish by GW182 can be divided into two distinct mechanisms. One is previously defined interaction between GW182 PAM2 domain and PABP, as mutations on PAM2 domain that abolish the GW182-PABP interaction partially impaired translation repression. In addition, these investigators are able to identify another domain downstream of PAM2 domain that contains new “PPPGL” motif (P-GL domain). Mutations on P-GL domains also impair GW182-mediated translational repression without affecting GW182-PABP interaction. This clearly suggests a PABP-independent silencing mechanism. Notably, combined mutations on PAM2 and P-GL domains show additive effects in impairing silencing activity suggests multiple mechanisms are involved in miRNA mediated translation repression (123). A figure from Mishima et al. is shown to better illustrate the PAM2 and P-GL domains on GW182 (Figure 4-1).

GW182 protein family is composed of three paralogs in mammals, gene named TNRC6A, B and C and they seems to have equal silencing potentials (18,19,22,35,41). Knocking down one of the three paralogs partially de-repressed silenced targets indicating their functional redundancy (22). Tethering experiments showed comparable results among all three paralogs (18-20,22,41). Their functional differences have not been explored. Intriguingly, TNRC6C seemed to be dispensable as HeLa cells express ten-fold less compared to HEK293 cells (24). Our data indicated that knockdown of TNRC6C did not significantly impair miRNA-mimic stability, implying functional difference of GW182 paralogs. However, all three paralogs can complement the

reduced miRNA stability when GW182 was knocked down once again showed that they are functional redundant.

Although more details need to be further investigated on miRNA-mediated translational repression versus mRNA destabilization, the current data taken together strongly suggest a pivotal role of GW182 in miRNA-mediated gene silencing.

GW182 May Play a Role in miRNA Stability by Directly Interacting with AGOs **MiRNA Levels Are Tightly Regulated in Multiple Steps of Their Biogenesis**

miRNAs are commonly transcribed from endogenous genomic loci and their biogenesis comprises multiple steps, including the processing of primary miRNAs (pri-miRNAs) by Drosha in the nucleus and precursor miRNAs (pre-miRNAs) by Dicer in the cytoplasm to become mature miRNAs. The guiding strands then directly associate with Argonaute (AGO) proteins, core components of the RISC, serving as address labels to guide RISC to downregulate their target mRNAs by imperfect sequence matches (2,4). Maintaining proper cellular level of miRNA is fundamental as aberrant expression of certain miRNAs has been described in many diseases, including cancer (73). Multiple regulations have been described on each step of miRNA biogenesis. For example, miRNA can be regulated on the transcriptional level similar as other protein coding genes. Given the ability of miRNA to regulate gene expression in a post-transcriptional manner, negative feedback loop is often observed (4). In a model of endotoxin tolerance, for instance, miR-146a and many cytokine transcripts such as TNF- α and IL-1 β are co-regulated by transcriptional factor NF- κ B. The elevated miR-146a then in turn down-regulates adaptor molecule IRAK1 and TRAF6 on NF- κ B signaling pathway to prevent overproduction of cytokines (125,126).

Since two RNase III type endonucleases Drosha and Dicer can process primary and precursor miRNAs, many regulations are also occurring on these steps by altering their protein levels, their enzymatic activities and specificities, their binding partners or even directly altering the base-pairing, to affect the mature miRNA outcomes (4).

Comparing to these advances, however, our knowledge of how mature miRNAs are regulated is lagging behind. Mature miRNA dynamic changes have been described in previous studies (Discussion, Chapter 3). The detailed mature miRNA turnover mechanism as well as the role of RISC components in this process remains unclear.

Deep sequencing data has now suggested a large pool of mature miRNA undergoing modifications that could potentially regulate their stability as well as their silencing potentials. These modifications included adenylation, uridylation, and 2'-O-methylation catalyzed by unique enzymes in plants and animals (4,74,127). Efforts have also been put to identify the ribonucleases that are responsible for miRNA degradation. Chen and colleagues first identified a class of enzymes defined as small RNA degrading nuclease (SDN) genes in Arabidopsis that could trigger miRNA decay from 3' to 5' end (87). Grosshans and co-workers discovered another ribonuclease XRN-2 that catalyzed the degradation of miRNA from 5' to 3' in *C. elegans* (88). A very recent study showed two exoribonucleases, exosome complex and QIP (a putative exonuclease identified in fungi (128)), are important for fungi small RNAs maturation and degradation in fungi (129). This is consistent with our data and others (79) using HEK293 cells that identified exosome complex as the 3'-5' "microRNase" (89).

RISC Components in Regulating Mature miRNA Stability

AGO proteins have long been shown to correlate with the mature miRNA level, possibly by direct association and protection (80). Recent progress confirms this

observation by showing AGO proteins directly stabilize miRNA and overexpression of wildtype AGO proteins or even endonucleolytic cleavage-inactive AGOs decelerate miRNA decay (81). However, are other RISC components also involved in regulating miRNA stability?

In Chapter 3, knockdown of GW182 did not impair endogenous Ago2 mRNA and protein level but reduced transfected miRNA-mimic stability. Exosome complex was responsible for reduced miRNA level only when endogenous GW182 was depleted. In vitro experiments also suggested miRNAs were more vulnerable to nuclease treatment in the absence of GW182. These observations for the very first time offered more insight of GW182 in protecting loaded miRNA by directly interacting with AGO proteins.

Why AGO proteins need GW182 to further protect their bound miRNA from being degraded by exoribonuclease? One viable model is that the miRNA maturation, unwinding, and loading process require the help of some exoribonucleases such as exosome complex, as observed in fungi (129). After the formation of complete miRISC, cells may then need to ensure functional miRNA to be protected. On the other hand, miRNA-AGO without binding GW182 may be treated as “defective” RISC and are being triggered for decay by exosome complex to help recycle AGO proteins as they have been proposed as “speed limiting” factors for miRNA silencing (80). A model of AGO-GW182 protecting miRNA from exoribonucleases was included in Chapter 3 (Figure 3-8).

Role of GW182 in miRNA Secretion via Secretory Vesicle Exosomes

It has been noticed that a wide spectrum of miRNAs exists in a variety of body fluid including serum, plasma, saliva, urine, and milk (108,130). The escape from being degraded by ribonucleases in these body fluids suggests certain protective mechanisms

for these miRNA. There are two current interpretations: i) miRNAs and other nucleic acids such as mRNA are packed into membrane bound secretory vesicles such as exosomes and actively secreted into extracellular space (83,131); ii) miRNAs actively secret in a vesicle-free manner but are protected by their associated proteins such as Ago2 or high-density lipoproteins (HDL) (132,133). Nonetheless, RISC components such as Ago2 and GW182 are inevitably involved in these two processes as they form complex with mature miRNAs. These secretory miRNAs have been shown to serve an important role in "wireless" cell-cell communication by regulating recipient cells gene expression (82,130,134,135).

Secretory vesicles include exosomes, microvesicles, ectosomes, membrane particles, exosome-like vesicles, and apoptotic vesicles that are defined by their difference in physicochemical characteristics (82). Among these vesicles, exosomes are 50-100 nm in diameter derived from endosome. It has long been thought to help promote antigen presentation between immune cells (136). Recent studies show exosomes are shed from almost all kinds of cells, including cancer cells, and they carried genetic materials, including mRNA and miRNA, that can alter recipient cell gene expression (83,85,131,137). These studies also show that exosomes containing miRNAs are not randomly packed but rather undergone a selective process through an unknown mechanism (130). The formation of exosomes relies on the endosomal sorting complex required for transport (ESCRT) complexes (138,139). Depletion of ESCRT impaired miRNA activity in both *Drosophila* and human cells, indicating the functional RISC were coupled to multivesicular bodies that can later form exosomes (84,109). Interestingly, Voinnet and colleagues showed a strong enrichment of GW182, but not

Ago2, in purified exosomes (84) whereas others demonstrated Ago2, but not GW182, was co-purified with exosomes (86). We have not been successful in detecting GW182 or Ago2 in purified exosomes following an established protocol (101). However, in Chapter 3, stable knockdown of GW182 in HEK293 cells showed reduced miRNA secretion via exosomes, suggesting GW182 may indeed regulate miRNA selection and secretion through exosomes.

A couple of reports have raised another possibility that miRNAs can be secreted in a vesicle-independent manner but protected by their bound proteins such as Ago2 or HDL proteins (132,133). In our results, we did observe a fraction of miRNA existing in exosomes-depleted soluble fraction. Although detailed protein components associated with vesicle-free miRNAs are still not clear, it is likely that GW182 helps Ago2 protecting vesicle-free miRNA. Future work will be needed to elucidate the protein identity and the functional difference between exosomes carrying and vesicle-free miRNAs. It has not been very clear how exosomes can specifically target recipient cells and how the exosomal miRNAs are released and become functional in recipient cells. A prospective is provided below to address the advantages and limitations of current studies on exosomes.

Prospective

In my dissertation study, I have shown that GW182 plays a critical role in silencing miRNA targets and protecting miRNA stability. GW182 has quickly evolved as a key repressor in RISC evidenced from numerous studies. Strong silencing effect has been observed when GW182 was brought to proximity of mRNA reporter. It is reasonable to postulate that the primary and direct function of GW182 resides on its silencing activity. Since GW182 physically binds to AGOs, my work suggests a novel role of GW182 in

protecting AGO bound miRNA. This finding indicates a miRNA quality control function that has not been described. However, miRNA levels are controlled in many steps of their biogenesis pathway; it might not be the prioritized function of GW182 in cellular content.

GW182 has been shown to reside in cytoplasmic foci called GW bodies or P-bodies (1). Disassembly of GWBs by depleting GWB components LSm1, LSm3, HPat, or Ge-1 did not reduce the ability of GW182 in silencing its tethered reporters (140). However, the possibility remains that there might still be “sub-microscopic foci” in these experiments. Our observations that the number and size of GWBs increase upon the introduction of siRNA or miRNA (34) suggests a functional relevance of GWBs in siRNA or miRNA induced gene silencing. Many mRNAs bound by miRNAs can form GWBs, possibly through some GW182 domains that play a role in aggregating miRNA bound proteins. The extraction and sequestration of mRNA from the cytoplasm may be a quick and highly efficient method in translation repression. Deadenylation/decay of mRNA is secondary to the biological need for quick repression of mRNA translation. It could also be possible that GWBs help protect miRNA stability by limiting their accessibility to exonucleases. However, GW182-KD cells used in Chapter 3 did not totally abolish the formation of GWBs in HEK293 cells possibly due to the incomplete knockdown efficiency and putative compensatory effects contributed by the abundance of TNRC6B and 6C. Further experiments need to be designed to test the role of GWBs in regulating mature miRNA stability.

Although numerous reports have shown that exosomal miRNA can be transferred into recipient cells to regulate their cellular activities, further experiments are still required to confirm this notion. Many studies use artificial reporters to evaluate the

exosomal miRNA in recipient cells without demonstrating their effect on endogenous targets. Others overexpress siRNA or miRNA in exosomes or use chemical compounds to stimulate exosomes secretion (130). Therefore, it is difficult to conclude absolutely that this process plays important roles in physiological conditions. GW182 or Argonaute proteins have been separately reported to be in exosomes (84,86) although our data did not confirm these observations. In our hand, knockdown of GW182 causes reduced miRNA secretion but apparently without affecting the number of exosomes. However, the regulation of quality and potency of exosomal RNA in shaping recipient cells remains questionable. The selectivity of exosomes in locating their putatively designated recipient cells is not clear. Most importantly, how GW182 regulated miRNA packing and secretion via exosomes and its impact on recipient cell silencing activity need to be addressed. Although there are increasing numbers of studies focusing on exosomes and it is promising, much more efforts are clearly needed to make it into an attractive potential therapeutic application.

In summary, my dissertation identifies and characterizes the GW182 silencing domains and defining a novel function of GW182 in regulating miRNA stability by binding to AGO proteins. These studies offer new insights in understanding the miRNA silencing mechanism.

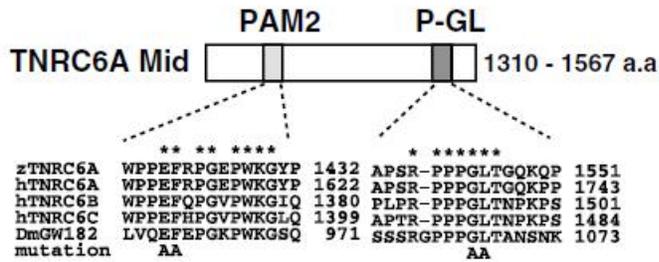


Figure. 4-1. Schematic map of GW182 two conserved domains PAM2 and P-GL. Sequence alignments are shown in comparison among zebrafish, human, and *Drosophila* GW182 proteins. Asterisks indicate the conserved amino acids among species. Alanine substitutes are shown to study the requirement of these two conserved domains in binding to PABP proteins and translational repression. This figure is adopted from Mishima et al. (2012). Translational inhibition by deadenylation-independent mechanisms is central to microRNA-mediated silencing in zebrafish. *Proc Natl Acad Sci U S A*, **109**, 1104-1109.

LIST OF REFERENCES

1. Eystathioy, T., Chan, E.K., Tenenbaum, S.A., Keene, J.D., Griffith, K. and Fritzler, M.J. (2002) A phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles. *Mol Biol Cell*, **13**, 1338-1351.
2. Filipowicz, W., Bhattacharyya, S.N. and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*, **9**, 102-114.
3. Eulalio, A., Triteschler, F. and Izaurralde, E. (2009) The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing. *RNA*, **15**, 1433-1442.
4. Krol, J., Loedige, I. and Filipowicz, W. (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet*, **11**, 597-610.
5. Huntzinger, E. and Izaurralde, E. (2011) Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet*, **12**, 99-110.
6. Yang, Z., Jakymiw, A., Wood, M.R., Eystathioy, T., Rubin, R.L., Fritzler, M.J. and Chan, E.K. (2004) GW182 is critical for the stability of GW bodies expressed during the cell cycle and cell proliferation. *J Cell Sci*, **117**, 5567-5578.
7. Jakymiw, A., Pauley, K.M., Li, S., Ikeda, K., Lian, S., Eystathioy, T., Satoh, M., Fritzler, M.J. and Chan, E.K. (2007) The role of GW/P-bodies in RNA processing and silencing. *J Cell Sci*, **120**, 1317-1323.
8. Meaux, S., van Hoof, A. and Baker, K.E. (2008) Nonsense-mediated mRNA decay in yeast does not require PAB1 or a poly(A) tail. *Mol Cell*, **29**, 134-140.
9. Anderson, P. and Kedersha, N. (2009) RNA granules: post-transcriptional and epigenetic modulators of gene expression. *Nat Rev Mol Cell Biol*, **10**, 430-436.
10. Erickson, S.L. and Lykke-Andersen, J. (2011) Cytoplasmic mRNP granules at a glance. *J Cell Sci*, **124**, 293-297.
11. Anderson, P. and Kedersha, N. (2006) RNA granules. *J Cell Biol*, **172**, 803-808.
12. Jinek, M. and Doudna, J.A. (2009) A three-dimensional view of the molecular machinery of RNA interference. *Nature*, **457**, 405-412.
13. Fabian, M.R., Sonenberg, N. and Filipowicz, W. (2010) Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.*, **79**, 351-379.

14. Rana, T.M. (2007) Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol*, **8**, 23-36.
15. Hutvagner, G. and Simard, M.J. (2008) Argonaute proteins: key players in RNA silencing. *Nat Rev Mol Cell Biol*, **9**, 22-32.
16. Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L. and Hannon, G.J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science*, **305**, 1437-1441.
17. Bartel, D.P. (2009) MicroRNAs: target recognition and regulatory functions. *Cell*, **136**, 215-233.
18. Baillat, D. and Shiekhattar, R. (2009) Functional dissection of the human TNRC6 (GW182-related) family of proteins. *Mol. Cell. Biol.*, **29**, 4144-4155.
19. 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.
20. Lian, S.L., Li, S., Abadal, G.X., Pauley, B.A., Fritzler, M.J. and Chan, E.K.L. (2009) The C-terminal half of human Ago2 binds to multiple GW-rich regions of GW182 and requires GW182 to mediate silencing. *RNA*, **15**, 804-813.
21. 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.
22. Zipprich, J.T., Bhattacharyya, S., Mathys, H. and Filipowicz, W. (2009) Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression. *RNA*, **15**, 781-793.
23. Chekulaeva, M., Filipowicz, W. and Parker, R. (2009) Multiple independent domains of dGW182 function in miRNA-mediated repression in *Drosophila*. *RNA*, **15**, 794-803.
24. Yao, B., Li, S., Jung, H.M., Lian, S.L., Abadal, G.X., Han, F., Fritzler, M.J. and Chan, E.K.L. (2011) Divergent GW182 functional domains in the regulation of translational silencing. *Nucleic Acids Res*, **39**, 2534-2547.
25. Fabian, M.R., Mathonnet, G., Sundermeier, T., Mathys, H., Zipprich, J.T., Svitkin, Y.V., Rivas, F., Jinek, M., Wohlschlegel, J., Doudna, J.A. *et al.* (2009) Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Mol Cell*, **35**, 868-880.
26. Jinek, M., Fabian, M.R., Coyle, S.M., Sonenberg, N. and Doudna, J.A. (2010) Structural insights into the human GW182-PABC interaction in microRNA-mediated deadenylation. *Nat Struct Mol Biol*, **17**, 238-240.

27. Braun, J.E., Huntzinger, E., Fauser, M. and Izaurralde, E. (2011) GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets. *Mol Cell*, **44**, 120-133.
28. Chekulaeva, M., Mathys, H., Zipprich, J.T., 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 Struct Mol Biol*, **18**, 1218-1226.
29. Fabian, M.R., Cieplak, M.K., Frank, F., Morita, M., Green, J., Srikumar, T., Nagar, B., Yamamoto, T., Raught, B., Duchaine, T.F. *et al.* (2011) miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT. *Nat Struct Mol Biol*, **18**, 1211-1217.
30. Eystathiou, T., Jakymiw, A., Chan, E.K., Seraphin, B., Cougot, N. and Fritzler, M.J. (2003) The GW182 protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies. *RNA*, **9**, 1171-1173.
31. Jakymiw, A., Lian, S., Eystathiou, T., Li, S., Satoh, M., Hamel, J.C., Fritzler, M.J. and Chan, E.K. (2005) Disruption of GW bodies impairs mammalian RNA interference. *Nat Cell Biol*, **7**, 1267-1274.
32. Lian, S., Jakymiw, A., Eystathiou, T., Hamel, J.C., Fritzler, M.J. and Chan, E.K.L. (2006) GW bodies, microRNAs and the cell cycle. *Cell Cycle*, **5**, 242-245.
33. Pauley, K.M., Eystathiou, T., Jakymiw, A., Hamel, J.C., Fritzler, M.J. and Chan, E.K. (2006) Formation of GW bodies is a consequence of microRNA genesis. *EMBO Rep*, **7**, 904-910.
34. Lian, S., Fritzler, M.J., Katz, J., Hamazaki, T., Terada, N., Satoh, M. and Chan, E.K. (2007) Small interfering RNA-mediated silencing induces target-dependent assembly of GW/P bodies. *Mol Biol Cell*, **18**, 3375-3387.
35. Li, S., Lian, S.L., Moser, J.J., Fritzler, M.L., Fritzler, M.J., Satoh, M. and Chan, E.K. (2008) Identification of GW182 and its novel isoform TNGW1 as translational repressors in Ago2-mediated silencing. *J Cell Sci*, **121**, 4134-4144.
36. Pauley, K.M., Satoh, M., Pauley, B.A., Dominguez-Gutierrez, P.R., Wallet, S.M., Holliday, L.S., Cha, S., Reeves, W.H. and Chan, E.K.L. (2010) Formation of GW/P bodies as marker for microRNA-mediated regulation of innate immune signaling in THP-1 cells. *Immunol. Cell Biol.*, **88**, 205-212.
37. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806-811.

38. Liu, J., Rivas, F.V., Wohlschlegel, J., Yates, J.R., 3rd, Parker, R. and Hannon, G.J. (2005) A role for the P-body component GW182 in microRNA function. *Nat Cell Biol*, **7**, 1261-1266.
39. Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, **75**, 843-854.
40. Tritschler, F., Huntzinger, E. and Izaurralde, E. (2010) Role of GW182 proteins and PABPC1 in the miRNA pathway: a sense of déjà vu. *Nat Rev Mol Cell Biol*, **11**, 379-384.
41. Eulalio, A., Helms, S., Fritsch, C., Fauser, M. and Izaurralde, E. (2009) A C-terminal silencing domain in GW182 is essential for miRNA function. *RNA*, **15**, 1067-1077.
42. Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degan, B., Muller, P. *et al.* (2000) Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA. *Nature*, **408**, 86-89.
43. Ding, L., Spencer, A., Morita, K. and Han, M. (2005) The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in *C. elegans*. *Mol Cell*, **19**, 437-447.
44. Schneider, M.D., Najand, N., Chaker, S., Pare, J.M., Haskins, J., Hughes, S.C., Hobman, T.C., Locke, J. and Simmonds, A.J. (2006) Gawky is a component of cytoplasmic mRNA processing bodies required for early *Drosophila* development. *J Cell Biol*, **174**, 349-358.
45. Eulalio, A., Rehwinkel, J., Stricker, M., Huntzinger, E., Yang, S.F., Doerks, T., Dorner, S., Bork, P., Boutros, M. and Izaurralde, E. (2007) Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. *Genes Dev*, **21**, 2558-2570.
46. Miyoshi, K., Okada, T.N., Siomi, H. and Siomi, M.C. (2009) Characterization of the miRNA-RISC loading complex and miRNA-RISC formed in the *Drosophila* miRNA pathway. *RNA*, **15**, 1282-1291.
47. Pillai, R.S., Artus, C.G. and Filipowicz, W. (2004) Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *RNA*, **10**, 1518-1525.
48. Chekulaeva, M. and Filipowicz, W. (2009) Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol*, **21**, 452-460.
49. Eulalio, A., Huntzinger, E. and Izaurralde, E. (2008) Getting to the root of miRNA-mediated gene silencing. *Cell*, **132**, 9-14.

50. Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G. and Tuschl, T. (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell*, **15**, 185-197.
51. Jaronczyk, K., Carmichael, J.B. and Hobman, T.C. (2005) Exploring the functions of RNA interference pathway proteins: some functions are more RISCy than others? *Biochem J*, **387**, 561-571.
52. Ding, L. and Han, M. (2007) GW182 family proteins are crucial for microRNA-mediated gene silencing. *Trends Cell Biol*, **17**, 411-416.
53. Chekulaeva, M., Parker, R. and Filipowicz, W. (2010) The GW/WG repeats of Drosophila GW182 function as effector motifs for miRNA-mediated repression. *Nucleic Acids Res*.
54. Eulalio, A., Huntzinger, E. and Izaurralde, E. (2008) GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat Struct Mol Biol*, **15**, 346-353.
55. Eulalio, A., Tritschler, F., Buttner, R., Weichenrieder, O., Izaurralde, E. and Truffault, V. (2009) The RRM domain in GW182 proteins contributes to miRNA-mediated gene silencing. *Nucleic Acids Res*, **37**, 2974-2983.
56. Lian, S.L., Li, S., Abadal, G.X., Pauley, B.A., Fritzler, M.J. and Chan, E.K. (2009) The C-terminal half of human Ago2 binds to multiple GW-rich regions of GW182 and requires GW182 to mediate silencing. *RNA*, **15**, 804-813.
57. Zekri, L., Huntzinger, E., Heimstadt, S. and Izaurralde, E. (2009) The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. *Mol. Cell. Biol.*, **29**, 6220-6231.
58. Chen, C.Y., Zheng, D., Xia, Z. and Shyu, A.B. (2009) Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. *Nat Struct Mol Biol*, **16**, 1160-1166.
59. Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P. and Izaurralde, E. (2006) mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev*, **20**, 1885-1898.
60. Till, S., Lejeune, E., Thermann, R., Bortfeld, M., Hothorn, M., Enderle, D., Heinrich, C., Hentze, M.W. and Ladurner, A.G. (2007) A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. *Nat Struct Mol Biol*, **14**, 897-903.
61. Ding, X.C. and Grosshans, H. (2009) Repression of *C. elegans* microRNA targets at the initiation level of translation requires GW182 proteins. *EMBO J*, **28**, 213-222.

62. Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engele, P., Lim, L.P. and Bartel, D.P. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*, **27**, 91-105.
63. Lewis, B.P., Burge, C.B. and Bartel, D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15-20.
64. Peters, L. and Meister, G. (2007) Argonaute proteins: mediators of RNA silencing. *Mol Cell*, **26**, 611-623.
65. Wu, L., Fan, J. and Belasco, J.G. (2008) Importance of translation and nonnucleolytic ago proteins for on-target RNA interference. *Curr Biol*, **18**, 1327-1332.
66. Ebert, M.S., Neilson, J.R. and Sharp, P.A. (2007) MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods*, **4**, 721-726.
67. Decker, C.J., Teixeira, D. and Parker, R. (2007) Edc3p and a glutamine/asparagine-rich domain of Lsm4p function in processing body assembly in *Saccharomyces cerevisiae*. *J Cell Biol*, **179**, 437-449.
68. Chu, C.Y. and Rana, T.M. (2006) Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. *PLoS Biol*, **4**, e210.
69. Minshall, N., Kress, M., Weil, D. and Standart, N. (2009) Role of p54 RNA helicase activity and its C-terminal domain in translational repression, P-body localization and assembly. *Mol Biol Cell*, **20**, 2464-2472.
70. Tritschler, F., Braun, J.E., Eulalio, A., Truffault, V., Izaurralde, E. and Weichenrieder, O. (2009) Structural basis for the mutually exclusive anchoring of P body components EDC3 and Tral to the DEAD box protein DDX6/Me31B. *Mol Cell*, **33**, 661-668.
71. Kahvejian, A., Svitkin, Y.V., Sukarieh, R., M'Boutchou, M.N. and Sonenberg, N. (2005) Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. *Genes Dev*, **19**, 104-113.
72. Pauley, K.M., Satoh, M., Pauley, B.A., Dominguez-Gutierrez, P.R., Wallet, S.M., Holliday, L.S., Cha, S., Reeves, W.H. and Chan, E.K. (2010) Formation of GW/P bodies as marker for microRNA-mediated regulation of innate immune signaling in THP-1 cells. *Immunol Cell Biol*, **88**, 205-212.
73. Croce, C.M. (2009) Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet*, **10**, 704-714.
74. Kai, Z.S. and Pasquinelli, A.E. (2010) MicroRNA assassins: factors that regulate the disappearance of miRNAs. *Nat Struct Mol Biol*, **17**, 5-10.

75. Hwang, H.W., Wentzel, E.A. and Mendell, J.T. (2007) A hexanucleotide element directs microRNA nuclear import. *Science*, **315**, 97-100.
76. Rissland, O.S., Hong, S.J. and Bartel, D.P. (2011) MicroRNA Destabilization Enables Dynamic Regulation of the miR-16 Family in Response to Cell-Cycle Changes. *Mol. Cell*, **43**, 993-1004.
77. Kim, Y.K., Yeo, J., Ha, M., Kim, B. and Kim, V.N. (2011) Cell Adhesion-Dependent Control of MicroRNA Decay. *Mol. Cell*, **43**, 1005-1014.
78. Krol, J., Buskamp, V., Markiewicz, I., Stadler, M.B., Ribi, S., Richter, J., Duebel, J., Bicker, S., Fehling, H.J., Schubeler, D. *et al.* (2010) Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs. *Cell*, **141**, 618-631.
79. Bail, S., Swerdel, M., Liu, H., Jiao, X., Goff, L.A., Hart, R.P. and Kiledjian, M. (2010) Differential regulation of microRNA stability. *RNA*, **16**, 1032-1039.
80. Diederichs, S. and Haber, D.A. (2007) Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell*, **131**, 1097-1108.
81. Winter, J. and Diederichs, S. (2011) Argonaute proteins regulate microRNA stability: Increased microRNA abundance by Argonaute proteins is due to microRNA stabilization. *RNA Biol*, **8**.
82. Thery, C., Ostrowski, M. and Segura, E. (2009) Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol*, **9**, 581-593.
83. Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J.J. and Lotvall, J.O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*, **9**, 654-659.
84. Gibbins, D.J., Ciaudo, C., Erhardt, M. and Voinnet, O. (2009) Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol*, **11**, 1143-1149.
85. Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y. and Ochiya, T. (2010) Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J. Biol. Chem.*, **285**, 17442-17452.
86. Zhang, Y., Liu, D., Chen, X., Li, J., Li, L., Bian, Z., Sun, F., Lu, J., Yin, Y., Cai, X. *et al.* (2010) Secreted monocytic miR-150 enhances targeted endothelial cell migration. *Mol. Cell*, **39**, 133-144.
87. Ramachandran, V. and Chen, X. (2008) Degradation of microRNAs by a family of exoribonucleases in Arabidopsis. *Science*, **321**, 1490-1492.

88. Chatterjee, S. and Grosshans, H. (2009) Active turnover modulates mature microRNA activity in *Caenorhabditis elegans*. *Nature*, **461**, 546-549.
89. Grosshans, H. and Chatterjee, S. (2010) MicroRNases and the Regulated Degradation of Mature Animal miRNAs. *Adv Exp Med Biol*, **700**, 140-155.
90. Sen, G.L. and Blau, H.M. (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol*, **7**, 633-636.
91. Karlowski, W.M., Zielezinski, A., Carrere, J., Pontier, D., Lagrange, T. and Cooke, R. (2010) Genome-wide computational identification of WG/GW Argonaute-binding proteins in *Arabidopsis*. *Nucleic Acids Res*, **38**, 4231-4245.
92. El-Shami, M., Pontier, D., Lahmy, S., Braun, L., Picart, C., Vega, D., Hakimi, M.A., Jacobsen, S.E., Cooke, R. and Lagrange, T. (2007) Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. *Genes Dev*, **21**, 2539-2544.
93. Fukaya, T. and Tomari, Y. (2011) PABP is not essential for microRNA-mediated translational repression and deadenylation in vitro. *EMBO J*, **30**, 4998-5009.
94. Tang, Y., Luo, X., Cui, H., Ni, X., Yuan, M., Guo, Y., Huang, X., Zhou, H., de Vries, N., Tak, P.P. *et al.* (2009) MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. *Arthritis Rheum*, **60**, 1065-1075.
95. Boss, I.W., Nadeau, P.E., Abbott, J.R., Yang, Y., Mergia, A. and Renne, R. (2011) A Kaposi's sarcoma-associated herpesvirus-encoded ortholog of microRNA miR-155 induces human splenic B-cell expansion in NOD/LtSz-scid IL2Rgammanull mice. *J. Virol.*, **85**, 9877-9886.
96. Damgaard, C.K., Kahns, S., Lykke-Andersen, S., Nielsen, A.L., Jensen, T.H. and Kjems, J. (2008) A 5' splice site enhances the recruitment of basal transcription initiation factors in vivo. *Mol Cell*, **29**, 271-278.
97. Eberle, A.B., Lykke-Andersen, S., Muhlemann, O. and Jensen, T.H. (2009) SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nat Struct Mol Biol*, **16**, 49-55.
98. An, D.S., Qin, F.X., Auyeung, V.C., Mao, S.H., Kung, S.K., Baltimore, D. and Chen, I.S. (2006) Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol Ther*, **14**, 494-504.
99. Chang, L.J. (2010) Lentiviral vector transduction of dendritic cells for novel vaccine strategies. *Methods Mol Biol*, **614**, 161-171.

100. Ikeda, K., Satoh, M., Pauley, K.M., Fritzler, M.J., Reeves, W.H. and Chan, E.K. (2006) Detection of the argonaute protein Ago2 and microRNAs in the RNA induced silencing complex (RISC) using a monoclonal antibody. *J Immunol Methods*, **317**, 38-44.
101. Thery, C., Amigorena, S., Raposo, G. and Clayton, A. (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*, **Chapter 3**, Unit 3 22.
102. Jagannath, A. and Wood, M.J. (2009) Localization of double-stranded small interfering RNA to cytoplasmic processing bodies is Ago2 dependent and results in up-regulation of GW182 and Argonaute-2. *Mol. Biol. Cell*, **20**, 521-529.
103. Logozzi, M., De Milito, A., Lugini, L., Borghi, M., Calabro, L., Spada, M., Perdicchio, M., Marino, M.L., Federici, C., Iessi, E. *et al.* (2009) High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One*, **4**, e5219.
104. Kiriakidou, M., Tan, G.S., Lamprinaki, S., De Planell-Saguer, M., Nelson, P.T. and Mourelatos, Z. (2007) An mRNA m7G cap binding-like motif within human Ago2 represses translation. *Cell*, **129**, 1141-1151.
105. Wang, Y., Juraneck, S., Li, H., Sheng, G., Wardle, G.S., Tuschl, T. and Patel, D.J. (2009) Nucleation, propagation and cleavage of target RNAs in Ago silencing complexes. *Nature*, **461**, 754-761.
106. Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., Rothballer, A., Ascano, M., Jr., Jungkamp, A.C., Munschauer, M. *et al.* (2010) Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell*, **141**, 129-141.
107. Yao, B., Li, S., Jung, H.M., Lian, S.L., Abadal, G.X., Han, F., Fritzler, M.J. and Chan, E.K. (2011) Divergent GW182 functional domains in the regulation of translational silencing. *Nucleic Acids Res*, **39**, 2534-2547.
108. Kosaka, N., Iguchi, H. and Ochiya, T. (2010) Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci*, **101**, 2087-2092.
109. Lee, Y.S., Pressman, S., Andress, A.P., Kim, K., White, J.L., Cassidy, J.J., Li, X., Lubell, K., Lim do, H., Cho, I.S. *et al.* (2009) Silencing by small RNAs is linked to endosomal trafficking. *Nat Cell Biol*, **11**, 1150-1156.
110. van Rooij, E., Sutherland, L.B., Qi, X., Richardson, J.A., Hill, J. and Olson, E.N. (2007) Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science*, **316**, 575-579.

111. Wang, Y., Sheng, G., Juranek, S., Tuschl, T. and Patel, D.J. (2008) Structure of the guide-strand-containing argonaute silencing complex. *Nature*, **456**, 209-213.
112. Schirle, N.T. and Macrae, I.J. (2012) The Crystal Structure of Human Argonaute2. *Science*.
113. Gregory, R.I., Chendrimada, T.P., Cooch, N. and Shiekhattar, R. (2005) Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell*, **123**, 631-640.
114. MacRae, I.J., Ma, E., Zhou, M., Robinson, C.V. and Doudna, J.A. (2008) In vitro reconstitution of the human RISC-loading complex. *Proc Natl Acad Sci U S A*, **105**, 512-517.
115. Maniataki, E. and Mourelatos, Z. (2005) A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. *Genes Dev*, **19**, 2979-2990.
116. Kawamata, T., Yoda, M. and Tomari, Y. (2011) Multilayer checkpoints for microRNA authenticity during RISC assembly. *EMBO Rep*, **12**, 944-949.
117. Cheloufi, S., Dos Santos, C.O., Chong, M.M. and Hannon, G.J. (2010) A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature*, **465**, 584-589.
118. Cifuentes, D., Xue, H., Taylor, D.W., Patnode, H., Mishima, Y., Cheloufi, S., Ma, E., Mane, S., Hannon, G.J., Lawson, N.D. *et al.* (2010) A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science*, **328**, 1694-1698.
119. Jiang, Z., Yu, N., Kuang, P., Chen, M., Shao, F., Martin, G., Chui, D.H., Cardoso, W.V., Ai, X. and Lu, J. (2012) Trinucleotide repeat containing 6a (Tnrc6a)-mediated microRNA function is required for development of yolk sac endoderm. *J Biol Chem*, **287**, 5979-5987.
120. Pillai, R.S., Bhattacharyya, S.N., Artus, C.G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E. and Filipowicz, W. (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*, **309**, 1573-1576.
121. Guo, H., Ingolia, N.T., Weissman, J.S. and Bartel, D.P. (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*, **466**, 835-840.
122. Bazzini, A.A., Lee, M.T. and Giraldez, A.J. (2012) Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. *Science*, **336**, 233-237.

123. Mishima, Y., Fukao, A., Kishimoto, T., Sakamoto, H., Fujiwara, T. and Inoue, K. (2012) Translational inhibition by deadenylation-independent mechanisms is central to microRNA-mediated silencing in zebrafish. *Proc Natl Acad Sci U S A*, **109**, 1104-1109.
124. Djuranovic, S., Nahvi, A. and Green, R. (2012) miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. *Science*, **336**, 237-240.
125. Taganov, K.D., Boldin, M.P., Chang, K.J. and Baltimore, D. (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A*, **103**, 12481-12486.
126. Nahid, M.A., Pauley, K.M., Satoh, M. and Chan, E.K.L. (2009) miR-146a is critical for endotoxin-induced tolerance: Implication in innate immunity. *J. Biol. Chem.*, **284**, 34590-34599.
127. Ji, L. and Chen, X. (2012) Regulation of small RNA stability: methylation and beyond. *Cell Res*.
128. Maiti, M., Lee, H.C. and Liu, Y. (2007) QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and facilitates conversion of duplex siRNA into single strands. *Genes Dev*, **21**, 590-600.
129. Xue, Z., Yuan, H., Guo, J. and Liu, Y. (2012) Reconstitution of an Argonaute-Dependent Small RNA Biogenesis Pathway Reveals a Handover Mechanism Involving the RNA Exosome and the Exonuclease QIP. *Mol Cell*.
130. Chen, X., Liang, H., Zhang, J., Zen, K. and Zhang, C.Y. (2012) Secreted microRNAs: a new form of intercellular communication. *Trends Cell Biol*, **22**, 125-132.
131. Skog, J., Wurdinger, T., van Rijn, S., Meijer, D.H., Gainche, L., Sena-Esteves, M., Curry, W.T., Jr., Carter, B.S., Krichevsky, A.M. and Breakefield, X.O. (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol*, **10**, 1470-1476.
132. Arroyo, J.D., Chevillet, J.R., Kroh, E.M., Ruf, I.K., Pritchard, C.C., Gibson, D.F., Mitchell, P.S., Bennett, C.F., Pogosova-Agadjanyan, E.L., Stirewalt, D.L. *et al.* (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci. U. S. A.*, **108**, 5003-5008.
133. Vickers, K.C., Palmisano, B.T., Shoucri, B.M., Shamburek, R.D. and Remaley, A.T. (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol*, **13**, 423-433.

134. Hu, G., Drescher, K.M. and Chen, X.M. (2012) Exosomal miRNAs: Biological Properties and Therapeutic Potential. *Front Genet*, **3**, 56.
135. Mittelbrunn, M. and Sanchez-Madrid, F. (2012) Intercellular communication: diverse structures for exchange of genetic information. *Nat Rev Mol Cell Biol*, **13**, 328-335.
136. Thery, C., Zitvogel, L. and Amigorena, S. (2002) Exosomes: composition, biogenesis and function. *Nat Rev Immunol*, **2**, 569-579.
137. Mittelbrunn, M., Gutierrez-Vazquez, C., Villarroya-Beltri, C., Gonzalez, S., Sanchez-Cabo, F., Gonzalez, M.A., Bernad, A. and Sanchez-Madrid, F. (2011) Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun*, **2**, 282.
138. Hurley, J.H. (2008) ESCRT complexes and the biogenesis of multivesicular bodies. *Curr Opin Cell Biol*, **20**, 4-11.
139. Hurley, J.H. and Hanson, P.I. (2010) Membrane budding and scission by the ESCRT machinery: it's all in the neck. *Nat Rev Mol Cell Biol*, **11**, 556-566.
140. Eulalio, A., Behm-Ansmant, I., Schweizer, D. and Izaurralde, E. (2007) P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. *Mol Cell Biol*, **27**, 3970-3981.

BIOGRAPHICAL SKETCH

Bing Yao was born in Qingdao, Shandong Province, People's Republic of China. He graduated from Qingdao No. 19 middle school in 1998, and then attended Ocean University of China in Qingdao, China, with the marine biotechnology major. He earned his Bachelor in Science in 2002. At this time, Bing developed his interests in molecular biology research and subsequently joined Dr. Zhenmin Bao's laboratory working on molecular genetics and breeding of scallops, aiming to establish a new scallop breeder that is resistant to certain viral infection. He received his Master in Science in 2005. In the fall of 2005, Bing received an opportunity to work in Dr. Lei Zhou laboratory at the University of Florida focusing on epigenetic regulation of irradiation-induced cell death in *Drosophila*. Bing became a graduate student in the Interdisciplinary Program in Biomedical Sciences (IDP) at the University of Florida in 2006, and married his beloved wife, Can Zhang, in the same year.

Bing decided to join Dr. Keith Robertson laboratory in 2007 studying functional characterization of the interplay between the DNA methylation machinery and Polycomb proteins in mammalian cells. He successfully passed his qualifying examination in late 2008. Due to the relocation of Dr. Robertson to another institution, Bing decided to face a new challenge in joining Dr. Edward Chan laboratory in the beginning of 2009, working on role of GW182 in inducing miRNA-mediated gene repression and regulating miRNA stability. After graduation, Bing plans on accepting a postdoctoral position in Dr. Peng Jin laboratory in Department of Human Genetics at the Emory University, where he can combine epigenetic regulation and miRNA together to investigate their interplay in human diseases.