

FUNCTIONAL DOMAIN CHARACTERIZATION OF GW BODY MARKER PROTEIN
GW182

By

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To my mother who constantly encourages me to pursue my dream; to my wife who supports me every day; especially to my high school chemistry teacher and my Ph.D. mentor who help me to reach two of the most important milestones in my career

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Abstract of Dissertation Presented to the Graduate School
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FUNCTIONAL DOMAINS CHARACTERIZATION OF GWB MARKER: GW182

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MicroRNAs (miRNAs) have emerged as key regulators of about 30% of all genes expressed in human. Executed by a group of ribonucleoproteins named miRNA-mediated silencing complex, most of the miRNAs induce translational repression due to its imperfect complementarity to target mRNA. Argonaute protein family Ago1-4 is the best characterized components in miRISC. The mechanism of miRNA-mediated translational repression remains unclear. The mRNAs targeted by miRNA were turned over in cytoplasmic foci named GW bodies. GW182, an 182kDa protein characterized by multiple glycine/tryptophan (GW) repeats, is important for GW body formation and miRNA-mediated gene silencing. Here, we aim to characterize the functional domains in GW182 and understand their importance in miRNA-mediated gene regulation. We first identified TNGW1, which contains trinucleotide repeats (TNR) in its mRNA, as a novel GW182 isoform. Knockdown of TNGW1 mRNA did not affect GW182 protein level, indicating GW182 was transcribed and translated independently from TNGW1. Using truncated constructs and glutathione-S-transferase pulldown assay, we discovered that GW182 and TNGW1 contained four non-overlapping regions which were able to interact with the C-terminal half of Ago2 and other Ago proteins. Mutagenesis study showed that GW motif may not be important for some of the GW182:Ago2 interaction. Further study found that GW182 and TNGW1 acted more directly than Ago2 in translational repression. The

repression effect caused by tethered GW182 and TNGW1 were not dependent on Ago2 protein. Mapping of different Ago proteins revealed that their abilities to cause repression were associated with their interaction with GW182. Lastly, functional studies narrowed down two non-overlapping regions in GW182 harboring inhibitory effect to luciferase reporters. These regions were also found associated with ribosomal protein RPLP0, but not RPLP1 or RPLP2, implying the presence of incomplete ribosomal stalk structures in the repression complex. Our finding proposed a model that GW182 acts as the repression trigger in miRNA related gene regulation, where the ribosomal stalk could potentially be the target in this event.

CHAPTER 1 INTRODUCTION

The Discovery of GW Body and Its Marker Protein GW182

In 2002, GW182 was identified and cloned by our laboratory as a novel protein using autoimmune serum from a patient with motor and sensory neuropathy (Eystathioy et al., 2002). It is a protein with a size of 182kDa and characterized by its multiple glycine/tryptophan (GW) repeats along the protein sequence. At that time, the only known domain in GW182 based on bioinformatics is the RNA recognition motif (RRM) in its C-terminus. GW182 is found to be associated with a specific subset of mRNAs and consistently enriched within unique cytoplasmic foci designated as GW bodies that are distinct from other known cytoplasmic organelles such as Golgi complex, endosomes, lysosomes, and peroxisomes (Eystathioy et al., 2002). Therefore, GW bodies were speculated as novel cytoplasmic foci related with mRNA storage and/or degradation. Morphologically, GW bodies are generally small, spherical, cytoplasmic foci of about 100-300 nm in diameter and devoid of a lipid bilayer membrane (Yang *et al.*, 2004). More interestingly, the number and size of GW bodies varies in different cell types and at different stages of the cell cycle (Yang *et al.*, 2004).

In 2003, Sheth and Parker report in yeast Dcp1-positive cytoplasmic foci as processing bodies (P bodies). P bodies were found enrich of mRNA degradation intermediates in addition to the 5'-3' mRNA decay factors (Sheth and Parker, 2003). Later study in mammalian cells shows RNA decay factors Dcp1 and LSm4 co-localize with GW182 in GW bodies (Eystathioy et al., 2003c) implying GW body is the mammalian counterpart of yeast P bodies. GW bodies are also shown to contain poly (A)⁺ RNA and dynamically disappear as mRNA breakdown was abolished (Cougot et al., 2004). Therefore, GW body are considered as the mammalian

analogues of P bodies and as the sites for active 5'-3' mRNA degradation, which are designated here provisionally as GW/P bodies (GWB).

GW182 is important for the assembly of GWB. In vitro gene knockdown of GW182 using short hairpin RNA (shRNA) plasmid results in disappearance of GWB (Yang *et al.*, 2004). Expression of a dominant negative construct of GW182 GW1Δ1 also disassemble GWB (Jakymiw *et al.*, 2005). Therefore, GW182 was proposed as the marker as well as the matrix protein of GWB.

The Functional Link between RNA Interference and GW Body

RNA interference (RNAi) is a conserved mechanism of gene regulation involved in multiple biological functions. In lower organisms, it was initially described as a genetic control mechanism implicated in virus resistance (Covey *et al.*, 1997; Ratcliff *et al.*, 1997), genome maintenance (Assaad *et al.*, 1993) and developmental control (Boerjan *et al.*, 1994). In 1998, RNAi was first characterized in *C. elegans* as a potent and sequence specific mechanism that silences endogenous genes by Andrew Fire and Craig Mello (Fire *et al.*, 1998), who later received the 2006 Nobel Prize in Physiology or Medicine for their contribution. The classical RNAi activity is triggered by small double-stranded RNAs including small interfering RNA (siRNA) and microRNA (miRNA). In siRNA-mediated silencing, the dsRNAs, which are formed in cells or are introduced into cells by viral infection or artificial expression, are processed by RNase III enzyme, Dicer, into ~20-bp double-stranded small interfering RNAs (siRNAs). The antisense strands of the siRNAs are then incorporated into siRNA-induced silencing complex (siRISC). Subsequently, siRISC binds to and cleaves target mRNA with complete complementary sequence to the siRNA (Filipowicz *et al.*, 2005). Argonaute 2 (Ago2) is the core component of RISC and harbors RNase H activity responsible for the cleavage of

target mRNA (Liu et al., 2004). Different from siRNA pathway, miRNAs are endogenous ~21-nt regulatory RNAs that are evolutionarily conserved in most species and are estimated to regulate ~30% of protein-encoding genes in human (Lewis *et al.*, 2005). MiRNAs are processed from endogenous precursor molecules folded into hairpin-like structure. The maturation of miRNAs includes two steps, both catalyzed by enzymes of the RNase III family, Drosha and Dicer. Drosha, working with its co-effector DGCR8, is responsible for the processing of primary miRNA transcripts (pri-miRNAs) to ~70-nt hairpins named precursor miRNAs (pre-miRNAs). Subsequently, Dicer, accompanied with TRBP, processes pre-miRNAs into mature miRNAs, which binds to the 3'-UTR of target mRNA by the seed sequence and regulates gene expression by increasing instability or repressing translation of target mRNA (Filipowicz *et al.*, 2005). The mature miRNA then incorporates into miRNA-induced silencing complex (miRISC) and target to the mRNA in a sequence specific manner. If the miRNA is highly complementary to the mRNA target, it can induce mRNA cleavage as siRNA (Hornstein et al., 2005) through the slicing activity of Ago2. However, if the miRNA is only partially complementary to the target mRNA, the miRISC will induce translational repression. In mammal, the only well characterized components in miRISC are the four Argonaute proteins, Ago1 to Ago4. However, the molecular mechanism of the miRNA-mediated translational repression remains unclear.

GWB is closely associated with RNAi activity. Firstly, the most important RNAi factors, Ago proteins and siRNA (Jakymiw et al., 2005; Liu et al., 2005a), are found enriched in GWB. Secondly, the size and number of GWB are related to RNAi activity. Increase in siRNA-mediated activities induced GWB formation (Lian et al., 2007) whereas inhibition of miRNA pathway led to disassembly of GWB (Pauley et al., 2006; Eulalio et al., 2007b). Thirdly, the GWB contain enriched 5'-3' mRNA degradation factors, including Xrn1 (5'-3' exonuclease),

Dcp2:Dcp1 (decapping enzyme), and LSM1-7 complex (stimulator of mRNA decapping) (Heyer et al., 1995; Bashkirov et al., 1997; Ingelfinger et al., 2002; van Dijk et al., 2002), which are responsible for miRNA-mediated mRNA degradation. Therefore, GWB is proposed as the biomarker of cell RNAi activity (Lian et al., 2007).

The Importance of GW182 in miRNA Function

Although multiple models were proposed by different groups (Filipowicz et al., 2008), the mechanism of how miRNAs trigger the translational repression remains unclear. However, because direct cleavage of the target mRNA is hindered in most of the cases, more protein factors need to be recruited to assure the repression function. We are one of the first two groups to describe the importance of GW182 in RNAi function (Jakymiw et al., 2005; Liu et al., 2005a). The importance of GW182 is further supported by the later studies and can be summarized as the following three aspects. First, GW182 tightly interacted with Argonaute proteins in human (Jakymiw et al., 2005; Liu et al., 2005a), and other species including *Drosophila* (Behm-Ansmant et al., 2006), *C. elegans* (Ding et al., 2005) and *Arabidopsis* (El-Shami et al., 2007). In addition, the interaction between GW182 and Ago2 was RNA-independent (Liu et al., 2005a) and proposed to depend on evolutionally conserved WG/GW motif (El-Shami et al., 2007; Till et al., 2007). A recent report showed the GW182:Argonaute interaction was essential for miRNA induced gene silencing and its subsequent mRNA decay in *Drosophila* (Eulalio et al., 2008b). Second, the translational repression effect was impaired when GW182 was knocked down (Liu et al., 2005a; Chu and Rana, 2006). Behm-Ansmant et al. also showed in *Drosophila* that tethering GW182 to the 3'-UTR of mRNA, which bypassed the requirement of miRNA, led to translational repression (Behm-Ansmant et al., 2006). Last, GW182 was important for GWB formation as it may form an optimal microenvironment for recruiting the RNA decay factors to

GWB. Tethering GW182 to mRNA induced RNA decay required 5'→3' RNA decay factors in *Drosophila* (Behm-Ansmant et al., 2006). In the absence of GW182, even the formation of GWB cannot be detected. Nevertheless, the inter-dependence among these proteins during translational repression remains unclear in mammalian system. With the elucidation of additional GWB components, questions are raised about how GW182, Argonaute and RNA decay factors contribute to the RNA induced gene silencing.

In the current study, we hypothesize that GW182 is an important molecule in the function of miRNA-mediated translation repression. By identifying a new isoform of GW182 and characterizing its functional domain responsible for Ago interaction and repression, we are able gain a better insight of the mechanism of translational repression in the molecular level. More importantly, it may lead to the development of new methods to control and adjust the miRNA-mediated gene regulation, which may be used to enhance RNAi-based therapy in the future.

CHAPTER 2
IDENTIFICATION AND CHARACTERIZATION OF TNGW1 AS THE NOVEL ISOFORM
OF GW182 AND THEIR FUNCTION IN GWB FORMATION AND TRANSLATIONAL
REPRESSION

Introduction

RNA interference (RNAi) is a potent post-transcriptional regulation mechanism for gene expression. It is triggered by small molecule RNAs, including small interfering RNA (siRNA) and microRNA (miRNA), and then executed by the RNA induced silencing complex (RISC) wherein targeted mRNA is degraded through the 5'→3' RNA decay pathway. GW bodies (GWB), also known as mammalian processing bodies, were found closely associated with RNAi and its related RNA turnover activities (Jakymiw et al., 2007; Eulalio et al., 2007a). Increase in siRNA-mediated activities induced GWB formation (Lian et al., 2007) whereas inhibition of miRNA pathway led to disassembly of GWB (Pauley et al., 2006; Eulalio et al., 2007b). Blocking the 5'→3' RNA decay before its initiation diminished GWB (Cougot et al., 2004) while blocking after its initiation increased the size and number of GWB (Sheth and Parker, 2003; Cougot et al., 2004; Andrei et al., 2005). A recent report demonstrated that the formation of GWB was the consequence of RNAi activities in *Drosophila* (Eulalio et al., 2007).

GW182, one of the marker proteins of GWB, was first identified in 2002 as a target protein of autoantibodies from a patient with motor and sensory neuropathy (Eystathioy et al., 2002). It is an 182kDa protein characterized by multiple glycine/tryptophan (G/W) repeats. GW182 is important for GWB formation and RNA induced gene silencing function even though it had no known enzymatic activity. Our earlier studies showed that knock-down of GW182 significantly disassembled GWB (Yang et al., 2004; Jakymiw et al., 2005) and impaired the efficiency of siRNA functions (Jakymiw et al., 2005). Other investigators reported that GW182 was more

important in miRNA function and closely associated with translational repression (Liu et al., 2005a; Chu and Rana, 2006). The formation of GWB from the sub-microscopic to microscopic level may occur during the process (Franks and Lykke-Andersen, 2007).

The gene name of GW182 in NCBI GenBank database is trinucleotide repeat containing 6A, or TNRC6A. Interestingly, the GenBank database predicts another isoform, which we have provisionally named trinucleotide GW1 (TNGW1), containing trinucleotide repeats (TNR) in its mRNA. Expansion of TNR is known to be related to a set of diseases, most notably those with neuropsychiatric features, such as Huntington's disease (Margolis et al., 1997). TNRC6A is one of the 20 trinucleotide repeat containing genes in the human genome but to date it has not been related to trinucleotide expansion diseases. In clinical studies, we showed that autoantibodies to GW182/GWB were associated with Sjögren's syndrome, mixed motor/sensory neuropathy, ataxia, and systematic lupus erythematosus (Eystathioy et al., 2003b; Bhanji et al., 2007). However, to date there are no published reports describing the expression of TNGW1 and therefore, we examine its expression and potential effect on translational repression.

Materials and Methods

Identification of TNGW1 mRNA

To identify the TNGW1 mRNA, polymerase chain reaction (PCR) amplification was performed on cDNA from HeLa, Hep-2 and HepG2 cell lines (ATCC, Manassas, VA), and adult human normal testis (BioChain, Hayward, CA) using primer TNRC-1: 5'-ATAATGCCAAGCGAGCTACAG-3' (nt248-268), and primer TNRC-2: 5'-AAGGGAAGTGCCATTCATACC-3' (nt1512-1492). PCR reactions used SureStart™ Taq DNA polymerase (Stratagene, Cedar Creek, TX) following manufacturer's protocol. The annealing temperature for PCR amplification was 54 C. The complete nucleotide sequence of the PCR products was determined in both strands using BigDye terminator sequencing at the

University of Florida Interdisciplinary Center for Biotechnology Research Sequencing Core Laboratory.

TNGW1 cDNA Cloning and Construction of Expression Plasmids

To construct the full-length TNGW1, PCR amplification was conducted on the human testis cDNA (BioChain) using primer TNRC-5a: 5'-TTTGGGAAGATCTATGAGAGAATTGGAAGCTAAAGCT-3' containing a synthetic Bgl II site sequence (underline) immediately upstream of TNGW1 ATG translational start site, and primer TNRC-2, which is downstream of an internal Kpn I site (nt1252). The 1.5kb PCR product was purified and digested with Bgl II and Kpn I to generate a 1.2kb fragment that was used to replace the 5' 500bp BamH I to Kpn I fragment in the full-length GW182 cloned in the pENTR vector; the BamH I restriction site was from the 5' linker sequence of the pENTR vector. Both the 1.2kb Bgl II-Kpn I fragment and the BamH I and Kpn I linearized plasmid of pENTR-GW182 were gel purified and then ligated at 16°C overnight to generate pENTR-TNGW1 with an 8.4kb insert. Expression vectors, enhanced green fluorescence (EGFP) tagged and glutathione-S-transferase (GST) tagged TNGW1 were generated using pENTR-TNGW1 and respective pDEST vectors via recombination using LR Clonase® II (Invitrogen, Carlsbad, CA) following the manufacturer's protocol.

To generate a construct to express a recombinant polypeptide containing the TNR (rTNR, aa1-204), pENTR-TNGW1 was first digested with BamH I (nt610) and Not I (3' end linker) to release a 6.5kb fragment consist of GW182. The overhangs of the vector encoding the N-terminus of TNGW1 was filled-in and then ligated at room temperature (RT) for 1 hour to generate the deletion construct pENTR-rTNR. Expression vectors for rTNR in pDEST17 (Invitrogen) and pDEST-EGFP were generated to produce 6XHis-rTNR in *E. coli* and EGFP-rTNR for expression in mammalian cells respectively.

The tethering assay plasmids including pCIneo-NHA vector, NHA-Ago2, firefly luciferase containing 5 BoxB structures (FL-5BoxB), Renilla luciferase containing 5 BoxB structures (RL-5BoxB), firefly luciferase (FL), and Renilla luciferase (RL) were gifts from Dr. Witold Filipowicz, Friedrich Miescher Institute for Biomedical Research, Switzerland (Pillai et al., 2004). To generate NHA-GW182 and NHA-TNGW1, the pCIneo-NHA vector was converted to gateway destination vector using the Gateway Vector Conversion System (Invitrogen). Then TNGW1 and GW182 were moved from corresponding pENTR vectors to the pCIneo-NHA gateway vector respectively by recombination. All DNA constructs were confirmed by direct DNA sequencing.

Generation of Antibodies Specific to the TNGW1 Isoform

The expression of recombinant 6XHis-rTNR protein in BL21 (DE3) E. coli and purification by nickel affinity chromatography was performed using Qiagen's protocol as previously described (Eystathioy et al., 2002). Two New Zealand White rabbits 6225 and 6226 were used to generate polyclonal antibodies using standard protocol by Lampire Biological Laboratories, Pipersville, PA. Pre-immune blood samples as well as samples collected after initial and booster injections were harvested and analyzed for reactivity. For the production of monoclonal antibodies (mAb), hyperimmunized BALB/c mice were used to generate hybridomas carried out by the University of Florida Interdisciplinary Center for Biotechnology Research Hybridoma Core Laboratory. Three mouse mAb (2E11, 5C8 and 2F11) were selected based on enzyme-linked immunosorbent assay and indirect immunofluorescence screening. All three mouse mAb were identified as IgG2a, κ antibodies.

Cell Culture and Transfection

HeLa and HEK 293 cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum in a 37°C incubator with 5% CO₂. Lipofectamine 2000 (Invitrogen) was used for

transient siRNA and DNA plasmid transfection following manufacturer's protocol. Briefly, the cultured cells were grown to 40-50% confluence and transfected with 100 nM siRNA. Cells were fixed or lysed 2 days after the transfection. In 3-day experiments, cells were fixed at day 1, day 2, and day 3 after transfection. The siRNA for TNGW1 (siTNR) was designed by using the online tool from the Dharmacon website and targets in the TNR region of TNGW1. The corresponding sequences are: sense strand 5'-UCGGUAUCCUCGUGAAGUATT-3'; antisense strand 5'-UACUUCACGAGGAUACCGATT-3'. The sequence of siRNA for EGFP (siGFP), Ago2 (siAgo2) and GW182 (siGW182) were reported in previous study (Lian et al., 2007). In DNA plasmid transfection experiments, cells were maintained at 70~90% confluence for transfection and harvested 24 or 48 hours after transfection and analyzed by immunofluorescence and/or western blot.

Immunoprecipitation (IP) and Western Blot Analysis (IP-WB)

IP-WB analysis was performed as described in detail in a previous study (Moser et al., 2007). In brief, for the IP step, human anti-GWB antibodies from the prototype serum 18033 (Mitogen Advanced Diagnostics Laboratory, University of Calgary, Calgary, AB, Canada) were chemically cross-linked to Protein A-Sepharose beads to prevent elution in the subsequent SDS-PAGE step. IP samples were resolved in a 6.5% gel SDS-PAGE with the low molecular proteins (<75kDa) run off the gel in order to achieve the optimal separation of two isoforms of GW182 and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Primary antibodies used in the western blot step included mouse antibodies anti-rTNR 2E11 (undiluted), 5C8 (undiluted), anti-GW182 4B6 (1:10) (Eystathioy et al., 2003a), anti-Hemagglutinin (HA) (1:1,000, Covance, Emeryville, CA), and rabbit antibodies, anti-rTNR 6225 (1:200), 6226 (1:200), anti-GW182 5182 (1:200), 6642 (1:200) and anti-EGFP (1:1000, Invitrogen). Secondary antibodies included either horseradish peroxidase (HRP)-goat anti-

human Ig (1:20,000; Sigma, St. Louis, MO), goat anti-rabbit immunoglobulin (1:20,000; Jackson ImmunoResearch, West Grove, PA), or goat anti-mouse immunoglobulin (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA). Bands were detected using the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ) or Supersignal Chemiluminescent system (Pierce Chemical, Rockford, IL). When necessary, nitrocellulose membranes were stripped using stripping buffer (100 mM, 2-mercaptoethanol, 2% SDS, 62.5mM Tris, pH 6.7) for 30 minutes at 65°C for further probing.

Addressable Laser Bead Immunoassay

A set of addressable beads bearing laser reactive dyes (Luminex, Austin, TX) were coupled to purified rTNR polypeptide and analyzed for antibody reactivity as previously described (Eystathioy et al., 2003a). The mouse mAb and rabbit sera were diluted in QUANTA Plex diluent (INOVA, San Diego, CA) to a final concentration of 1:100. Thirty microliters of QUANTA Plex diluent was added to each well followed by 10 µl of the diluted sample and then incubated on an orbital shaker for 30 minutes at RT. This was followed by the addition of 40 µl of phycoerythrin-conjugated species specific anti-IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:50 to each well and incubated on the orbital shaker for an additional 30 minutes. The reactivity of the antigen-coated beads was determined on a Luminex 100 dual-laser flow cytometer (Luminex, Austin, TX). Each assay included negative and positive controls and results were expressed as median fluorescent units (MFU).

Characterization of Anti-rTNR Antibodies by Synthetic Peptide Epitope Mapping

To characterize the specific reactivity of anti-rTNR antibodies generated, membranes containing in situ synthesized sequential 15mer peptides offset by five amino acids and representing the region-terminal domain of the TNGW1 protein (Table S1) were prepared (Eve Technologies, Calgary, AB, Canada) as previously described (Selak et al., 2003; Eystathioy et al.,

2003a). The dehydrated membranes were prepared for immunoblotting by an initial 10 minutes incubation in 100% ethanol followed by rehydration in Tris-buffered saline (TBS; 10 mM Tris-HCl pH 7.6, 150 mM NaCl) for 10 minutes at RT. The membranes were blocked in 2% milk/TBS overnight at 4°C and incubated with various primary antibodies at the appropriate dilution for 1.5 hours at RT on a shaker. Following 3 washes of 5 minutes each with 2% milk/TBS, appropriate HRP-conjugated secondary antibodies diluted in 2% milk/TBS as described above were incubated with the membranes for 45 minutes at RT on a shaker. Membranes were washed 3 times with TBS for 2 minutes each and the bound antibodies were detected using the enhanced chemiluminescence kit (Amersham Biosciences). The same stripping method was used for the peptide membrane as described in the western blot section.

Indirect Immunofluorescence Assay

HEp-2 slides (ImmunoConcepts, Sacramento, CA) or HeLa cells grown as a monolayer were used to perform indirect immunofluorescence assay as described (Jakymiw et al., 2005). Primary antibodies used included: mouse anti-rTNR 2F11 (undiluted culture supernatant), rabbit anti-rTNR 6226 (1:200), rabbit anti-GST (1:1000) (provided by Dr. Sayeski, University of Florida), rabbit anti-Dcp1a (1:500) (provided from Dr. Lykke-Andersen, University of Colorado), human anti-GWB sera 18033 (1:6000) and IC6 (1:1000, Mitogen Advanced Diagnostics Laboratory, University of Calgary). Secondary antibodies include Alexa Fluor 488 (1:400, Invitrogen), Alexa Fluor 568 (1:400, Invitrogen), and Cy5 (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA) conjugated goat antibodies to IgG of corresponding species (human, rabbit or mouse). Goat anti-mouse IgG2a TRITC (1:50, Southern Biotech, Birmingham, AL) and goat anti-mouse IgG1 488 (1:400, Invitrogen) were used specifically in 2F11 and 4B6 dual staining assay. The slides were mounted by using VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Southfield, MI). Fluorescent images

were captured with a Zeiss Axiovert 200M microscope (Carl Zeiss, Jena, Germany and processed using Adobe Photoshop (Adobe Systems, San Jose, CA). Magenta was used as a pseudo-color in images when needed.

Tethering Assay Using the Dual Luciferase System

HEK 293 cells seeded at 75-80% confluence were transfected with 600ng DNA plasmid of NHA vector, NHA-Ago2, NHA-GW182 or NHA-TNGW1 plus targeted luciferase (either 150ng FL-5BoxB or 10ng RL-5BoxB) and control luciferase (50ng RL or 100ng FL) plasmid using Lipofectamine2000. Cells were harvested 48 hours after transfection and the FL and RL activities were measured using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) following the manufacturer's protocol. Relative luciferase activities (ratio of targeted luciferase activities over control luciferase activities) were first calculated as described in the laboratory of Dr. Filipowicz (Pillai et al., 2004) and the translational repression was calculated based on a recent study (Lytle et al., 2007). Briefly, FL-5BoxB/RL activity in NHA vector transfected (control) group was regarded as 0% translational repression. The repression levels of other experimental groups were calculated by the percentage reduction of relative luciferase activities compared to that in NHA control group. All data were collected in 3 to 6 independent experiments for statistical analysis. The expressions of all NHA constructs were monitored by western blot as shown in Fig. 2-9B.

Quantification of mRNA Degradation Using Quantitative RT-PCR

Total RNA samples from tethering assays were extracted from HEK 293 cell lysates by using RNeasy Mini Kit (Qiagen, Valencia, CA). RNase-Free DNase Set (Qiagen) was applied to eliminate the potential DNA contamination. Samples were analyzed in duplicate by quantitative RT-PCR using SYBR-Green Master mix (Applied Biosystems, Foster City, CA). The relative mRNA levels of FL-5BoxB/RL were calculated by $\Delta\Delta C_t$ method. The melting curve in each

individual measurement was monitored to guard against non-specific amplification. The FL-5BoxB/RL mRNA levels of NHA-Ago2, NHA-GW182 and NHA-TNGW1 were compared to the mRNA level in NHA vector transfected control group, which was defined as 0% mRNA degradation, and calculated the corresponding mRNA degradation. Sequences of primers for RL were: forward 5'-TCCTACGAGCACCAAGACAAGA-3', reverse 5'-GATCACGTCCACGACACTCTCA-3'. Sequences of primers for FL were: forward 5'-GCGACCAACGCCTTGATT-3', reverse 5'-TCCCAGTAAGCTATGTCTCCAGAA-3'. In siRNA tethering assay, the mRNA levels of Ago2 and TNGW1/GW182 were measured using TaqMan® Fast Universal Master Mix (Applied Biosystems) with the corresponding TaqMan® Gene Expression Assay (Ago2, Hs00293044_m1; TNRC6A, Hs00379422_m1 and 18S rRNA, 4310893E, Applied Biosystems).

Results

TNGW1 Was a Novel Isoform of Human GW182

The TNRC6A/GW182 gene locates on human chromosome 16p11.2. The first reported protein isoform of this gene, GW182, has distinct regions enriched in glycine (G) and tryptophan (W) repeats referred to as the GW-rich regions (Eystathioy et al., 2002). GW182 also has a glutamine/asparagine (Q/N)-rich region (Decker et al., 2007) in the middle and a classic RNA recognition motif (RRM) near the C-terminus. The predicted novel form, TNGW1 (Fig. 2-1A), is a protein of about 210 kDa containing a TNR Q-repeat domain (aa93-127) in its N-terminus. The mRNA of TNGW1 contains 5 additional exons upstream of the putative AUG start codon of GW182 (Fig. 2-1B). The TNR Q-repeat domain is encoded by the 5th exon of TNGW1 mRNA and its corresponding nucleotide and amino acid sequence are shown in Fig. 2-1C. Interestingly, based on the genomic sequence data analysis using the University of California Santa Cruz Genome Browser software, the translation initiation sites of these two isoforms are predicted to

be about 60kb apart (Fig. 2-1B). Sequence alignment analysis showed that the N-terminus of TNGW1 was conserved among human, rat, and mouse with some degree of diversity in the Q-repeat region (Fig. 2-1D). Similar domains were not identified in *Drosophila* or *C. elegans* or the two other human homologues TNRC6B and TNRC6C.

Based on the predicted sequence of TNGW1 in the NCBI GenBank database, the RT-PCR assay was designed to verify its existence at the mRNA level using primer sets flanking the unique region of TNGW1 (nt248-1492). The anticipated 1.2kb PCR bands were amplified from cDNA samples of HeLa, HEP-2, HepG2 cells and human testis (Fig. 2-2). The 1.2kb PCR products from HeLa, HEP-2 and human testis were gel-purified, submitted for direct DNA sequencing and verified to be identical to the NCBI reference sequence (NM_014494.2). These results demonstrated that the TNGW1 mRNA, containing an in-frame junction between the novel 5' exons and GW182, could be detected in at least 2 human cancer cell types and a normal adult tissue.

After experimentally verifying that the mRNA of TNGW1 was expressed, we were interested to determine whether GW182 and TNGW1 proteins were both expressed. Antibodies specifically recognizing TNGW1 were developed to complement the previously generated anti-GW182 antibodies (Eystathioy et al., 2002; Eystathioy et al., 2003a). A recombinant polypeptide containing the TNR Q-repeat domain (rTNR, aa1-204) was generated to immunize two rabbits 6225 and 6226. The polyclonal antibodies isolated from both rabbits showed strong reactivity to rTNR by addressable laser beads immunoassay (Fig. 2-3A) or by western blot (Fig. 2-3B) but did not cross-react with GW182 (Fig. 2-3B). Pre-immune antibodies from rabbit 6225 and 6226 as well as the two rabbit polyclonal antibodies to GW182, 5182 and 6642, did not show reactivity to rTNR (Fig. 2-3A, B). Furthermore, three mouse mAb identified as 2E11,

2F11, and 5C8 were generated to rTNR after initial screening and subsequent subcloning. As demonstrated by the addressable laser bead assay, all three anti-rTNR mAb showed high MFU indicating strong reactivity to rTNR (Fig. 2-3C). In contrast, mAb to GW182 (GW182 4B6) and mAb to TNRC6B (GW2 25) had remarkably low MFU which was comparable to culture supernatant controls (Fig. 2-3C). Since there are more than 20 TNR containing genes in the human genome (Margolis et al., 1997), antibodies generated against rTNR could potentially crossreact with Q-repeat sequences of other TNR containing genes. Therefore, additional studies were performed to determine the specificity of each anti-rTNR antibody using synthetic peptide arrays spanning the first 300 amino acids of TNGW1 (Fig. 2-3D). Both the rabbit 6225 and 6226 and human anti-GWB serum 18033 recognized multiple 15mer peptides including those in TNR Q-repeat domain. In contrast, all three mouse anti-rTNR mAb recognized a relatively narrow set of the peptides (peptide 9-11, Table S1) resided outside of TNR Q-repeat domain. In summary, our data demonstrated that all the anti-rTNR antibodies generated recognized specific sequence within N-terminus of TGWN1. The mouse mAb reacted highly specific to TNGW1 with a lower risk of crossreacting with other TNR-containing gene products.

Detection of endogenous GW182 and TNGW1 proteins by standard western blot was challenging because the protein levels were usually very low. Hence, separating and distinguishing these high molecular mass proteins required careful optimization. To demonstrate the specific expression of these proteins in HeLa cells, IP-WB was carried out by using the human serum 18033, an anti-GWB serum known to contain antibodies to GW182, Ago2, and Ge-1, to enrich these protein complexes from HeLa cell lysates prior to western blot detection. If TNGW1 is present along with GW182 in HeLa cells as predicted from the RT-PCR data (Fig. 2-2), anti-GW182 antibodies will recognize two bands because TNGW1 includes the entire

sequence of GW182. As expected, the two rabbit polyclonal antibodies to GW182 (5182 and 6642), and the mouse mAb to GW182 (4B6) recognized both forms of GW182 proteins where the faster-migrating band considered to be GW182 was the predominant isoform (Fig. 2-4A, left panel). In support of this conclusion, the anti-rTNR mAb (2E11, Fig. 2-4A and 5C8, Fig. 2-4B) as well as rabbit anti-rTNR sera (6225 and 6226, Fig. 2-4B) recognized only the slower-migrating TNGW1. These data confirmed that both TNGW1 and GW182 proteins were expressed in HeLa cells.

There are three possibilities as to how TNGW1 and GW182 are expressed in cells. The first is that TNGW1 and GW182 are independently translated from two individual mRNA transcripts derived from chromosome 16 with different transcriptional start sites separated by ~60kb. The second possibility is that TNGW1 and GW182 are translated from the same mRNA with two different AUG start sites governed by respective Kozak's consensus sequences (Kozak, 1991). The third possibility is that GW182 is a post-translationally processed product of TNGW1. To address these possibilities, we designed siRNA specific for TNGW1 mRNA (siTNR) to examine the effect of suppressing TNGW1 mRNA on the expression of TNGW1 and GW182 protein. The knock-down effect of siTNR was initially validated by demonstrating its repression on the expression of co-transfected EGFP-TNGW1 but no effect on the expression of co-transfected EGFP-GW182 in HeLa cells (data not shown). Forty-eight hours after siTNR transfection, western blot analysis of cell lysates showed that only the TNGW1 band disappeared whereas the GW182 band remained the same (Fig. 2-4C, lane 1) as compared to the untreated (Fig. 2-4C, lane 4) or mock transfected controls (Fig. 2-4C, lane 3). These observations indicated that TNGW1 was derived from its own unique mRNA. However, if GW182 was post-translationally processed from TNGW1 as considered above, the GW182 band in the siTNR

transfected (Fig. 2-4B, lane 1) would have represented stable processed products under these experimental conditions. Contradicting to this possibility, transfection of siRNA targeting the common region of TNGW1 and GW182 (siGW182) resulted in the disappearance of both isoforms (Fig. 2-4B, lane 2). This data supported the conclusion that GW182 was not processed from TNGW1 and that TNGW1 and GW182 was transcribed independently. In summary, our data demonstrated that TNGW1 and GW182 are distinct at both transcription and translation levels.

Intracellular Localization of TNGW1 and Its Relationship with Other GWB Components

GW182 is one of the accepted marker proteins of GWB (Eystathioy et al., 2002) and has been shown important for GWB formation (Jakymiw et al., 2005; Lian et al., 2007). Since TNGW1 shares the same amino acid sequence as GW182, except for the N-terminal domain including the TNR Q-repeat region, we were interested to examine the intracellular location of TNGW1 and its role in GWB formation. The immunofluorescence staining of mouse monoclonal anti-rTNR 2F11 on HEp-2 cells showed GWB staining that was also recognized by the human anti-GWB serum 18033 (Fig. 2-5A, arrows). Notably, both mouse anti-rTNR 2F11 (Fig. 2-5A) and rabbit anti-rTNR 6226 (Fig. 2-6A) stained a subset of about 30% GWB. In contrast, anti-GW182 mAb 4B6 stained more, although not all, GWB recognized by 18033 (Fig. 2-6B). The data implies that the amount of TNGW1 and GW182 may vary in different GWB even when they are present in GWB. We therefore examined this hypothesis by performing dual staining with anti-rTNR 2F11 and anti-GW182 4B6 in the same HEp-2 cells (Fig. 2-5B). Visualized by IgG subclass-specific secondary antibodies, anti-GW182 4B6 stained apparently more GWB than anti-rTNR 2F11, while all 2F11 staining colocalized with 4B6 staining. The results support the hypothesis that TNGW1 may be absent, or at least in very low abundance in a subset of GWB, where only GW182 is present as the predominant isoform. However, given the

limitation of antibodies, we could not rule out the possibility that the immunoreactive region of these isoform were obscured by the presence of one or more additional GWB component. Nevertheless, our data indicated that TNGW1 resided in a subset of GWB and this clearly demonstrated heterogeneity in GWB contributed by GW182 gene products.

GW182 was once proposed to be a matrix protein in GWB because it was required for the assembly of these foci (Yang et al., 2004), which also harbored multiple proteins including Ago2, Dcp1a, Ge-1, and RAP55 (Jakymiw et al., 2007). Therefore, we decided to determine whether the extra N-terminal domain in TNGW1 would affect the localization of other GWB components to GWB. Immunofluorescence assay was performed on HeLa cells where EGFP-Ago2 was co-transfected with either GST-GW182 or GST-TNGW1. Fig. 2-7A shows that both GST-GW182 and GST-TNGW1 are enriched in cytoplasmic foci together with EGFP-Ago2, indicating the extra N-terminal region of TNGW1 did not interfere with the localization of TNGW1 or Ago2 to GWB. Furthermore, Fig. 2-7B shows that in cells transfected with EGFP-TNGW1 alone the EGFP-labeled GWB were also co-stained with anti-Dcp1a antibodies and human serum IC6 containing antibody to Ge-1 and RAP55 (Bloch et al., 2006). Notably, transfected cells with either low or high expression of EGFP-TNGW1 did not apparently affect the localization of endogenous Dcp1a and Ge-1/RAP55 to the TNGW1-containing foci suggesting that TNGW1 could efficiently substitute for putative GW182 functions such as recruitment of Ago2 and formation of foci enriched in RNA decay factors.

TNGW1 Is Not Essential for the Formation of GWB

As shown in previous studies, GW182 is essential for the formation of microscopically visible GWB. However, since the existence of TNGW1 was not appreciated at that time, previous conclusions were based on the knock-down of both TNGW1 and GW182. Since we showed that siTNR achieved almost complete knock-down of TNGW1 without affecting the

level of GW182 (Fig. 2-4B), we could determine whether TNGW1 knock-down affects the formation of GWB. SiTNR was transfected into HeLa cells and the changes of GWB were monitored at day 0, 1, 2 and 3. SiGW182 was transfected side by side into HeLa cells as a control and the formation of GWB was monitored by co-staining with rabbit anti-Dcp1a and human anti-GWB serum 18033. Consistent with the western blot data (Fig. 2-4C), 2 days after transfection, the siGW182 transfection led to the disassembly of most GWB presumably due to knock-down of both TNGW1 and GW182 (Fig. 2-8). In contrast, 2 and 3 days after siTNR transfection, microscopic GWB were still detected by both anti-Dcp1a and serum 18033. This observation demonstrated that TNGW1 was not important for GWB formation under these experimental conditions.

TNGW1 and GW182 Exert Strong Repression Effect in Ago2 Mediated Translational Silencing

To explore the effect of translational repression attended by TNGW1 compared to GW182 or Ago2, we adopted the tethering assay from the work of Pillai et al. (Pillai et al., 2004). An N-terminal tag λ N-HA (NHA) polypeptide was fused to TNGW1, GW182 and Ago2. The λ N tag binds the 5BoxB secondary structures harbored in the 3'-UTR of the FL-5BoxB RNA resulting in a tethering effect of the tagged protein to the 3'-UTR. The repression effect in human 293 cells was evaluated by comparing the FL-5BoxB activities among different experimental groups relative to the untargeted RL activities (Fig. 2-9A) using the method described in a previous study (Lytle et al., 2007). After 48 hours of transfection, FL-5BoxB activity was repressed by 46% when Ago2 was tethered to the reporter. Interestingly, tethered TNGW1 or GW182 induced strong repression on reporter (67.6% and 65.3%, respectively) which was 46.9% or 41.3% stronger than that induced by Ago2, respectively (Fig. 2-10A). Comparison of the corresponding FL-5BoxB and RL mRNA levels by quantitative RT-PCR assay showed that both

tethered TNGW1 and GW182 was accompanied by a 23.7% and 24.5% reduction in FL-5BoxB mRNA, respectively, whereas tethered Ago2 had an associated 50.8% reduction in FL-5BoxB mRNA (Fig. 2-10B). Therefore, the analysis of translation efficiencies of FL-5BoxB mRNA in each experimental group calculated using the formula described in previous study (Lytle et al., 2007) showed that the tethered TNGW1 and GW182 reduced the translational efficiency of FL-5BoxB mRNA (42.5% and 46.0% respectively) to a significantly greater extent than tethered Ago2 (109.7%), which repressed the FL-5BoxB activity with a lower abundance of reporter mRNA at 48 hours (Fig. 2-10C). Previously, Pillai et al. have shown that tethering Ago2 to RL reporter with 5BoxB in 3'-UTR mainly induced translational repression in HeLa cells (Pillai et al., 2004). Our observations, however, indicated that tethered Ago2 repressed FL-5BoxB activity was accompanied by a reduced level of reporter mRNA in 293 cells. The discrepancy observed in RNA level may be caused by the usage of different cell lines (293 vs. HeLa), or a difference in mRNA turnover between FL and RL reporters.

With the interesting finding that TNGW1 and GW182 exerted a stronger translational repression effect than Ago2, the next issue was to address the inter-dependence of these proteins in translational repression. The same tethering assay was performed in HeLa with the introduction of siRNA transfection to knock-down either Ago2 or GW182 prior to the co-transfection of tethering protein and reporter constructs. Surprisingly, the repression effect by NHA-Ago2 was totally abolished in cells with GW182 knock-down (Fig 2-11A). In contrast, the repression effect of tethered GW182 or TNGW1 was not affected by knock-down of Ago2 (Fig. 2-11B). The observations that tethered Ago2 was required GW182/TNGW1 for translational suppression whereas tethered GW182 did not require Ago2 for suppression was reproducible when either FL-5BoxB or RL-5BoxB was used as the reporter (Fig. 2-12). Our data implied that

both TNGW1 and GW182 contributed further direct effect on repression than Ago2 and they were required for Ago2 mediated translational silencing.

Discussion

Expression of Human TNRC6A/GW182 Gene and Its Effect on GWB Formation

In this study, we have identified TNGW1 as a novel 210kDa isoform of GW182 with both proteins highly enriched in GWB. As predicted in the NCBI GenBank database, the amino acid sequences of TNGW1 and GW182 are identical with the exception that TNGW1 has an extra 253aa-polypeptide in its N-terminus. TNGW1 was expressed in several human cancer cell lines and human testis along with the reported short isoform GW182. Our data demonstrated that TNGW1 and GW182 were expressed independently and GW182 was the predominant gene product. The expression of their mRNAs is possibly due to alternative splicing, alternative promoters and transcriptional start sites. The expression level of each isoform under different cellular conditions with potentially different RNAi activities and the factors determining their expression need further investigation.

Both TNGW1 and GW182 were highly enriched in GWB but TNGW1 was detected only in about one third of endogenous GWB. Knock-down of TNGW1 did not noticeably disrupt GWB formation indicating that TNGW1 was not required for the formation of many GWB. Both TNGW1 and GW182 exerted similar translational repression effect in the tethering assay. These data implied that TNGW1 might be functionally redundant and GW182 plays a more important role in the formation of GWB. However, this interpretation needs to be solidified by determining the effect of TNGW1 on GWB formation in the absence of GW182. Since an efficient method to knock-down GW182 without affecting TNGW1 is not currently available, it remains unclear whether TNGW1 alone can substitute for all putative function for GW182. Given that TNGW1 contains the whole amino acid sequence of GW182, it is likely that TNGW1

is capable of most functional characters of GW182. With the extended N-terminal region containing TNR Q-repeat domain, the full functional characteristics for TNGW1, in addition to GW182, remain to be determined.

Interdependence of Ago2 and TNGW1/GW182 in miRNA-Mediated Translational Repression

In mammalian system, Ago2 is considered the most important factor in the RISC because it binds siRNA and miRNA as well as being the only factor harboring the slicing activity responsible for siRNA-induced silencing (Liu et al., 2004; Yuan et al., 2005). However, the mechanism for miRNA-mediated repression remains unclear. In the presence of incomplete complementarity between anti-sense strand miRNA and its target mRNA, the “slicing” function of Ago2 is interfered, where Ago2 may need to recruit multiple factors to secure its repression effect on the targeted mRNA and possibly induce the subsequent mRNA degradation. GW182 has been reported as more important in gene silencing when slicing activity is limited, for example, in miRNA-mediated silencing (Liu et al., 2005a; Chu and Rana, 2006). In the present study, we extended the understanding of their interdependence by tethering TNGW1, GW182, or Ago2 to the 3'-UTR of a luciferase reporter mRNA and comparing their relative potentials in translational repression. Our data demonstrated that both TNGW1 and GW182 exerted stronger translational repression than Ago2. Furthermore, the repression effect of tethered Ago2 was sensitive to the presence of TNGW1 and/or GW182, while repression by tethered TNGW1 or GW182 did not require Ago2. These observations suggested that either TNGW1 or GW182 has a more direct impact on translational repression than Ago2. Although it is possible that other Argonaute proteins could substitute for Ago2 when it was knock-down, the functional importance of TNGW1 and/or GW182 was obvious even though human cells may also have two homologues of GW182, TNRC6B and TNRC6C. In miRNA-mediated translational silencing,

the miRNA loaded in Ago2 can direct GW182 to their targeting mRNA and repress its translation. Hence, when TNGW1 or GW182 was tethered to the reporter mRNA via λ N tag, it may bypass the requirement of miRNA-Ago2 guidance. Consistent with this observation, Eulalio et al. have shown that the interaction between GW182 and Argonaute proteins was essential for miRNA mediated translational repression and mRNA decay in *Drosophila* (Eulalio et al., 2008b). However, whether the repression effect is caused directly by TNGW1 and/or GW182 or the additional factors recruited in later stages of the process requires further investigation.

In summation, our data suggest the following scenario with a putative order in the sequence of events. With the guidance of miRNA, Ago2 is able to target to the 3'-UTR of a specific mRNA. GW182/TNGW1 is subsequently enriched to the 3'UTR due to its interaction with Ago2. Once GW182/TNGW1 is brought to the 3'-UTR, the translational suppression is triggered either directly by GW182/TNGW1 or by other factors further recruited to the complex. In the tethering assay, tethered GW182/TNGW1 did not require miRNA and Ago2 for translational suppression because their function is substituted by the interaction of the λ N tag and BoxB sequence. As more studies of translational repression are reported (Filipowicz et al., 2008; Eulalio et al., 2008a), the importance of GW182 in each step may be further characterized in future studies.

The Functional Differences of GW182 Isoforms and the Heterogeneity of GWB

It is possible that TNGW1 and GW182 are redundant protein products of the human TNRC6A gene in some aspects of translational repression process. Our data showed that they both formed cytoplasmic foci that colocalized with other RNAi related and mRNA decay factors. As demonstrated by the functional assays tested in the current study, both isoforms induced translational repression and mRNA degradation to a similar extent. However, the hypothetical

redundancy between TNGW1 and GW182 may be due to our limited understanding of their functions. A similar case could also be made for the human Argonaut protein family that is comprised of at least 4 Argonaute proteins (Ago1-4) that share over 90% sequence similarity. Except for Ago2, known as the catalytic engine of RISC (Liu et al., 2004), the biological functions and significance of Ago1, 3 and 4 are not well understood.

One distinguishing feature of TNGW1 is that it is only localized to a subset of GWB in HEp-2 cells. This specific localization of TNGW1 is likely related to its unique N-terminal polypeptide domain that is not found in GW182. This N-terminal domain may be responsible for interacting with or recruiting protein factors to help with translational suppression. It is also possible that this unique N-terminal domain can affect the protein folding of TNGW1 and somehow interfere with its interaction with Argonaute proteins. The fact that TNGW1 functional capacity is similar to GW182 makes it important to further investigate the potential differences in their functions.

Our data confirmed there was heterogeneity in GWB in terms of TNGW1 and GW182 distributions, which is consistent with the observations from recent reports (Jakymiw et al., 2007; Moser et al., 2007). The importance of this heterogeneity could be a reflection of different stages of GWB assembly. However, it could also reflect the diversity in the functional status of GWB, which are closely related to miRNA-mediated function. When targeted by miRNA, most mRNAs may enter the accelerated turnover process whereas some may not. A recent report showed that two luciferase reporter mRNAs carrying different 3'-UTR were degraded at different rates when both were translationally repressed (Eulalio et al., 2008b). Under stress conditions, the translational efficiency of some miRNA targeted mRNAs were reported to be up-regulated (Bhattacharyya et al., 2006a; Vasudevan et al., 2007), which required these targeted

mRNA to remain stable during miRNA-targeting. Interestingly, the determining factors in the turnover of targeted mRNA seemed not only depend on the miRNA targeting sequence but also on the proteins that were recruited during the silencing process (Bhattacharyya et al., 2006b). Since the formation of GWB was shown to be a consequence of miRNA activity (Pauley et al., 2006; Eulalio et al., 2007b), it is not surprising that there are multiple functional roles for these cytoplasmic foci. The identification of the TNGW1 as a novel isoform of GW182, and the heterogeneous distribution of TNGW1, GW182, and other RNAi factors in GWB (Jakymiw et al., 2007), will provide us a better understanding of the RNAi process at molecular cell biology level.

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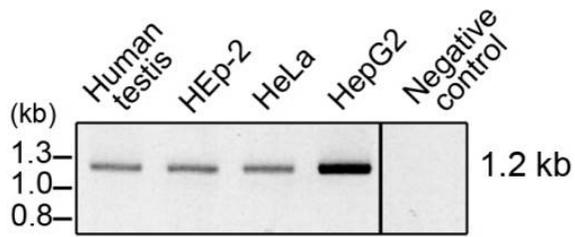


Figure 2-2. TNGW1 mRNA containing the TNR exon detected in human testis and different cell lines using RT-PCR. Primer set flanking the TNR region of TNGW1 mRNA (362-1626nt) amplified 1.2 kb bands from cDNA samples of human testis tissue, HEp-2, HeLa and HepG2 cells. The amplified 1.2 kb products from HEp-2, HeLa and human testis tissue were subsequently purified and their consensuses to reference sequence were confirmed by direct DNA sequencing.

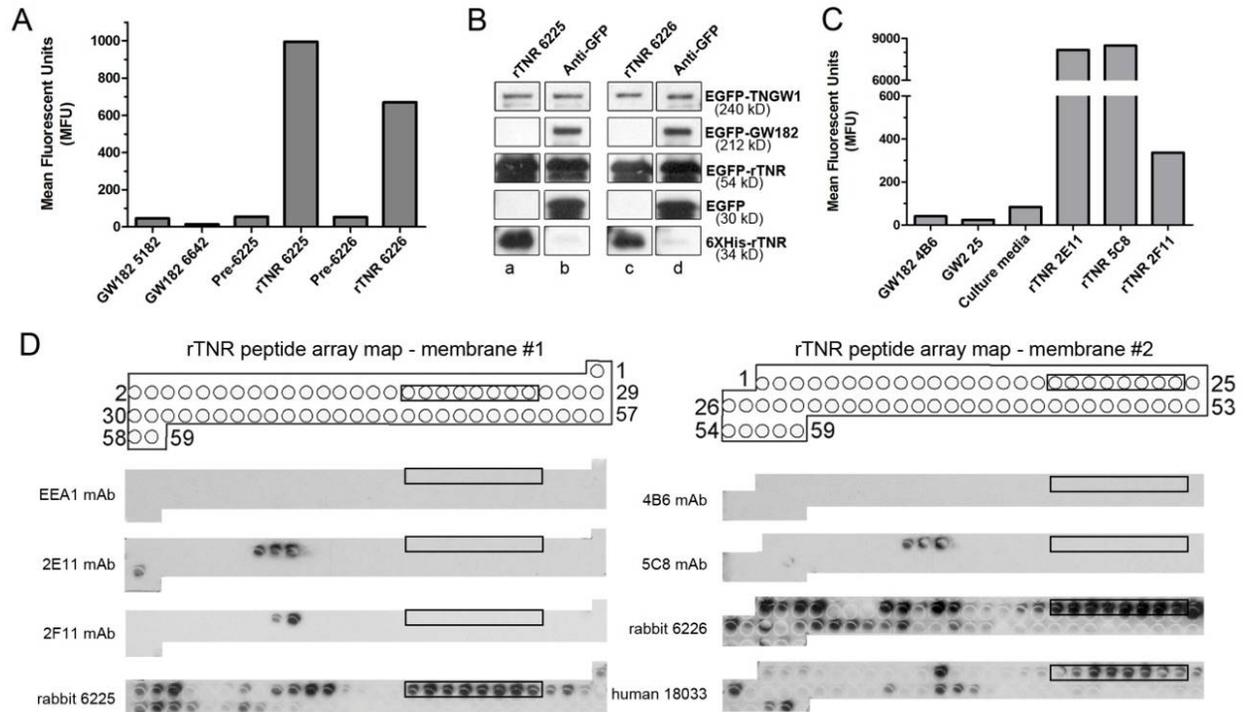


Figure 2-3. Production and characterization of polyclonal and monoclonal antibodies specific to the TNGW1 isoform. A) Rabbit polyclonal anti-rTNR antibodies strongly reacted with rTNR polypeptide coated in addressable laser bead immunoassay. Compared to 2 rabbit anti-GW182 antibodies (5182 and 6642) and the pre-immune rabbit sera, rabbit anti-rTNR sera 6225 and 6226 strongly reacted with rTNR-coated beads. B) Rabbit anti-rTNR antibodies recognized only TNGW1 but not GW182 in western blot. HeLa cells transfected with EGFP-tagged TNGW1, GW182, TNR and EGFP vector were harvested 24 hours after transfection. These cell lysates together with recombinant 6XHis-tagged TNR were analyzed by western blot using rabbit anti-rTNR sera 6225 and 6226. The membranes shown in column a and c were first blotted with rabbit anti-rTNR antibodies, stripped, and then re-blotted with anti-EGFP antibodies to confirm the recombinant proteins were expressed (column b and d, respectively). C) Reactivity of mouse anti-rTNR mAb with rTNR polypeptide in addressable laser bead immunoassay. Three mouse mAb 2E11, 5C8 and 2F11 showed strong MFU to rTNR, whereas control mAb anti-GW182 4B6, anti-GW2-25 and culture medium showed little or no reactivity. Note that mAb 2E11 and 5C8 showed ~20 folds higher MFU than 2F11. D) Peptide array mapping of antibody reactivity to TNR region. Two duplicate peptide array membranes containing 59 sequential 15-amino acid peptides, each with 5-amino acid-offset, spanning the TNR region were synthesized and used to examine antibody specificity. Membrane #1 (left) was probed sequentially with anti-EEA1 control mAb, anti-rTNR mAb 2E11, 2F11 and rabbit anti-rTNR 6225. Membrane #2 was probed sequentially by anti-GW182 mAb 4B6, anti-rTNR mAb 5C8, rabbit anti-rTNR 6226 and human serum 18033. The black box shows that the peptide strings reside in TNR Q-repeat domain as indicated by underline in Figure 2-1C. All rabbit and human sera showed reactivity to multiple peptides. The 3 mouse anti-rTNR mAb recognized relatively narrow region outside of the TNR Q-repeat domain.

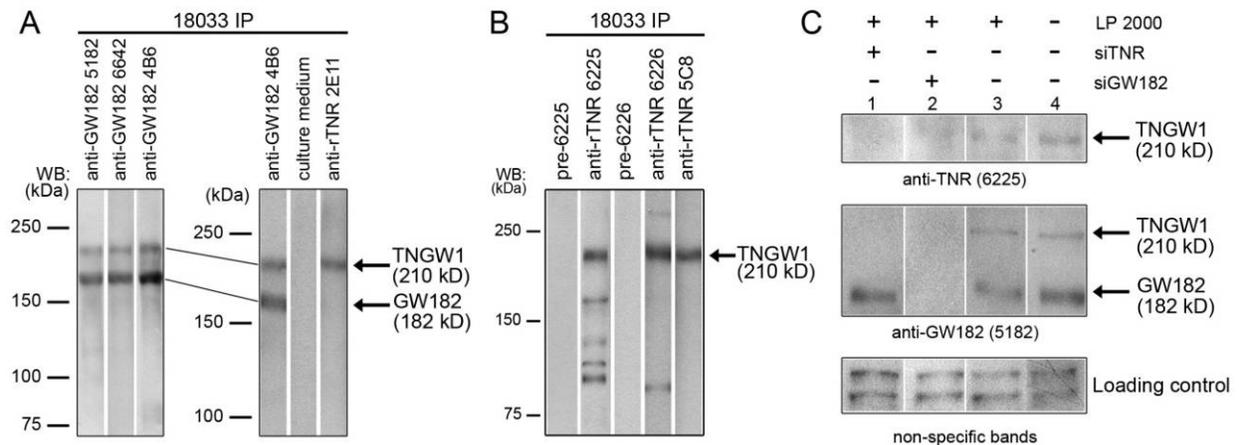


Figure 2-4. TNGW1 and GW182 were independent products of human TNRC6A gene. A) Expression of both TNGW1 and GW182 were detected in HeLa cells. GWB components were enriched by IP using human anti-GWB serum 18033 and analyzed by western blot using rabbit anti-GW182 antibodies 5182 and 6642, mouse anti-GW182 mAb 4B6 and anti-rTNR mAb 2E11. All 3 anti-GW182 antibodies recognized 2 bands migrating about 210 and 180 kDa, where anti-rTNR 2E11 only recognized the 210 kDa band regarded as TNGW1, the novel isoform of GW182. B) TNGW1 was detected by multiple anti-rTNR antibodies. 18033 IP samples were also examined by rabbit polyclonal antibodies 6225, 6226, and mouse mAb 5C8 generated to the N-terminal domain rTNR. A consistent 210 kDa band was detected by each of these antibodies regarded as TNGW1. C) TNGW1 knockdown using siRNA specific to the TNGW1 did not affect the level of GW182 in HeLa cells. HeLa cells were transfected with siRNA specific to TNGW1 (siTNR) or both forms (siGW182) using lipofectamine 2000 (LP 2000). After 48 hours of transfection, cells were lysed and analyzed by western blot using rabbit anti-rTNR serum 6225 or anti-GW182 serum 5182. Compared to mock transfection using lipofectamine 2000 (LP2000) and untreated control groups, siGW182 knock-down both TNGW1 and GW182 whereas siTNR only knock-down TNGW1.

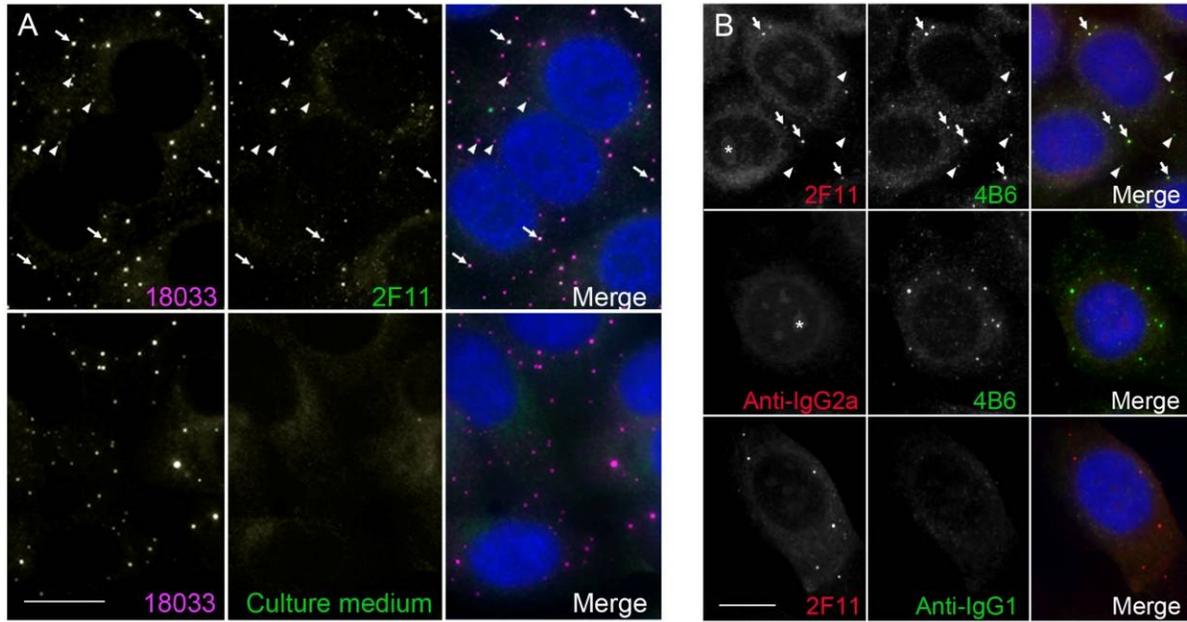


Figure 2-5. TNGW1 resided in a subset of GWB (part1). A) Mouse monoclonal anti-rTNR antibody 2F11 stained a subset of GWB recognized by prototype serum 18033. HEp-2 cells were stained with mouse mAb 2F11, which stained a subset of GWB detected by serum 18033 (arrows) but did not recognize other GWB (arrowheads). Culture medium was used as a negative control. Bar, 10 μ m. B) Mouse monoclonal anti-rTNR antibody 2F11 stained a subset of GWB recognized by monoclonal anti-GW182 antibody 4B6. HEp-2 cell staining was performed similarly with anti-rTNR 2F11 (IgG2a) and anti-GW182 4B6 (IgG1) and different fluorochrome conjugated mouse IgG subclass specific secondary antibodies to evaluate the distribution of TNGW1 in GW182-positive GWB. Anti-rTNR 2F11 stained some of the foci detected by 4B6 (arrows) but not others (arrowhead). The second and third row of images show controls with either 4B6 or 2F11 and stained with both secondary antibodies to demonstrate secondary antibody specificities. The asterisk shows nonspecific nucleolar staining detected by the anti-IgG2a secondary antibodies. Bar 10 μ m.

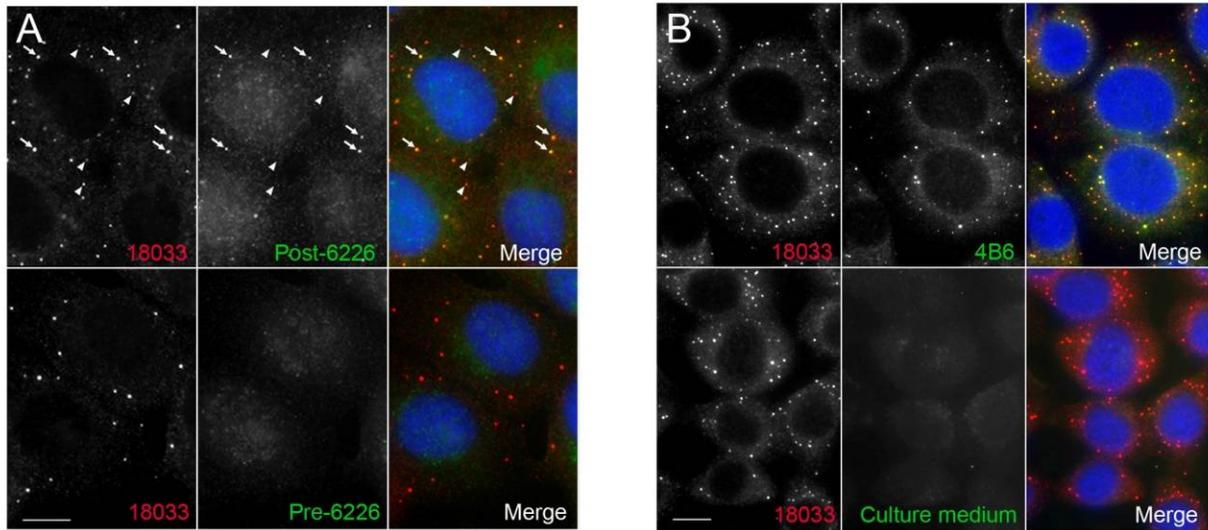


Figure 2-6. TNGW1 resided in a subset of GWB (part 2). A) Rabbit polyclonal anti-rTNR serum 6226 stained a subset of GWB in HEp-2 cells. Anti-rTNR (Post-6226) stained a subset of GWB recognized by human serum 18033 (arrows) whereas the pre-immune serum (Pre-6226) from the same rabbit did not have any GWB staining. Note some GWB (arrowheads) are not recognized by anti-rTNR. The diffuse nuclear and cytoplasmic staining for rabbit 6226 was not related to TNGW1 because similar staining was observed with the pre-immune serum. Bar, 10 μ m. B) Anti-GW182 mAb 4B6 co-stained the majority of GWB recognized by human anti-GWB prototype serum 18033 in HEp-2 cells. Culture medium was used as a negative control. Bar, 10 μ m.

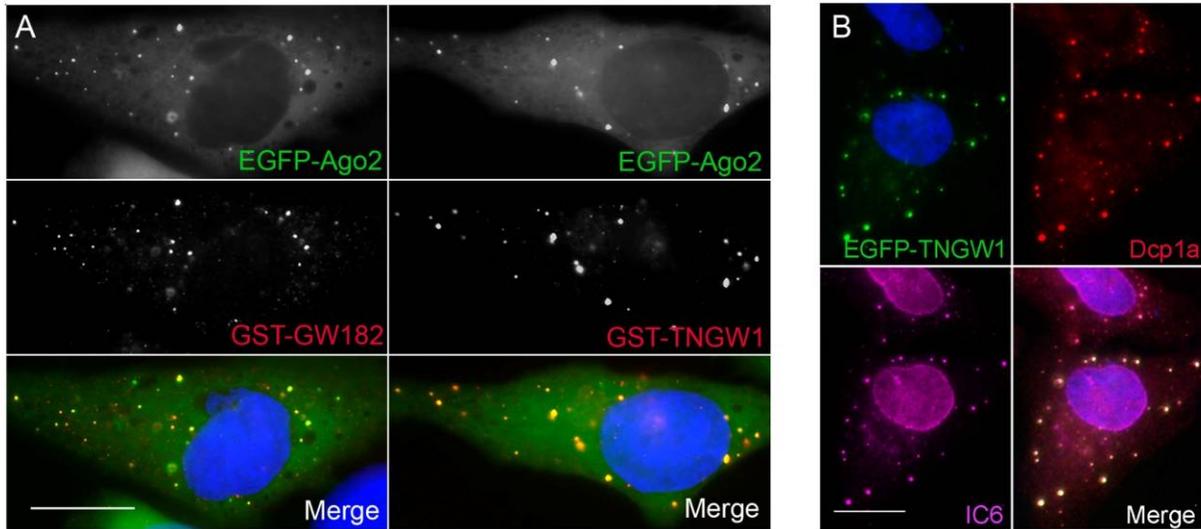


Figure 2-7. Intracellular localization of TNGW1 with other GWB components. A) Both TNGW1 and GW182 colocalized to GWB with transfected Ago2. HeLa cells transfected with either GST tagged GW182 or TNGW1 (red) and EGFP-Ago2 (green) were fixed and analyzed by indirect immunofluorescence assay 24 hours after transfection. Both TNGW1 and GW182 formed cytoplasmic foci and colocalized with Ago2. Bar, 10 μ m. B) Transfected TNGW1 colocalized with RNA decay factors enriched in GWB. HeLa cells transfected with EGFP-TNGW1 were fixed 24 hours after transfection and analyzed by indirect immunofluorescence assay using rabbit anti-Dcp1a (red) and human serum IC6 (magenta, recognizes Ge-1, RAP55 and unrelated nuclear envelop protein). The transfected EGFP-TNGW1 formed cytoplasmic foci that were co-stained by both anti-Dcp1a and IC6 antibodies. Bar, 10 μ m.

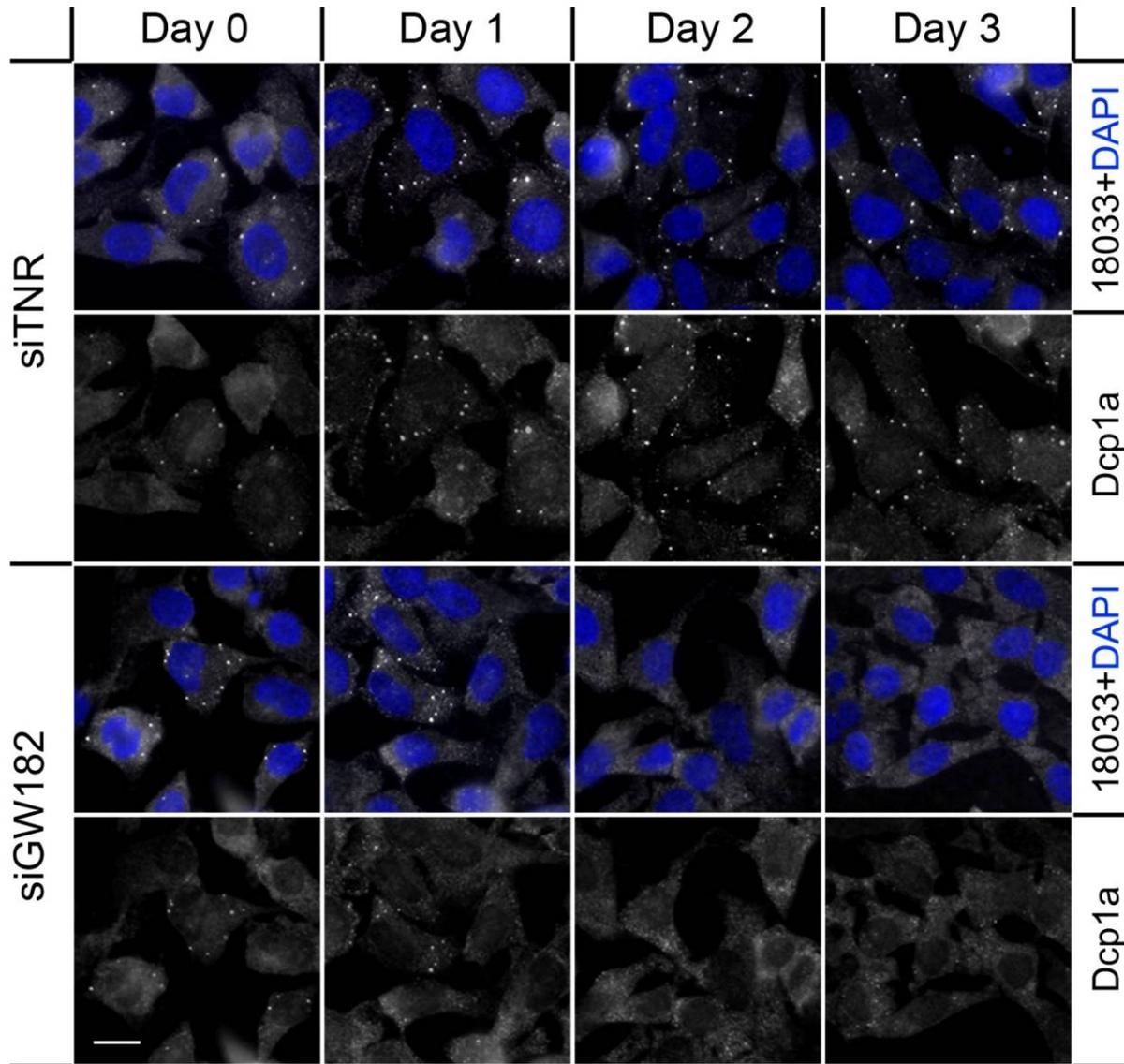


Figure 2-8. Knockdown of TNGW1 has no apparent effect on the assembly of GWB in HeLa cells. HeLa cells were transfected with siTNR or siGW182 and then harvested at 0, 1, 2 or 3 days after transfection. Indirect immunofluorescence assay was performed by using rabbit anti-Dcp1a (red) and human anti-GWB serum 18033 (green) to examine the effect of either siRNA on GWB formation. Bar, 10 μ m.

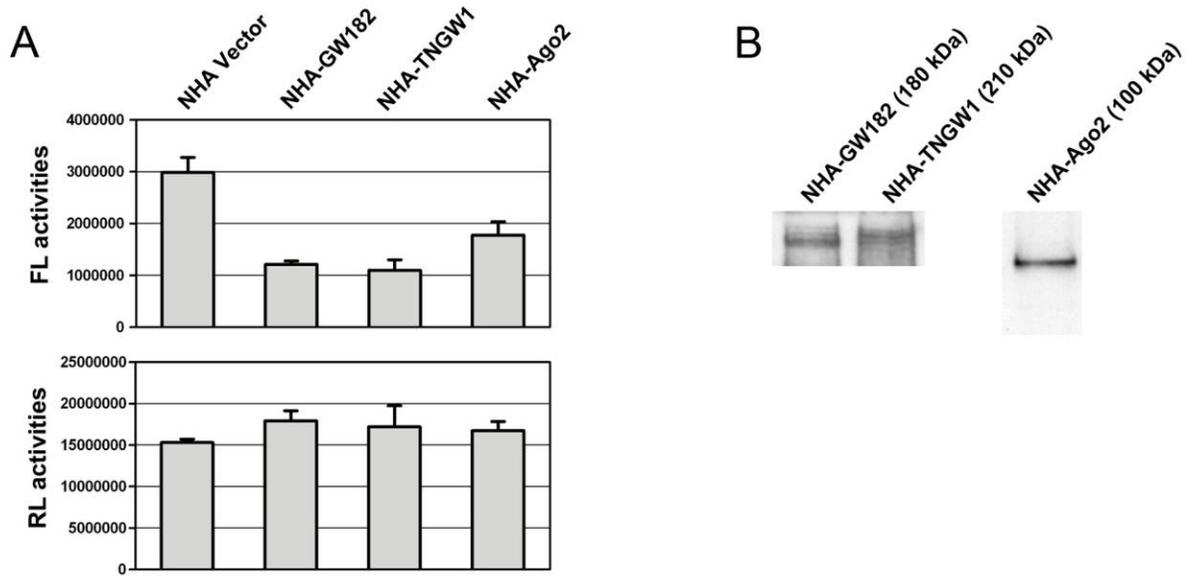


Figure 2-9. Dual luciferase assay measurement and NHA constructs expression in 293 cells. A) Absolute reading of either FL or RL activities in the tethering assay performed in 293 cells (Fig. 2-10). Error bars show standard deviations. B) Western blotting data showed the expression of each NHA tagged construct in the tethering assay.

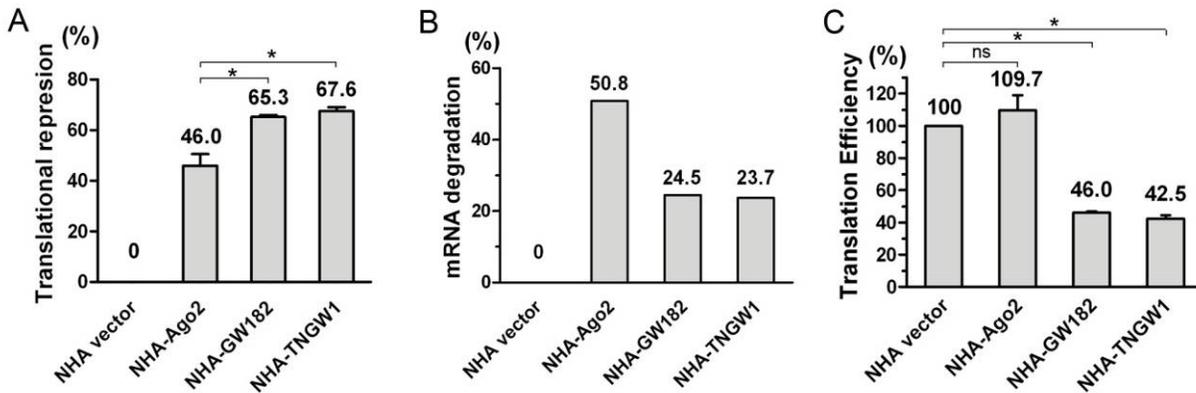


Figure 2-10. Tethered TNGW1 and GW182 exerted translational repression to a greater extent than Ago2. A) Both tethered TNGW1 and GW182 exerted stronger repression effect than tethered Ago2. Assays for both luciferase activities were performed 48 hours after transfection. Tethered TNGW1 and GW182 showed 67.6% and 65.3% translation repression effect to FL-5BoxB reporter, which was significantly higher than tethered Ago2 respectively (asterisk $P < 0.05$, t test). Measured FL-5BoxB activities were normalized to corresponding RL activities. All translation repression effects were estimated by the differences of FL-5BoxB/RL activity compared to that in NHA vector transfected group. Error bars represent standard deviations. B) Tethered TNGW1 or GW182 induced less reporter mRNA reduction than tethered Ago2. The mRNA levels from the same assay were measured by SYBR-green quantitative real time PCR. The degradations of FL-5BoxB mRNA were determined by the reductions of the mRNA level between experimental groups and NHA vector transfected group. All FL-5BoxB mRNA levels were normalized to RL mRNA to minimize the experimental errors. C) Tethered TNGW1 and GW182 strongly reduced the translation efficiency of the reporter compared to tethered Ago2. Translation efficiencies of FL-5BoxB in different groups were calculated by the ratio of relative FL-5BoxB activities to their mRNA levels. In 293 cells, tethered Ago2 did not significantly (ns, t test) alter the FL-5BoxB reporter translation efficiency. However, tethered TNGW1 and GW182 reduced the translation efficiency FL-5BoxB reporter by 57.5% and 54.0% respectively (asterisk, $P < 0.01$, t test). Error bars represent standard deviations.

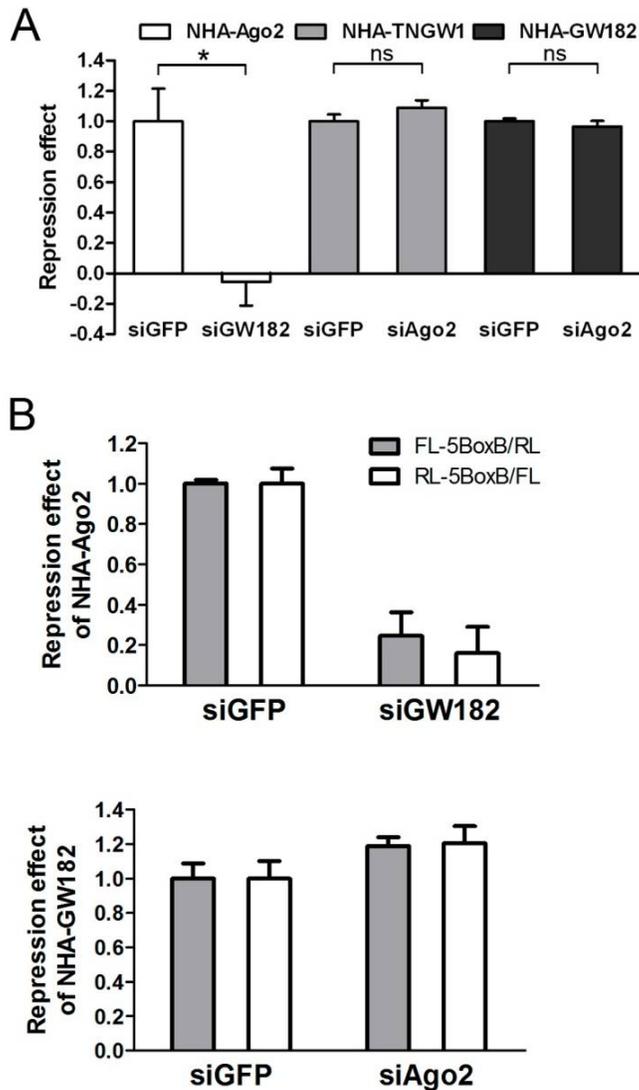


Figure 2-11. Translational repression of tethered Ago2 required endogenous TNGW1 and/or GW182. A) The repression effect of tethered Ago2 was abolished when TNGW1 and GW182 were knocked down while tethered TNGW1 or GW182 maintained repression in the absence of Ago2. HeLa cells were transfected with different siRNA 24 hours prior to the transfection of NHA-tagged constructs and RL-5BoxB/FL reporters. The translation repression effect related to tethered Ago2, TNGW1 or GW182 was determined by reduction of RL activity and the repression effect in siRNA to EGFP (siGFP) transfected group was normalized as 1. The repression effect caused by NHA-Ago2 was abolished when cells were treated with siGW182 (asterisk, $P < 0.01$, t test). Knock-down of Ago2 (siAgo2) caused no significant (ns, t test) effect on tethered GW182 or TNGW1 induced translational repression. Error bars represent standard deviations. B) Tethered Ago2-mediated gene silencing required TNGW1 and/or GW182 regardless either FL-5BoxB or RL-5BoxB was used as targeted reporter. Similar experiments were performed as described previously except for the use of RL-5BoxB as reporter together with FL reporter as co-transfection quantitative control. The repression effect of tethered Ago2 was abolished consistently when FL-5BoxB or RL-5BoxB was used as reporter while tethered GW182 maintained its repression to both reporters in the absence of Ago2.

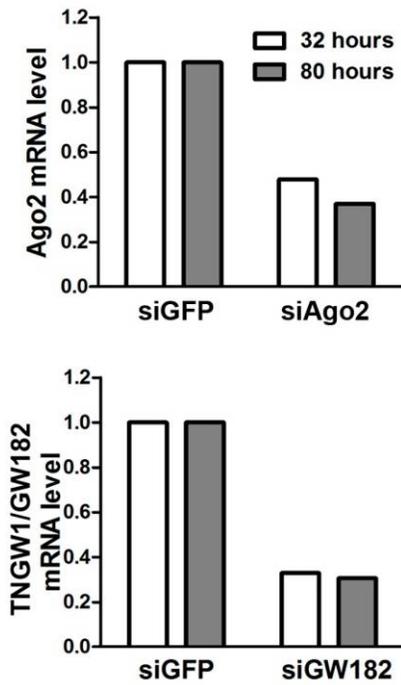


Figure 2-12. Quantitative real time PCR showed corresponding siRNA knock-down effect in the tethering assay. The mRNA levels of Ago2 and TNGW1/GW182 were determined by Taqman® real time RT-PCR after siRNA treatment in the tethering assay (Figure 2-11). Both Ago2 and TNGW1/GW182 were reduced over 60% 80 hours after siRNA treatment.

CHAPTER 3
THE C-TERMINAL HALF OF AGO2 BINDS TO MULTIPLE GW-RICH REGIONS OF
GW182 AND REQUIRES GW182 TO MEDIATE SILENCING

Introduction

MicroRNA (miRNA)-mediated gene silencing is an important post-transcriptional regulation which controls in part the half lives of mRNA targets. In this regulation, miRNA binds to the 3'-UTR of mRNA leading to translational inhibition, mRNA degradation, and mRNA sequestration (Nilsen, 2007). MiRNAs are evolutionarily conserved in most of species and are estimated to regulate ~30% of protein-encoding genes in human (Lewis et al., 2005; Filipowicz et al., 2008).

There are four human Ago proteins that include Ago1 to Ago4, which are the core components of silencing effector complexes and are known to bind single-stranded miRNA. These Ago proteins share greater than 80% identity and are primarily characterized by PAZ and PIWI domains. The PAZ domain contains a binding pocket for the 3' overhanging nucleotides of miRNA. Interestingly, despite highly conserved sequences, only the PIWI domain of Ago2 harbors RNase H-type activity and, therefore, Ago2 also functions in siRNA-mediated slicing of mRNA targets (Liu et al., 2004; Yuan et al., 2005). Tethering Ago proteins to the 3'-UTR of mRNA mimicked miRNA function and effected translational repression (Pillai et al., 2004).

GW182 is important for miRNA-mediated translational silencing and interacts with Ago2. GW182 contains several glycine/tryptophan-rich (GW-rich) regions, a glutamine/asparagine-rich (Q/N-rich) domain, and a C-terminal RNA recognition motif (RRM) (Eystathiou et al., 2002; Decker et al., 2007). GW182 localized to and was essential for the formation of GW bodies (GWB, also known as mammalian P bodies) (Yang et al., 2004; Schneider et al., 2006), cytoplasmic structures closely linked to mRNA decay (Sheth and Parker, 2003; Eystathiou et al., 2003c) and the miRNA/siRNA pathway (Jakymiw et al., 2005; Lian et al., 2006; Pauley et al.,

2006; Lian et al., 2007). Knockdown of GW182 greatly impaired miRNA-mediated gene silencing and subsequent mRNA degradation (Rehwinkel et al., 2005; Liu et al., 2005a). Interestingly, Ago proteins, miRNAs, and mRNAs targeted by miRNAs all colocalized with GW182 in GWB (Jakymiw et al., 2005; Pillai et al., 2005; Sen and Blau, 2005; Liu et al., 2005b). Furthermore, GW182 interacted with Ago2 and this interaction was conserved from plants to human (Ding et al., 2005; Jakymiw et al., 2005; Liu et al., 2005a; Behm-Ansmant et al., 2006; El-Shami et al., 2007; Till et al., 2007). However, the role of GW182 and the importance of GW182-Ago2 interaction in translational repression remain unclear. In the current study, we mapped the GW182-Ago2 interaction and investigated the possible role of this interaction in miRNA-mediated silencing in human.

Materials and Methods

Construction of Deletion Constructs of GW182 and Ago2

The details of constructing GW1 Δ 1 (aa254-751, formerly known as GW182 Δ 1), TNR (aa1-204), Ago2 (aa1-860) and PIWI (aa478-860) were described previously (Jakymiw et al., 2005; Li et al., 2008). GW1 Δ 1a (aa254-503), GW1 Δ 1b (aa502-751), GW1 Δ 7 (aa1034-1962), GW1 Δ 12 (aa895-1211) and GW1 Δ 1a truncated constructs (W1-2, W2-3, W3-4, W4-5) were cloned from cDNA of full length GW182 or its deletion constructs by PCR (see Table. 3-1 for information of primers and conditions). The human Ago3 mutant (Ago3m) in pCMV-SPORT6 vector was obtained from Invitrogen (Carlsbad, CA, Clone number: CS0DB008YP10). Ago3m sequence was cloned by PCR (see Table. 3-1 for primers and condition) to adapt to Gateway cloning system (Invitrogen). The products from the above PCR reactions were then cloned into pDONR207 (Invitrogen) using the Gateway BP recombination reaction as per the manufacturer's instructions (Invitrogen). To construct pENTR-GW1 Δ 5 (aa1670-1962), pENTR-GW182 was

digested with *Sall* (5' end linker) and *SpeI* (nt5008) to release a 4.2Kb vector fragment containing the C-terminus of GW182. The overhangs of this fragment were filled in and then ligated. To construct pENTR-GW1Δ10 (aa566-1343), phrGFP-KIAA1460 was digested with *XhoI* (5' end linker) and *SmaI* to release a 2.3Kb fragment, which was then subcloned into the *Sall* and *EcoRV* sites of pENTR2B (Invitrogen). The cDNA of GW1Δ1a mutant (tryptophan > alanine, W>A) was directly synthesized and subcloned in pUC57 vector by GenScript Corp (Piscataway, NJ) using the sequence as shown in Fig. 3-8. The mutated gene sequence was moved to pDONR207 vector (Invitrogen) using Gateway BP recombination for further applications. To construct pENTR-PAZ (aa1-480), pENTR-Ago2 was digested with *XhoI* (nt1467) and *XhoI* (3' end linker) to generate the vector fragment containing N-terminal half of Ago2, which was purified and then ligated. To construct pENTR-Ago1, EST clone pBluescript hAgo1 was first digested with *BamHI* (3' end linker) and the overhang was filled in to generate a blunt 3' end. Then the digested product was cut by *KpnI* (5' end linker) to generate a 4.0 kb fragment which was subcloned into the *KpnI* and *EcoRV* sites of pENTR1A (Invitrogen). To construct pENTR-Ago4, EST clone pBluescript hAgo4 was digested with *SmaI* (5' end linker) and *ScaI* (3' end linker) to generate a 3.5 Kb fragment which was then subcloned into the *DraI* and *EcoRV* sites of pENTR1A (Invitrogen). All of the variants used in current study were subcloned into Gateway compatible GST, GFP, 3xFlag or NHA (Li et al., 2008) vectors by using Gateway LR recombination reaction (Invitrogen). pIreSneo-Flag/HA Ago3 was obtained from Thomas Tuschl (Meister et al., 2004) through Addgene. The tethering assay plasmids including pCneo-NHA vector, NHA-Ago2, Renilla luciferase RL-5BoxB and FL were kind gifts from Dr. Witold Filipowicz, Friedrich Miescher Institute for Biomedical Research, Switzerland (Pillai et al., 2004). All DNA constructs used in this study were confirmed by direct DNA sequencing.

Antibodies

Rabbit anti-Ago2 and rabbit anti-GST were gifts from Dr. Tom Hobman (University of Alberta, Edmonton, Canada) and Dr. Peter Sayeski (University of Florida, Gainesville, USA), respectively. Mouse monoclonal anti-HA was purchased from Covance (Emeryville, CA). Mouse monoclonal anti-Flag M2 and anti-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-GFP was purchased from Invitrogen Corporation (Carlsbad, CA).

Plasmid Transfection, GST Pull-down, and Western Blot Analysis

HeLa cells were cultured in DMEM containing 10% fetal bovine serum in a 37°C incubator with 5% CO₂. HeLa cells were grown to 90-100% in 6-well plate at the day of transfection. GST-tagged construct was singly transfected or co-transfected with other tagged constructs into HeLa cells using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions for 24 h. For detection of expression of GST-tagged proteins in whole cell lysate, the cells were lysed in Laemmli sample buffer directly. For GST pull-down assay, the cells were lysed with NET/NP40 buffer (150mM NaCl, 5mM EDTA, 50mM Tris, pH 7.4, 0.3% NP40) with Complete Protease Cocktail Inhibitor (Roche Diagnostics) and then sonicated at 20% amplitude for 10 sec for 3 times on ice. The GST pull-down assay in Figure 3-4 was performed under conditions that followed a published protocol (Till et al. 2007). Afterwards, the lysates were centrifuged at 13,200 rpm for 5 min. The pellets (insoluble fractions) were lysed in Laemmli sample buffer directly. The soluble fractions were incubated with Glutathione SepharoseTM 4B (GE Healthcare) and mixed at 4°C for 2 h for GST pull-down. After the incubation, the beads were washed with NET/0.3% NP40 buffer for four times and the samples eluted in Laemmli sample buffer. The soluble fraction of cell lysates (input), GST pull-down samples, whole cell lysates, and insoluble fractions were separated on 10% polyacrylamide gel and transferred to

nitrocellulose. Western blotting was performed as described previously (Lian et al., 2007). The dilutions of primary antibodies were: 1:1000 for anti-GST, 1:400 for anti-Flag, 1:1000 for anti-GFP, and 1:500 for anti-Ago2, 1:1000 for anti-HA.

Indirect Immunofluorescence

Cells were fixed and permeabilized as described previously (Jakymiw et al., 2005; Lian et al., 2007). The dilution of anti-Flag antibody was 1:1000.

Tethering Assay Using a Dual Luciferase System

HeLa cells were grown to about 90~100% confluence in 24-well plate at the day of transfection. To determine the effect of tethering PIWI (aa478–860) and PAZ (aa1–480), the cells were transfected with 0.1ng of constructs expressing reporter Renilla luciferase (RL-5BoxB), 100ng of control firefly luciferase (FL) plus 700ng of NHA tag, NHA-Ago2, NHA-PIWI, or NHA-PAZ using Lipofectamine 2000 (Invitrogen) for 48 h as per the manufacturer's instructions. Cells were harvested 48 hour after transfection and the FL and RL activities were measured using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) following the manufacturer's protocol. Relative luciferase activities (ratio of targeted luciferase activities over control luciferase activities) were calculated as described previously (Pillai et al., 2004). Briefly, FL/RL activity in NHA vector transfected (control) group was regarded as 0% translational repression. The repression levels of other experimental groups were calculated by the percentage reduction of relative luciferase activities compared to that in NHA control group. The assay was performed in triplicates and repeated for 2 to 3 times. To detect the expressions of tethered NHA-tagged proteins, the above cell lysates were mixed at 1:1 ratio with Laemmli sample buffer and western blot was performed as described in “Western Blot Analysis” of Materials and Methods.

RNA Interference and Quantitative Real Time PCR

The sequence of siRNA for GW182 or for GFP was described previously (Lian et al., 2007). To determine how GW182-knockdown affects the Ago2- or PIWI-mediated suppression, cells were grown to 30-50% confluence and were transfected with 100 nM of siRNA for GW182, or siRNA for GFP as a negative control using Lipofectamine 2000 (Invitrogen). Thirty hours later, these cells were transfected again with constructs expressing reporter RL-5BoxB, control FL and NHA tagged constructs as described above. Forty-eight hours after the second transfection, total RNA was extracted from HeLa cells using RNeasy Mini Kit (Qiagen, Valencia, CA). RNase-Free DNase Set (Qiagen) was applied to eliminate potential DNA contamination. The relative mRNA level of GW182 was measured in duplicate using $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) and TaqMan® Fast Universal Master Mix (Applied Biosystems) with the corresponding TaqMan® Gene Expression Assay (TNRC6A, Hs00379422_m1, Applied Biosystems). The level of 18S rRNA was measured as internal control (18S rRNA, 4310893E, Applied Biosystems). The melting curve in each individual measurement was monitored to guard against non-specific amplification.

Results

GW182-Ago2 Interaction Was Important for the Localization of Ago2 in Cytoplasmic Foci

Previous studies have shown that Ago2 colocalized with GW182 in cytoplasmic GWB and GW182 was essential for the formation of these foci (Yang et al., 2004; Jakymiw et al., 2005). However, the driving force for the localization of Ago2 to GWB remains unknown. Based on the above data that the GW182 fragment GW1 Δ 10 formed insoluble complexes with Ago2 and PIWI, but not with PAZ, we hypothesized that the GW182-Ago2 interaction is crucial for Ago2 to localize to GWB. To examine this hypothesis, Flag-Ago2, -PIWI or -PAZ was co-expressed with GFP-GW1 Δ 10 in HeLa cells and Flag-PIWI or -PAZ were expressed alone as controls (Fig.

3-2). Flag-Ago2 was shown to colocalize with GFP-GW1 Δ 10 in cytoplasmic foci whereas singly expressed Flag-PIWI or -PAZ were diffusely distributed in the cytoplasm (Fig. 3-2, e, g). Interestingly, co-expression of GFP-GW1 Δ 10 with Flag-PIWI dramatically changed the distribution of Flag-PIWI, which was recruited to cytoplasmic foci and colocalized with GFP-GW1 Δ 10 (Fig. 3-2, b, f, k). In contrast, co-expressing GFP-GW1 Δ 10 with Flag-PAZ did not recruit the diffusely distributed Flag-PAZ to cytoplasmic GFP-GW1 Δ 10-positive foci (Fig. 3-2, c, h, m). This data supported that the contention that interaction of GW182 with the C-terminal half of Ago2 mediated the localization of Ago2 to GWB.

Ago2 Bound to Multiple Non-Overlapping GW-Rich Regions of GW182

Since the GW182 fragments, GW1 Δ 1 and GW1 Δ 10, were both shown to bind Ago2 and these two fragments have overlapping 186aa, it is possible that the overlapping region of GW182 (aa566-751) is the primary site for the GW182-Ago2 interaction. To examine this possibility, deletion constructs GW1 Δ 1a (aa254-503) and GW1 Δ 1b (aa502-751) were generated with the latter covering the overlapping region of GW1 Δ 1 and GW1 Δ 10 (Fig. 3-1A). In addition, other deletion constructs GW1 Δ 7 (aa1034-1962) and GW1 Δ 5 (aa1670-1962) were used to investigate whether regions of GW182 other than GW1 Δ 1 and GW1 Δ 10 bound Ago2. GFP-GW1 Δ 1a, -GW1 Δ 1b, -GW1 Δ 7, or -GW1 Δ 5 was co-expressed with GST-tagged Ago2 fragment PIWI in HeLa cells and a GST pull-down assay was performed to examine the interaction. As a negative control, GFP-GW1 Δ 1 was co-expressed with GST-tagged fragment N1, the N-terminal aa51-779 of a completely unrelated protein hZW10 (Famulski et al., 2008). Unexpectedly, GFP-GW1 Δ 1a, -GW1 Δ 1b, -GW1 Δ 7, and -GW1 Δ 5 were all co-precipitated with GST-PIWI (Fig. 3-3A, lanes 7-9, 14). Another GW182 truncated construct GW1 Δ 12, which contains the reported ortholog-conserved GW-rich region (aa1074-1144) (Till et al. 2007), was also able to co-precipitate Ago2

(Fig. 3-3B). Interestingly, GW1 Δ 1a, GW1 Δ 1b, GW1 Δ 12, and GW1 Δ 5 are non-overlapping fragments and thus this data showed that at least 4 separate regions of GW182 could bind Ago2. Moreover, the deletion constructs that bound Ago2 all contained a GW-rich region whereas TNR, the only deletion construct that did not bind Ago2, lacked a GW-rich region. In summary, the GW182 deletion constructs containing GW-rich regions all bound to the C-terminal half of Ago2 indicating that multiple regions of GW182 mediated the interaction of GW182 with Ago2 and that GW repeats might be an key element for Ago2-binding.

Tryptophan Residues of GW1 Δ 1a Were Not Required for Interaction With Ago2

Previous studies from two groups have shown that short synthetic peptides containing one to two WG/GW was able to interact with Ago protein (El-Shami et al. 2007; Till et al. 2007). The interaction was significantly reduced when either one of the tryptophans was mutated to alanine (Till et al. 2007). We noted that GW1 Δ 1a, unlike GW1 Δ 1b and GW1 Δ 5, lacks sequence homology with the reported orthologue-conserved sequence (see Discussion). It is possible that the interaction of GW1 Δ 1a with Ago2 is different from that of the other GW182 truncated constructs. To examine if any of the 5 tryptophans in GW1 Δ 1a play important roles in Ago2-binding, four sequential truncated constructs were designed to span the sequence of GW1 Δ 1a and each containing two tryptophans (Fig. 3-4A). Interestingly, all these truncated constructs were co-precipitated by GST-PIWI (Fig. 3-4B). In addition, to examine whether tryptophan is essential for the GW1 Δ 1a-PIWI interaction, all of the 5 tryptophans in GW1 Δ 1a were mutated to alanine (W>A) (Fig. 3-4A). Surprisingly, the W>A mutation of GW1 Δ 1a did not abolished its ability to co-precipitate with PIWI fragment (Fig. 3-4C). In summary, the GW1 Δ 1a fragments containing two tryptophans all bound to the C-terminal half of Ago2. However, loss of

tryptophan in GW1 Δ 1a did not disrupt the GW1 Δ 1a-PIWI association, implying that tryptophan might not be an essential feature for mediating the interaction of GW182 with Ago.

The Interaction of Ago2 With GW182 Was Conserved In Other Human Ago Proteins

There are four Ago proteins in human cells that share a high degree of sequence similarity. To examine whether Ago1, Ago3, and Ago4 also interact with GW182, GFP-Ago1, -Ago3, -Ago3m, or -Ago4 was co-expressed with GST-tagged GW182 fragments GW1 Δ 1 or GW1 Δ 10 in HeLa cells and a GST pull-down assay was performed. Ago3m is a splicing variant of Ago3 and is missing aa757-823, the C-terminal 66aa of PIWI domain. Interestingly, human Ago1, Ago3, and Ago4 bound GW1 Δ 1 and GW1 Δ 10 (Fig. 3-5A, B). Ago3m did not bind GW1 Δ 1 or GW1 Δ 10 indicating that the C-terminus of that cognate PIWI domain was required for the binding to GW182 (Fig. 3-5A, B). Notably, both GFP-Ago1 (Fig. 3-5A, lane 4) and Flag-Ago1 (Fig. 3-5B, lane 4) bound GST-GW1 Δ 1 demonstrating that different N-terminal fusion tags did not affect the binding of Ago1 with GW182. In summary, the interaction of human GW182 with Ago2 was observed with other human Ago proteins and the C-terminal region of PIWI domain was critical for the interaction of GW182 with Ago3.

Tethering C-Terminal Half of Ago2 to the 3'-UTR of mRNA Recapitulated Ago2-Mediated Silencing Which Required the Presence of GW182

It was reported that tethering Ago2 to the 3'-UTR of mRNA causes repression of protein synthesis (Pillai et al., 2004). Because the C-terminal half of Ago2 bound GW182 whereas the N-terminal half of Ago2 did not, we examined whether the C-terminal half of Ago2 was able to mediate silencing when tethered to the 3'-UTR of mRNA. The dual luciferase and tethering assay was used as described previously (Pillai et al., 2004). In this assay, the reporter Renilla luciferase (RL) contains five 19-nt BoxB hairpin structures in the 3'-UTR of its mRNA (RL-5BoxB). The λ N peptide, which is derived from λ phage and binds to BoxB structures with high

affinity (Legault et al., 1998), was fused to the N-terminus of HA tagged Ago2, PIWI, or PAZ. In this way, Ago2, PIWI, or PAZ was brought directly to the 3'-UTR of mRNA bypassing the requirement for miRNA. Interestingly, tethered PIWI was attended by almost as much repression as tethered full-length Ago2 (Fig. 3-6A). In contrast, tethered PAZ was totally devoid of the repression function of Ago2 (Fig. 3-6A). This data indicated that the functional domain mediating silencing lay within the C-terminal half of Ago2. The repression effect of other human Ago proteins was also examined. Both Ago1 and Ago4 exerted a similar repression effect on the reporter as Ago2 and PIWI (Fig. 3-6A). Interestingly, Ago3m, which lost the interaction with GW182, was not able to induce repression. These data supported the conclusion that the repression effect mediated by the tethered construct might be associated with its interaction with GW182. To examine whether GW182 is required for Ago2- or PIWI-mediated repression, siRNA was used to knockdown GW182 before Ago2, PIWI or PAZ was tethered to the reporter mRNA. Very interestingly, both Ago2- and PIWI-mediated repression was significantly reduced upon GW182-knockdown (Fig. 3-6B). The GW182-knockdown was confirmed by quantitative real time PCR (Fig. 3-6C). In summary, tethering the C-terminal half of Ago2 to the 3'-UTR of mRNA recapitulated the repression function of Ago2 and this repression required GW182.

Discussion

Formation of GW182 and Ago Protein Complexes

Two studies have identified that one GW-rich region capable of binding Ago2 is conserved in the plant and yeast orthologs of GW182, and that the GW repeat within this region is critical for GW182-Ago2 interaction (El-Shami et al. 2007; Till et al. 2007). Consistent with these studies, our data showed that multiple human GW-rich regions were able to bind Ago2 (Fig. 3-7). GW182 fragments GW1 Δ 10 (aa566-1343), GW1 Δ 7 (aa1034-1962), and GW1 Δ 12 (895-

1211) containing the ortholog-conserved GW-rich region (aa1074-1144) (Till et al. 2007) were shown to bind Ago2 (Fig. 3-7). In addition, our data showed that at least four non-overlapping regions of GW182 could independently bind Ago2 and, interestingly, three of these Ago2-binding fragments are outside of the ortholog-conserved GW-rich region (Fig. 3-7). Sequence alignment analysis showed that 27aa and 23aa residues of the ortholog-conserved GW-rich region shared 40.7% and 34.8% identity with the GW1 Δ 5 and GW1 Δ 1b, respectively (Fig. 3-7). However, there was no significant sequence identity between the ortholog-conserved region and GW1 Δ 1a. Further analysis of GW1 Δ 1a showed the 4 sub-regions, two of which did not overlap, were all capable of binding Ago2 and, surprisingly, the 5 Trp residues within GW1 Δ 1a were not apparently important for the interaction with Ago2. Our data implied that different GW-rich regions were capable of binding Ago proteins but that the requirement for tryptophan residues varies. Future studies of the crystal structure of the Ago2-GW182 complex should enhance our understanding of how these two molecules interact with each other.

Since our data also indicated that GW182 can bind multiple Ago proteins, it is possible that the different Ago proteins incorporate into the same complex and contribute to the formation of functional translational silencing complexes. It is yet to be determined if the function of the silencing complex depends on which Ago proteins it contains. In support of our speculation that GW182 helps to stabilize the binding of multiple Ago-miRNA complexes to the 3'-UTR of target mRNA for more efficient translational repression, it was reported that more closely-spaced miRNA binding sites in the 3'-UTR of target mRNA led to more efficient miRNA-mediated translational repression (Grimson et al. 2007). It is also possible that GW182 simultaneously binds to Ago-miRNA complexes on several different mRNAs and this GW182-Ago interaction may be the driving force for the assembly of submicroscopic and microscopic GWB. This

hypothesis is supported by current observations that the GW1 Δ 1 GW182 fragment or the PIWI Ago2 fragment could mediate GW182-Ago2 interaction and by our previous data that overexpression of either of these two constructs disassembled GWB, possibly due to disruption of GW182-Ago2 interaction by a dominant-negative effect (Jakymiw et al. 2005).

C-terminal Half of Ago2 Preserved the Silencing Function of Ago2 Probably Because It Maintained the Interaction With GW182

Our GW182-Ago2 interaction mapping showed that the C-terminal half of Ago2 (aa478-860) was sufficient for the binding with GW182 whereas the N-terminal half (aa1-480) was not required. Interestingly, only the C-terminal half of Ago2 preserved the silencing function of Ago2 when directly brought to the 3'-UTR of target mRNA. The silencing function mediated by Ago2 or the C-terminal half of Ago2 was abolished upon GW182-knockdown. Our data strongly suggested that interaction of Ago2 with GW182 is critical for the silencing process mediated by Ago2 at the 3'-UTR of target mRNA. This hypothesis is also supported by two recent studies where overexpressing the Ago-binding fragment of yeast or *Drosophila* ortholog of GW182 greatly disrupted GW182-Ago interaction and significantly impaired miRNA-mediated silencing in vitro and in vivo (Eulalio et al. 2008; Till et al. 2007).

Furthermore, our recent study showed that the repression effect caused by tethering GW182 was independent of Ago2 (Li et al., 2008) and our data herein again suggested that Ago2 is not the “final repressor” because its silencing function relied greatly on GW182. Interestingly, the PIWI domain of Ago2 was reported to be responsible for the interaction with Dicer (Tahbaz et al. 2004). It would be interesting to determine if Dicer and GW182 compete for the binding to Ago2 through the PIWI domain.

Based on the data from current study, we propose a model for miRNA-mediated gene silencing in which, after miRNA guides Ago-miRNA complex to the 3'-UTR of target mRNA,

Ago protein recruits GW182 to stabilize Ago-miRNA-mRNA binding and represses translation. In addition, GW182-Ago2 interaction recruits Ago2 to GWB, which accumulate many Ago2-miRNA-mRNA complexes and become centers for miRNA-mediated silencing. We speculate that the ability of GW182 to potentially bind multiple Ago proteins may contribute to aggregation and formation of the cytoplasmic foci GW/P bodies, which have been implicated to be critical components of miRNA activity.

*This work was accepted by Journal RNA for publication Feb 2009 and is in press now.

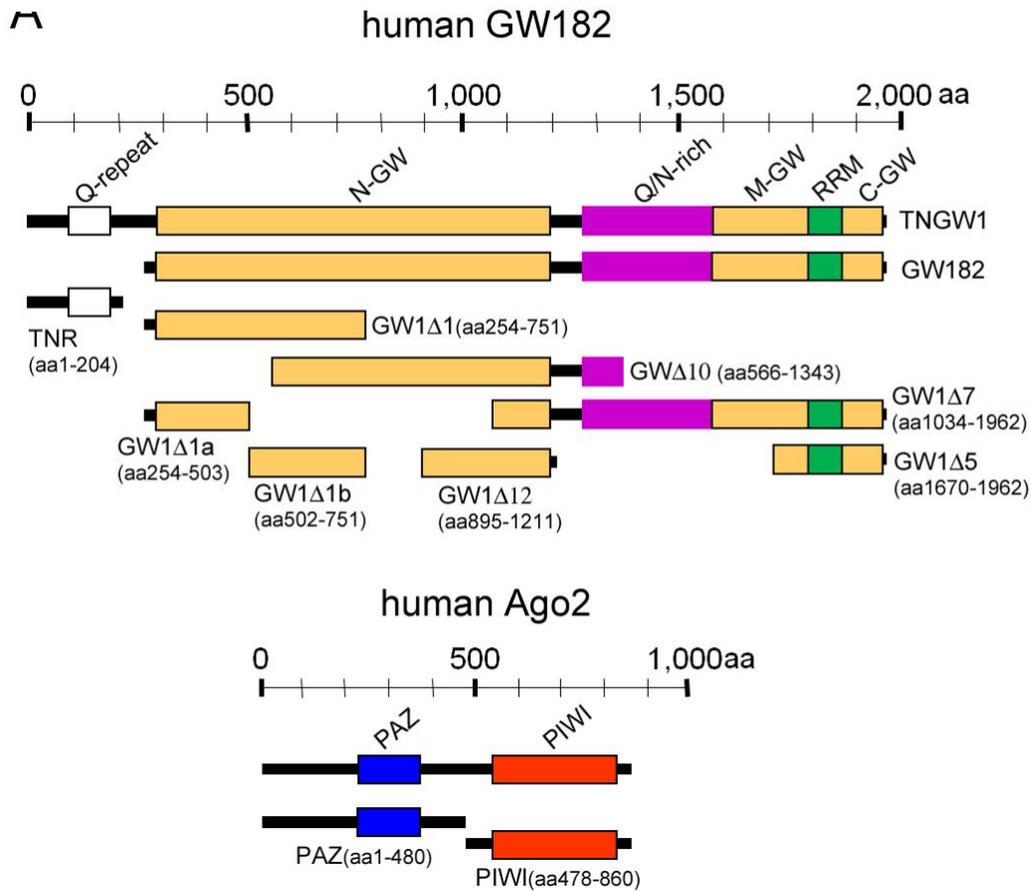


Figure 3-1. Schematic of human GW182 and Ago2 deletion constructs using in this study. Amino acid residues of GW182 constructs are referenced to the TNGW1, the longer isoform of GW182 (GenBank Accession NM_014494.2). Q-repeat, glutamine repeat (box in white); Q/N-rich, glutamine/asparagine-rich region (box in magenta); RRM, RNA recognition motif (box in green); GW-rich, glycine/tryptophan-rich region (boxes in yellow); N-GW, N-terminal GW-rich region; M-GW, middle GW-rich region; C-GW, C-terminal GW-rich region. Human Ago2 contains two conserved domains: PAZ domain (box in blue) and PIWI domain (box in red).

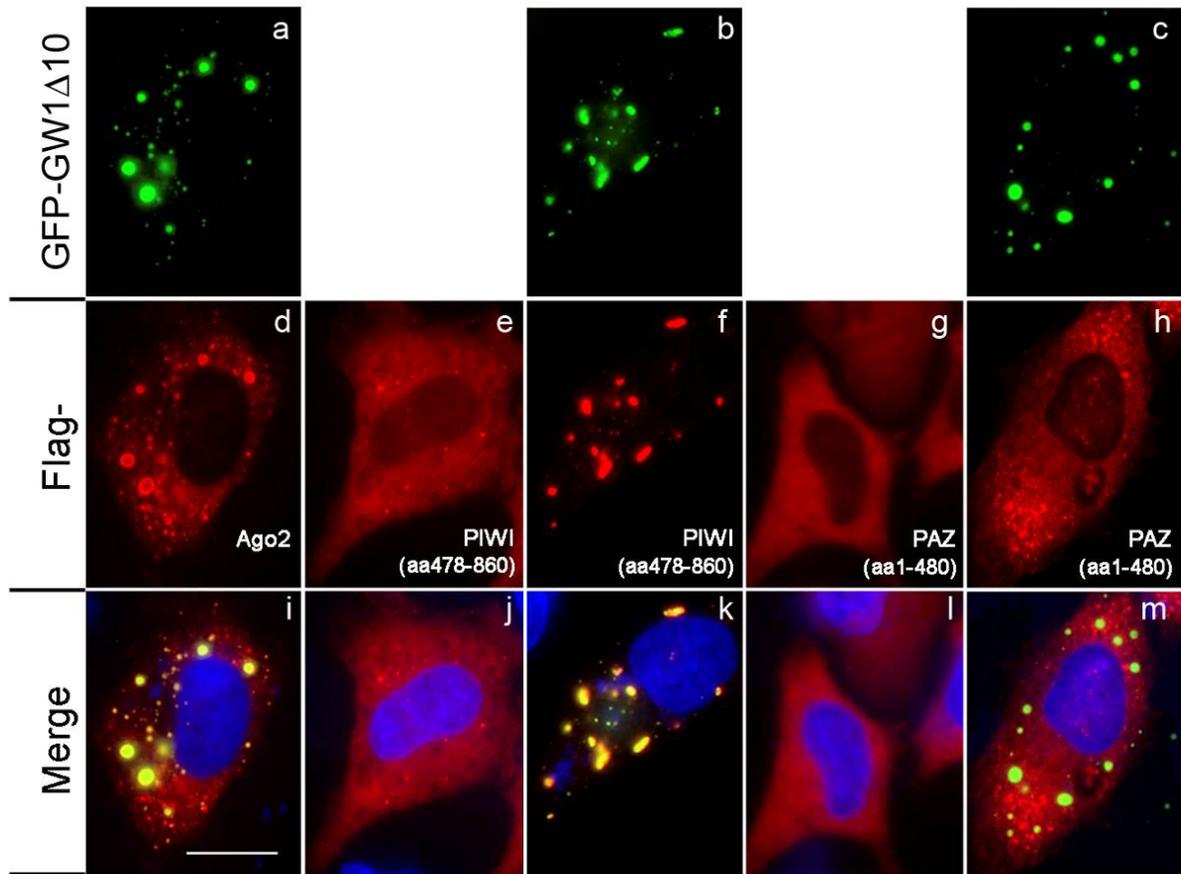


Figure 3-2. GW182 fragment GW1 Δ 10 (aa566-1343) recruited Ago2 to cytoplasmic foci by interacting with the C-terminal half of Ago2. GFP-GW1 Δ 10 (green, a-c) was co-transfected with Flag-Ago2 (d), PIWI (aa478-860, f) or PAZ (aa1-480, h) into HeLa cells. As controls, Flag-PIWI (e) or Flag-PAZ (g) was singly transfected. The cells were stained with anti-Flag antibody (red, d-h). Panels in the bottom row are the merged images (i-m). Nuclei were counterstained with DAPI (blue). Scale bar, 10 μ M.

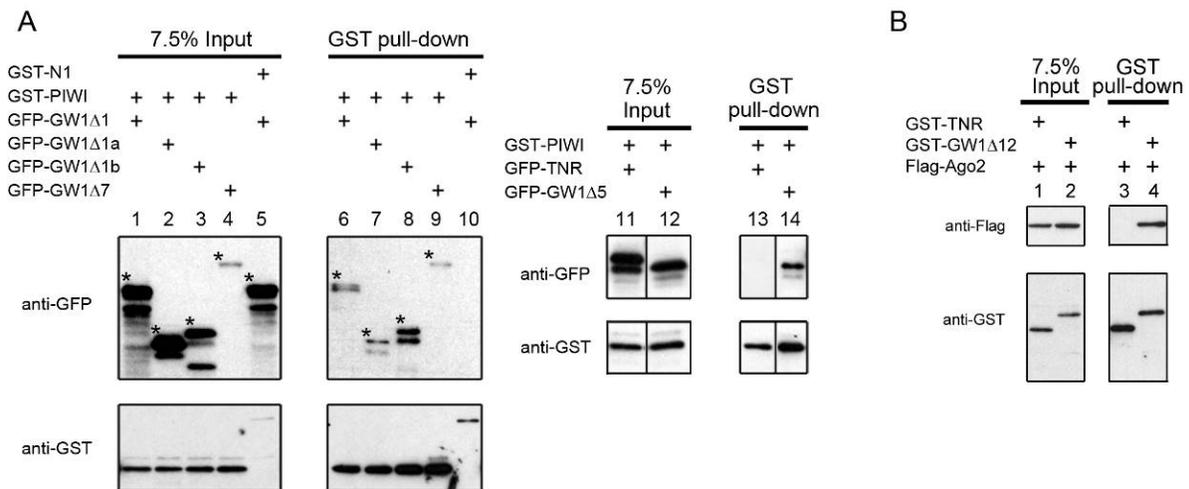


Figure 3-3. The C-terminal half of Ago2 bound to multiple non-overlapping GW-rich regions of GW182. A) GW182 fragments co-precipitated with C terminal half of Ago2. GST-PIWI (aa478-860) was co-transfected with GFP-tagged GW1Δ1 (lane 1), GW1Δ1a (lane 2), GW1Δ1b (lane 3), GW1Δ7 (lane 4), TNR (lane 11), or GW1Δ5 (lane 12) into HeLa cells. Similar to positive control GW1Δ1 (lane 6), GW1Δ1a (lane 7), GW1Δ1b (lane 8), GW1Δ7 (lane 9), GFP-GW1Δ5 (lane 14), but no GFP-TNR (lane 13), were detected in GST-PIWI precipitates. GST-tagged N1, N-terminal fragment from an unrelated protein hZW10, was co-transfected with GFP-GW1Δ1 (lane 5) as a negative control and no interaction was detected (lane 10). Asterisks indicate the corresponding GFP-tagged constructs in western blot. B) GW182 fragment GW1Δ12 co-precipitated with Ago2. Flag-Ago2 was co-transfected with GST-tagged TNR (lanes 1, 3) or with GW1Δ12 (lanes 2, 4), which contains the conserved sequence for Ago2 interaction. Flag-Ago2 was co-precipitated with GST-tagged GW1Δ12 but not TNR.

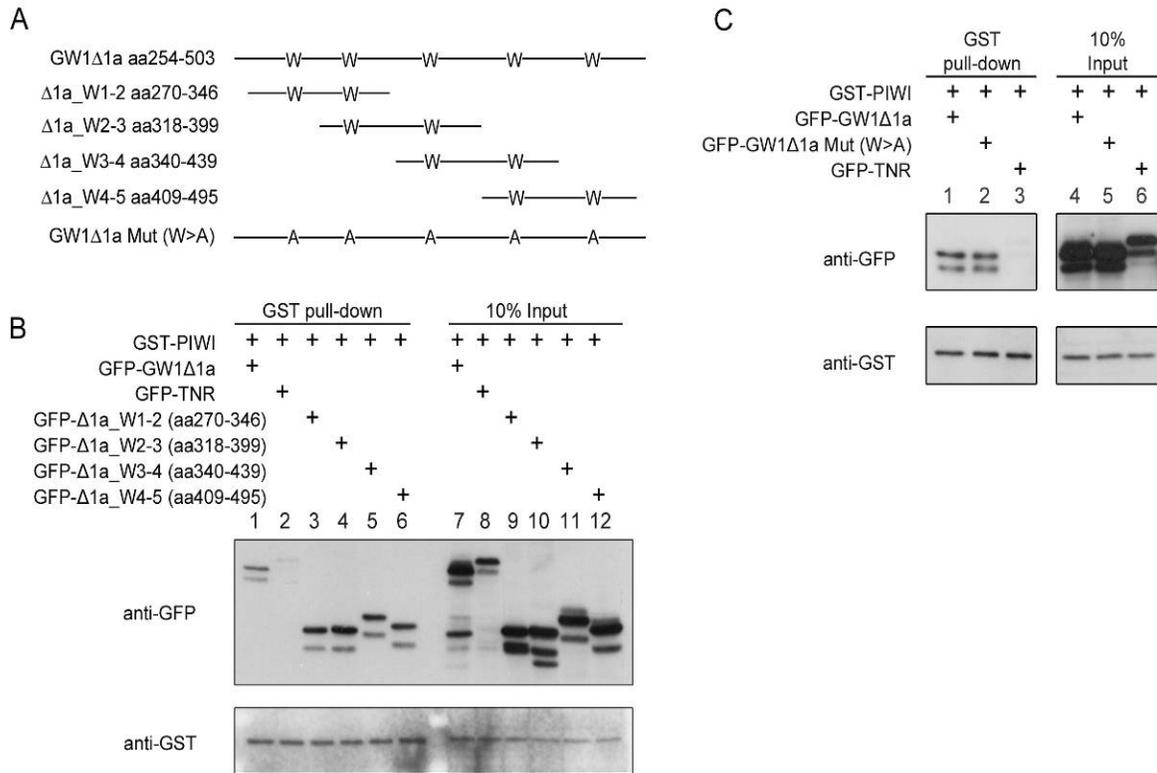


Figure 3-4. Four subregions of GW1Δ1a are capable of binding Ago2 and the interaction between GW1Δ1a and PIWI was not dependent on the five tryptophan residues. A) Schematics of the 4 overlapping fragments of GW1Δ1a each containing 2 tryptophan residues and the mutant designed with all 5 tryptophans substituted with alanine. B) Truncated constructs of GW1Δ1a containing any two continuous tryptophans were able to co-precipitate with PIWI. GST-PIWI was transfected with GFP tagged TNR, GW1Δ1a and its truncated constructs: Δ1a_W1-2 (aa270-346, contains the first and second tryptophans of GW1Δ1a), W2-3 (aa318-399, contains the second and third tryptophans), W3-4 (aa 340-439, contains the third and fourth tryptophan) and W4-5 (aa 409-495, contains the fourth and fifth tryptophans). Compared to GFP-TNR (lane 2), all truncated constructs of GW1Δ1a (lane 3-6) co-precipitated with GST-PIWI as full length GW1Δ1a did (lane 1). C) GW1Δ1a mutant without tryptophan still co-precipitated with PIWI. A GW1Δ1a mutant with all tryptophan (W) mutated to alanine (A) (GW1Δ1a Mut (W>A), lane 2) was co-transfected with GST-PIWI into HeLa cell. Compared to wild type GW1Δ1a (lane 1), GW1Δ1a mutant was co-precipitated with PIWI at the comparable level. GFP-TNR (lane 3) served as a negative control in the GST pull-down assay.

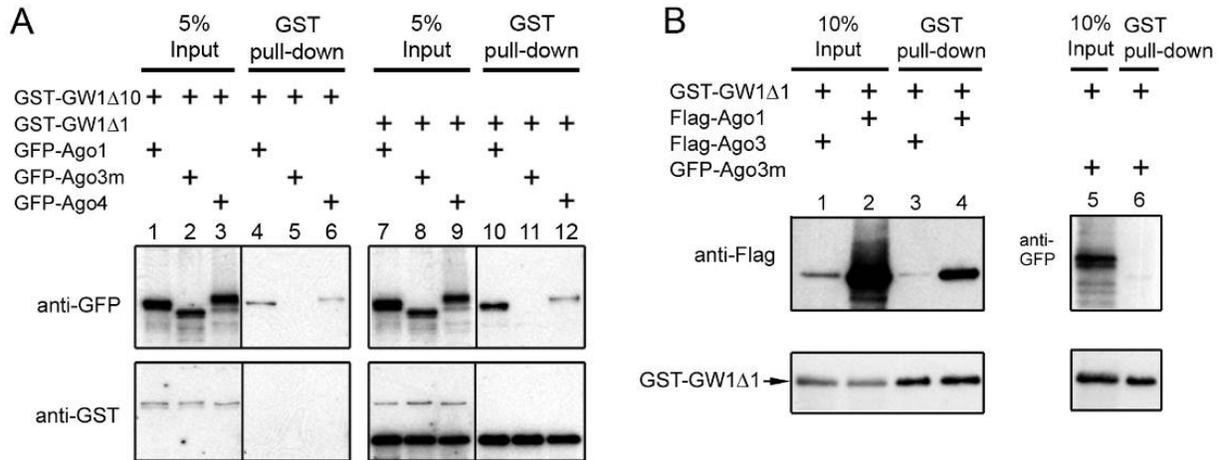


Figure 3-5. Both GW182 fragments GW1 Δ 1 and GW1 Δ 10 co-precipitated with other human Ago proteins. A) Ago 1 and Ago4, but not Ago3 mutant, co-precipitated with GW182 fragments GW1 Δ 1 and GW1 Δ 10. GFP-tagged Ago1, Ago3m (Ago3 mutant), or Ago4 was co-transfected with GST-GW1 Δ 10 (lanes 1-6) or GST-GW1 Δ 1 (lanes 7-12). Ago3m is missing an exon (aa757-823), the C-terminal 66aa of the PIWI domain compared to the reference sequence (NM_024852.2). Both Ago1 (lanes 4,10) and Ago4 (lanes 6,12) were pulled down by GST-GW1 Δ 10 or GST-GW1 Δ 1. In comparison, Ago3m was absent from either pull-down (lanes 5,11). B) Ago3 co-precipitated with GW182 fragment GW1 Δ 1. Flag-Ago3 (lanes 1,3) or -Ago1 (lanes 2,4) was co-transfected with GST-GW1 Δ 1 (lanes 1-4). GFP-Ago3m (lanes 5-6) was co-transfected with GST-GW1 Δ 1 as a negative control.

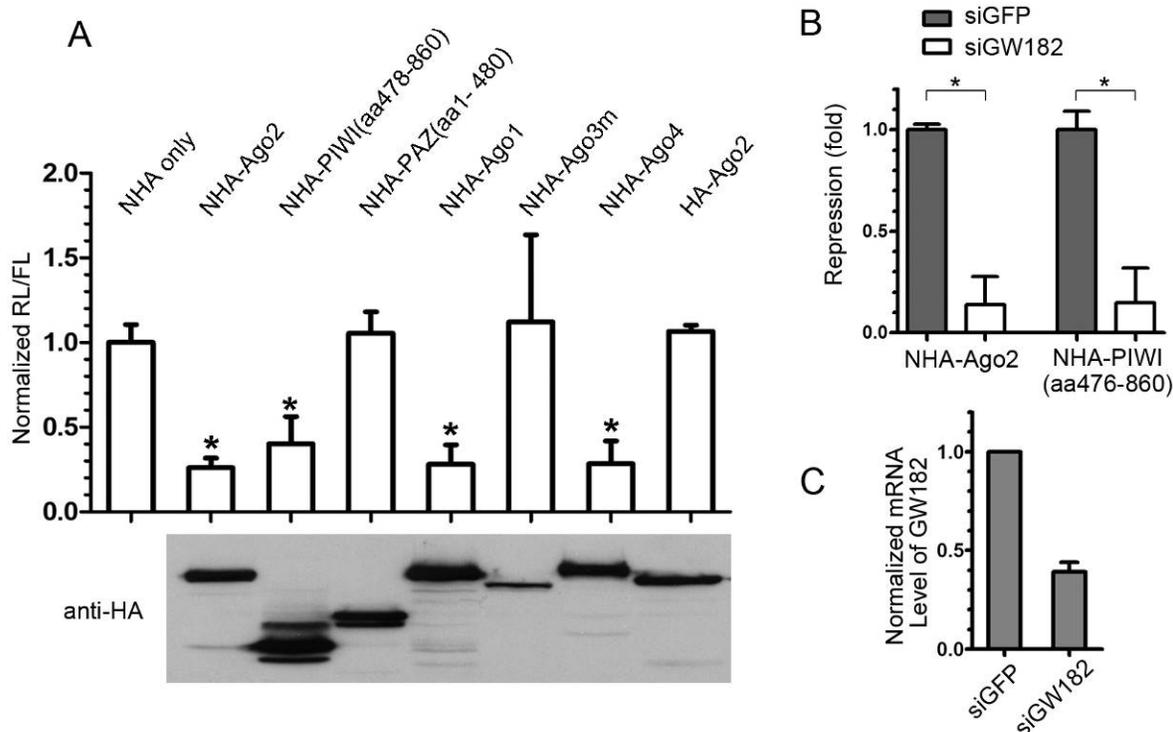


Figure 3-6. Gene silencing mediated by tethered C-terminal half of Ago2 required GW182. A) Tethered PIWI (aa478-860) down-regulated protein synthesis to the same extends of other Ago proteins. HeLa cells were transfected with constructs expressing the RL-5BoxB reporter, control FL reporter, and indicated NHA-tagged proteins. Bar graphs represent normalized mean values of RL/FL activities with standard errors. The RL/FL values in cells with tethered NHA-tagged Ago2, PIWI, Ago1 and Ago4 were significantly reduced compared to the value in NHA only group, which was normalized as 1. The NHA tagged PAZ, Ago3m, or HA-Ago2 did not show repression effect on the reporters. The expression of fusion proteins were determined by Western Blot using anti-HA mAb and are indicated below the bar graphs. The assay was repeated a minimum of 3 times. Asterisks indicate groups have significant difference with NHA only group (unpaired t test, $p < 0.0001$). No significant difference was shown between any two groups with asterisk (unpaired t test, $p > 0.05$). B) Translational repression mediated by tethered Ago2 or PIWI (aa478-860) was greatly impaired upon GW182-knockdown. HeLa cells were transfected with siRNA for either GW182 (siGW182) or GFP (siGFP). Thirty hours later, cells were transfected again with constructs expressing reporter RL-5BoxB, control FL reporter, and the same NHA-tagged proteins as indicated in panel A. Bar graphs represent the reduction of RL/FL in cells with tethered NHA-Ago2 or NHA-PIWI compared to those in cells with tethered NHA. The reduced values of RL/FL in cell transfected with siGFP were set as 1. Error bars indicate standard errors. The assay was performed in triplicates and was repeated for 2 times. *significant difference (unpaired t test, $p < 0.01$). C) GW182-knockdown by siRNA was confirmed by quantitative real time PCR. The bar graphs represent normalized mRNA level of GW182 with standard errors. The mRNA level of GW182 in cells transfected with GFP-siRNA was set as 1. The experiment was performed in triplicates.

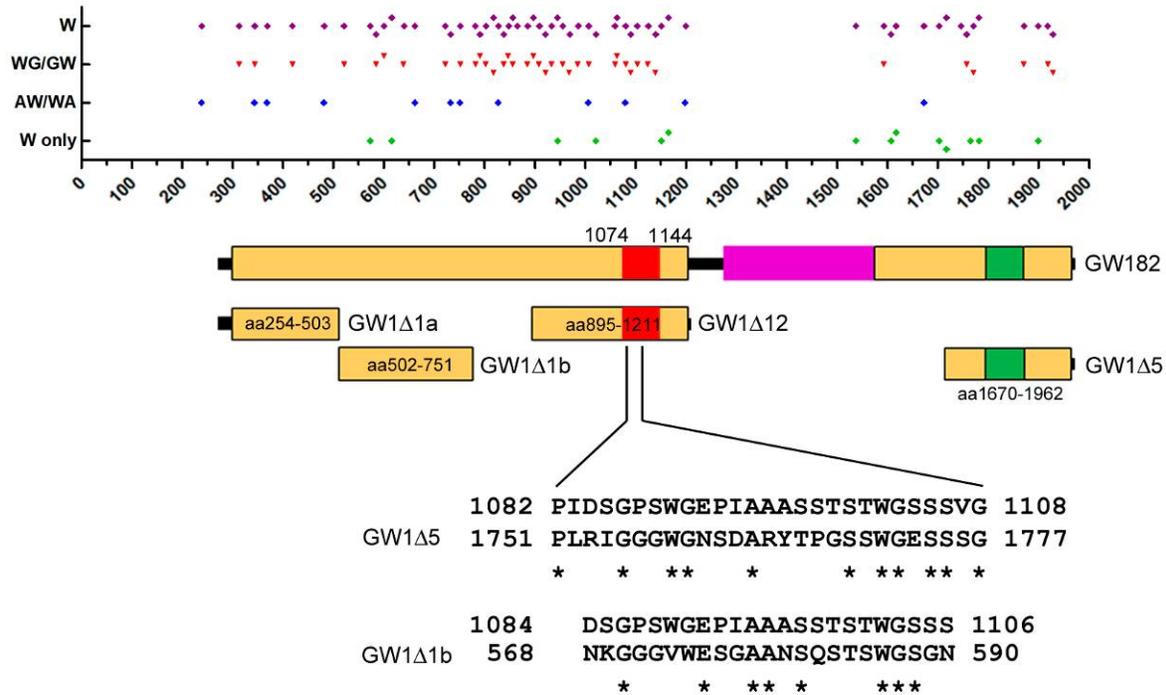


Figure 3-7. At least three non-overlapping GW-rich regions that are different from the ortholog-conserved GW-rich region can independently bind Ago2. The dot graph on top indicates the distribution of tryptophan in GW182. W, every tryptophan (magenta diamond); WG/GW, a glycine right adjacent to tryptophan (red triangle); AW/WA, an alanine right adjacent to tryptophan (blue diamond); W only, no glycine or alanine right adjacent to tryptophan (green diamond). The majority of the tryptophans are adjacent to either a glycine or alanine. The schematic of GW182 is indicated below the dot graph. GW-rich region, box in yellow; ortholog-conserved GW-rich region (aa1074-1144, (Till et al. 2007)) box in red. Compared to GW1Δ12, which contains the ortholog-conserved region, GW1Δ1a, GW1Δ1b and GW1Δ5 are the three non-overlapping regions identified in the current study that binds Ago2. The amino acid sequence alignment between GW1Δ5/ GW1Δ1b and ortholog-conserved GW-rich region was performed using ExpASY website tool. * represent sequence identity.

DNA sequence: (750nt)

G GGG ACA AGT TTG TAC AAA AAA GCA GGC TTC

atggatgctg attctgcctc cagttctgaa tcagagagaa acatcactat catggcttca
gggaacacag gtggtgaaaa agatggcctt cggaatagca ctggacttgg tccccaaac
aagttttag taggtagcag cagcaataat gtgggccatg gaagtagtac tgggccagcc
ggtttttccc atggagccat aataagcaca tgtcaggtct ctgtggatgc tctgaaagc
aaatctgaaa gtagcaaaa tagaatgaat gctgccggca ctgtaagttc tcatcaaat
ggaggggtaa atccaagcac tttgaattca gtagcaacc atggtgccgc cccagtatta
gagaacaatg gacttgccct aaaagggcct gtagggagtg gtagttctgg cattaatatt
cagtgcagta ctataggcca gatgcctaac aatcagagta ttaactctaa agtgagtgg
ggttctacc atggtaccgc cgggaagcct caggaaactt gtgaatctga agtaagtgg
acacagaagg tttcattcag tggtaacct caaaatatta cactgaaat gactggacca
aataacacta ctaactttat gacctctagt ttaccaaact ccggttcagt gcagaataat
gagctgccta gtagtaacac aggggccgcc cgtgtgagca caatgaatca tctcagatg
caggctccat caggatgaa tggcacttcc

TGA ACC CAG CTT TCT TGT ACA AAG TGG TCC

Corresponding protein sequence: (aa254-503, 250aa total)

MDADSASSESEERNITIMASGNTGGEKDGLRNSTGLGSQNKFVVGSSSNVGHGSSTGPA
GF^ASHGAIISTCQVSDAPESKSESSNRMNAAGTVSSSSNGGLNPSTLNSASNHGAA^APVL
ENGLALKGPVSGSSGINIQCSTIGQMPNNQSINSKVS^AGGSTHGTA^AGSLQETCESEVSG
TQKVSFSGQPQNITTEMTGPNNTTFNMTSSLPNSG^ASVQNNELPSSNTGA^ARVSTMNHPQM
QAPSGMNGTS

Figure 3-8. Sequence of synthesized GW1Δ1a Mut (W>A). The underlined sequences were designed to incorporate the recombination sites into pDONR207 vector (Invitrogen). Yellow highlights indicate sequences that were mutated from “tgg” to “gcc” in DNA sequence and tryptophan (W) to Alanine (A) in protein sequence.

Table 3-1. Primer sequences and PCR conditions for constructs used in the current study

Constructs		Primer sequence*	T _m (first 2 cycles)	T _m (next 30 cycles)
GW1Δ1a	Forward	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAACG</u> CCATGGATGCTGATTCT	55.7 °C	62.5 °C
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTGGGAAG TGCCATTCATACCTG		
GW1Δ1b	Forward	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACTT</u> CCCTTTCTCACCTTAGCA	55.2 °C	62.4 °C
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTGGCCTC TGTCCCATTTGTCAGT		
GW1Δ7	Forward	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAAG</u> ACCAGCAAGCACAGGTACA	55.7 °C	63.3 °C
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTTAGGCA ACATCAAGGCATAG		
GW1Δ12	Forward	<u>AAAGCAGGCTTCACTTGGGAAACAACATA</u>	55.2 °C	59.8 °C
	Reverse	<u>AGAAAGCTGGGTTC</u> AGGCTGGTGAGTCTCTCGAAA AAC		
Ago3m	Forward	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGG</u> AAATCGGCTCCGCAGGACCC	51.0 °C	57.0 °C
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAGA CCTTGGCCCCAC		
1Δ1a_W1-2	Forward	<u>AAAAAGCAGGCTCCAAAGATGGCCTTCGGAATA</u>	53.0 °C	58.9 °C
	Reverse	<u>AGAAAGCTGGGTTC</u> ATGAATTCAAAGTGCTTGGAA TT		
1Δ1a_W2-3	Forward	<u>AAAAAGCAGGCTTTGGAGCCATAATAAGCACAT</u>	51.3 °C	57.6 °C
	Reverse	<u>AGAAAGCTGGGTTC</u> AAATACTCTGATTGTTAGGCA TC		
1Δ1a_W3-4	Forward	<u>AAAAAGCAGGCTTTCATCAAATGGAGGGTTA</u>	51.9 °C	57.4 °C
	Reverse	<u>AGAAAGCTGGGTTC</u> AGATAGTGTTATTTGGTCCAG TCA		
1Δ1a_W4-5	Forward	<u>AAAAAGCAGGCTCTAAAGTGAGTGGTGGTTCTAC</u>	51.7 °C	57.3 °C
	Reverse	<u>AGAAAGCTGGGTTC</u> AGTGCCATTCATACCTGAT		

The underlined sequences were designed to incorporate the recombination sites for Gateway cloning system. Squares show the artificial stop codons for the truncated constructs.

CHAPTER 4 IDENTIFICATION OF TRANSLATIONAL REPRESSION DOMAINS IN GW182

Introduction

RNA interference (RNAi) was found in the last decade as an important mechanism of gene regulation at the post-translational level. Given the ability to knock down essentially any gene of interest, RNAi via small interfering RNAs (siRNA) has generated a great deal of interest in both basic and applied biology. Different from siRNA, which aims to regulate one gene in a time, a single microRNA (miRNA) has the potential to alter the expression of hundreds of proteins at the same time (Baek et al., 2008; Selbach et al., 2008). Increasing numbers of studies have linked miRNA to many biological functions and disease pathogenesis and this prompts the possibility of using miRNA as tools in gene therapy.

Significant works were carried out by the Bartel's group to characterize the sequence requirement for efficient targeting and regulation of miRNA (Lewis et al., 2005; Grimson et al., 2007; Friedman et al., 2009). However, the molecular basis of the miRNA-mediated translational repression is poorly understood. The Argonaute (Ago) family, including Ago1 to Ago4, was the most well characterized factors in the miRNA-induced silencing complex (miRISC) (Peters and Meister, 2007), where they interact with miRNA and recognize the target mRNA. After that, more protein factors are recruited to induce the subsequent repression. Multiple candidates are proposed to play an important role in the miRNA-mediated translational repression. Among these, GW182 is thought to be a conserved factor that retains the importance in miRNA-mediated repression across different species. Knockdown of GW182 impairs miRNA function in human (Liu et al., 2005a), drosophila (Behm-Ansmant et al., 2006) and *C. elegans* (Ding et al., 2005). An important feature of GW182 in this process is its conserved ability to interact with Ago proteins (Ding et al., 2005; Jakymiw et al., 2005; Liu et al., 2005a; Behm-

Ansmant et al., 2006; Till et al., 2007). A study in drosophila showed that the interaction between GW182 and Ago protein is essential for miRNA-mediated translational repression (Eulalio et al., 2008b). In our previous study, we showed that GW182 is able to interact with Ago2 in multiple regions (Lian et al., 2009). In addition, we showed that GW182 acts downstream of Ago2 to induce the translational repression effect (Li et al., 2008; Lian et al., 2009). These bring our interest to extend our understanding of how GW182 induce the repression. In the current study, we map the repression domain in GW182 and dissect the molecular mechanism of the translational repression event.

Materials and Methods

Plasmids

The cDNA of TNGW1, GW182, TNR, GW1Δ1, GW1Δ1a, GW1Δ1b, GW1Δ10, GW1Δ12, GW1Δ7, GW1Δ5, Ago2 and PIWI were constructed as described in previous studies (Jakymiw et al., 2005; Li et al., 2008; Lian et al., 2009). The TNGW1 N-terminal construct 1-565 is generated by restriction enzyme digestion (HpaI and SmaI) on TNGW1 and ligation using pENTR-TNGW1. GW1Δ8 and GW1Δ11 were generated by polymerase chain reaction (PCR) using GW182 cDNA as the template. The primers for cloning GW1Δ8 are: forward- GGGGACAAGTTTGTACAAAAAAGCAGGCTTCaTTAGACAGAATGGCAATCC, reverse- GGGGACCACTTTGTACAAGAAAGCTGGGTTAGGCAACATCAAGGCATAG. The primers for cloning GW1Δ11 are: forward- AAAAAGCAGGCTTCACTTGGGGAAACAACATA, reverse- AGAAAGCTGGGTTCACTCTGGTGAGTCTCTCGAAAA. All of the variants used in current study were subcloned into Gateway compatible GST, GFP, 3xFlag, or NHA (Li et al., 2008) vectors by using Gateway LR recombination reaction (Invitrogen). The tethering assay plasmids

including pCneo-NHA vector, NHA-Ago2, Renilla luciferase (RL) and Firefly luciferase (FL) with or without 5 Boxb (5Bb) structure were kind gifts from Dr. Witold Filipowicz, Friedrich Miescher Institute for Biomedical Research, Switzerland (Pillai et al., 2004). All DNA constructs used in this study were confirmed by direct DNA sequencing.

Antibodies

Rabbit anti-Ago2, rabbit anti-GST and human anti-P serum 6181 were gifts from Dr. Tom Hobman (University of Alberta, Edmonton, Canada), Dr. Peter Sayeski and Dr. Minoru Satoh (University of Florida, Gainesville, USA), respectively. Mouse monoclonal anti-HA was purchased from Covance (Emeryville, CA). Mouse monoclonal anti-Flag M2 was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-GFP was purchased from Invitrogen Corporation (Carlsbad, CA).

Cell Culture and Plasmid Transfection

HeLa and HEK293 cells were cultured in DMEM containing 10% fetal bovine serum in a 37°C incubator with 5% CO₂. Cells were grown to 90-100% in 6-well or 24-well plate at the day of transfection. The plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instruction. The tethering assays were performed in 24-well plate format, where 600ng NHA tagged construct was co-transfected with either 10ng RL-5Bb/100ng FL or 100ng FL-5Bb/10ng RL in 293 cells. Cells were harvested 48 hours after transfection for further experiments. For the GST pulldown assay, 2ug of GST-tagged construct was co-transfected with 2ug of either GFP or Flag tagged construct into HeLa cells. HeLa cells were harvested 24 hours after transfection for further experiments.

GST Pull-down and Western Blot Analysis

For GST pulldown assay, transfected cells were lysed with 300 ul NET/NP40 buffer (150mM NaCl, 5mM EDTA, 50mM Tris, pH 7.4, 0.3% NP40) with Complete Protease Cocktail

Inhibitor (Roche Diagnostics) and then sonicated at 20% amplitude for 10 sec for 3 times on ice. Afterwards, the lysates were centrifuged at 13,200 rpm for 5 min. A fraction of soluble cell lysate was mixed with Laemmli sample buffer as input for western blot analysis. About 200 ul of the soluble fractions were incubated with Glutathione SepharoseTM 4B (GE Healthcare) and mixed at 4°C for 2 h for GST pulldown. After the incubation, the beads were washed with NET/0.3% NP40 buffer for four times and the samples eluted in Laemmli sample buffer. The input and GST pull-down samples were separated on 10% polyacrylamide gel and transferred to nitrocellulose. Western blotting was performed as described previously (Lian et al., 2007). The dilutions of primary antibodies were: 1:1000 for anti-GST, 1:400 for anti-Flag, 1:1000 for anti-GFP.

Tethering Assay and Dual Luciferase Assay

HEK293 cells were harvested 48 hours after transfected with tethering constructs and dual luciferase reporters. The FL and RL activities were measured using Dual-Luciferase[®] Reporter Assay System (Promega, Madison, WI) following the manufacturer's protocol. Relative luciferase activities (ratio of targeted luciferase activities over control luciferase activities) were calculated as described previously (Pillai et al., 2004). The repression effect was estimated by the activity lost of the luciferase containing 5Bb structure in each group compared to the one transfected with NHA vector (Li et al., 2008). The repression effects of each constructs were normalized to the one of TNGW1, which was standardized as 1. The assay was performed multiple times as indicated in Fig. 4-2a. For quantifying the expression levels of different NHA tagged constructs, cell lysates from representative luciferase assay was mixed directly with Laemmli sample buffer and separated in 4-20% HCl-Tris Ready Gel (Biorad, Hercules, CA). Samples were then transferred to membrane and performed western blot as described above. To avoid the narrow dynamic sensitivity of tradition film system, bands visualized by enhanced

chemiluminescence assay were captured by Geliance 600 (PerkinElmer, Waltham MA) to obtain the most optimized image. The results were then analyzed by GeneTools software (PerkinElmer) to quantify the amount of protein expressed in individual assay.

Results

GW182 contained two putative, non-overlapping regions harboring the repression effect in tethering assay.

To dissect the repression function of tethered GW182, we decided to first narrow down the region responsible for the repression effect in tethering assay. Multiple truncated constructs of GW182 were generated covering the full length protein (Fig. 4-1). All these constructs were adapted to the tethering assay as reported in previous studies (Pillai et al., 2004; Li et al., 2008) and examined their repression effects accordingly. The relative repression effects caused by different GW182 truncated constructs emerged into three groups: 1) no repression effect, which included 1-565, TNR, GW1 Δ 1 and QN; 2) high repression effect comparable to full length protein, which included GW1 Δ 10, GW1 Δ 12, GW1 Δ 8, GW1 Δ 7 and GW1 Δ 5; 3) low to moderate repression effect, which included GW1 Δ 11. Interestingly, the result revealed that there was more than one region able to induce repression effect when it was tethered to the 3'-UTR of the reporter mRNA. These regions located in the middle and the C-terminal domains of GW182. GW1 Δ 12 and GW1 Δ 5 were the smallest, non-overlapping constructs residing in these regions. Since GW1 Δ 12 and GW1 Δ 5 were reported to interact with Ago proteins (Chapter 3), a reasonable interpretation is that their repression abilities were related to Ago proteins. Contradicting to this hypothesis, GW1 Δ 1, an N-terminal truncated construct of GW182 which strongly interacted with all four human Ago proteins (Lian et al., 2009), was not able to induced repression effect in the tethering assay. It implied that Ago proteins were not the direct effectors for repression.

To achieve a better characterization of the potencies of inducing translational repression in different regions of GW182, the repression effects observed in the tethering assay were normalized to the expression levels of the tethering constructs. From previous representative tethering assays, six cell lysates transfected with NHA tagged truncated constructs (TNR, GW1 Δ 1, GW1 Δ 11, GW1 Δ 12, QN and GW1 Δ 5), which covered the full length TNGW1, were selected and loaded equally as in the dual luciferase assay and separated in SDS-PAGE. The relative expression levels of transfected NHA tagged constructs were determined by western blot using the anti-HA monoclonal antibody (Fig. 4-2B). Based on the protein amounts of different NHA constructs, TNGW1 full length protein was much more potent, per molecule, in inducing repression when it was tethered to the mRNA of reporter (Fig. 4-2C). Although GW1 Δ 12 and GW1 Δ 5 were able to inducing repression effects in previous tethering assay, their repression potencies per molecule were only about 15% and 28%, respectively, relative to that of the full length protein of TNGW1. One potential limitation in this analysis is the low transfer efficiency of TNGW1 compared to GW1 Δ 12 and GW1 Δ 5 which are lower molecular weight truncated constructs. However, incomplete transfer of high molecular protein was not observed because there was no visible remaining dye-labeled molecular marker of 250kDa on the gel after the electrotransfer overnight. Therefore, the repression potency of GW182, including its isoform TNGW1, is considerably higher than the potency of GW1 Δ 12 and GW1 Δ 5 added together. This data implied that the two regions, GW1 Δ 12 and GW1 Δ 5, may fit into better architectural positions in the full length protein to achieve an enhanced repression effect in translation.

Endogenous acidic ribosomal protein P0, but not P1 or P2, was specifically associated with the complexes of Ago2 and GW182 truncated constructs GW1 Δ 12 and GW1 Δ 5.

After identifying two non-overlapping regions in GW182 that harbored repression effect, the next question we asked is how they triggered the repression. Conserved protein domain

analysis based on bioinformatics tools from NCBI website indicated two well-defined domains residing in these regions. An RNA recognition motif (RRM) locates in the middle of GW1Δ5, while an Ago hook, which is a conserved peptide stretch able to bind the PIWI domain in Ago proteins (pfam10427), resides in GW1Δ12. In addition, a sequence string residing in GW1Δ12 from aa953 to aa994 showed modest similarity to 60S acidic ribosomal protein P1 (RPLP1), which is a component of ribosomal stalk and interacts with the other two ribosomal proteins, P0 (RPLP0) and P2 (RPLP2) (Zurdo et al., 2000; Gonzalo et al., 2001).

To examine the possibility that GW182 can interact with ribosomal P proteins, and also distinguish it from its ability to interact with Ago proteins, we applied the GST pulldown assay established from our previous study (Lian et al., 2009). Consistent with our previous study, GW1Δ1a, GW1Δ1b, GW1Δ12 and GW1Δ5 were associated with Ago2 (Fig. 4-3A), although the interaction capabilities of GW1Δ1a and GW1Δ5 appeared to be weaker than observed when these constructs were fused to the GFP tag (Fig. 4-3B) in our current and previous study (Lian et al., 2009). Most interestingly, when the pulldown samples were probed with a human anti-P serum 6181, P0 was found associated with GW1Δ12 and GW1Δ5 but not with other constructs (Fig. 4-3A). In contrast to the strong interaction of GW1Δ1b to Ago2 protein, GW1Δ1b did not pull down P0 protein. The data showed that the association of P0 to the Ago2:GW182 construct was not because of the presence of Ago2 protein, but the specific regions of GW182 constructs. To our surprise, the rest of the ribosomal stalk components, P1 and P2, were absent in GW1Δ12 and GW1Δ5 complex (Fig. 4-3A). This implied that P0 was the only component existing in the pulldown complex, which provided strong evidence that it was not present either as part of assembled ribosomal stalk construct, or as part of 80S ribosome. These interactions between P0 and GW1Δ12 or GW1Δ5 were also observed when we used GST-PIWI to pull down GFP tagged

GW182 truncated constructs (Fig 4-3B). This data again confirmed that Ago2 was not sufficient to recruit P0 to the complex. The presences of GW1 Δ 12 or GW1 Δ 5, or at least the combination of them with Ago protein, were important to interact with P0. Interestingly, GW1 Δ 12 and GW1 Δ 5 were shown as the regions harboring repression effects in our current study. These findings suggested that the P0 protein of the ribosomal stalk could be the target of translational inhibition when these GW182 truncated constructs were tethered to the 3'-UTR of mRNA.

Discussion

Unlike that the Ago interacting capability identified in four non-overlapping regions of GW182 containing glycine and tryptophan repeats, the regions responsible for translational repression effect are relatively concentrated in two separated regions of about 300 amino acid residing in the middle and C-terminal domains of GW182. Interestingly, there are three studies published recently about the repression regions of GW182 protein: drosophila GW182 (dGW182) (Eulalio et al., 2009; Chekulaeva et al., 2009) and the human TNRC6C (Zipprich et al., 2009). All of them reported that the C-terminal domain containing RNA recognition motif to be highly important for translational repression. However, another study in drosophila found that there are three regions in dGW182 able to induce repression effect (Chekulaeva et al., 2009). Although this study is in agreement with the other two studies to some degree, it actually brings out the possibility that GW182 can potentially interact with itself so that many regions of GW182 can trigger the repression by recruiting the endogenous GW182 protein. Our data, which is focus on human GW182 protein, also identify that the C-terminal domain of GW182 showing repression effect. Furthermore, another repression region is identified in the middle region of GW182. Further studies need to be carried out to characterize the repression mechanisms of these two

regions. Whether their repression function depends on the endogenous GW182 protein will need to be clarified.

In our study, we have shown that some regions in GW182 such as GW1 Δ 1 can interact with Ago proteins but unable to induce translational repression. Besides re-confirming the findings that Ago proteins alone are not sufficient to induce repression (Li et al., 2008; Lian et al., 2009), it remains unclear why GW182 contains more regions which can interact with Ago proteins than those that can induce repression. One possibility is that by having more Ago interacting region, GW182 can interact with more than one Ago protein at a time. Studies from Bartel's group have showed that mRNA containing multiple miRNA target sites in its 3'UTR are repressed more efficiently than the one containing fewer miRNA target sites (Grimson et al., 2007). More importantly, closely spaced miRNA target sites often act synergistically and result in stronger repression than those separated farther apart. It implies that a new mechanism may be triggered to enhance repression effect when miRNA sites are close to each other. With its considerable size of 182 kDa and its multiple Ago interacting sites, GW182 may play an important role in this mechanism, in which it connects miRNA:Ago2 complexes that are close to each other to form a more stable complex. It may also constitute a larger obstacle locating close to the 5' cap of the mRNA to hinder the translational initiation and eventually induce GW body formation. Alternatively, the multiple Ago interacting sites may aim to increase the chance, and more importantly, modulate the proper interaction between GW182 and Ago2. More precisely, the Ago interacting abilities appears to vary in different regions of GW182. If we can imagine the wide-spread Ago interacting regions in GW182 as a funnel, where the deepest point is the strongest site interacting with Ago, all Ago proteins that "fall" into the funnel will eventually reach the deepest point unless it is already occupied. When the Ago protein is placed in the

“best” position along GW182, its bound mRNA will be exposed to the repression domains in GW182 and trigger the downstream event of repression. This hypothesis is less preferred than the first one since the enhanced effect is observed when miRNA target sites are placed close to each other. However, this alternative explanation can account for having more Ago interacting sites than repression sites if GW182 is not folded to allow interaction with multiple Ago at the same time.

Our data also show reproducibly that the ribosomal stalk protein P0, but not P1 or P2, interact specifically with GW1 Δ 12 and GW1 Δ 5, and not other GW182 deletion constructs. More interestingly, the GW182 truncated constructs associated with P0 are the same constructs that are able to induced repression effect in the tethering assay. It implies that the interaction with P0 is related to the repression capability of GW182. The ribosomal stalk is known as a distinct lateral protuberance located in the large ribosomal subunit (60S subunit) and is essential for the ribosome activity (Tchorzewski et al., 2003). It is composed of two heterodimers of P1 and P2 (Hagiya et al., 2005; Grela et al., 2008), with P1 binding to P0 (Zurdo et al., 2000; Gonzalo et al., 2001), which is, in turn, attached to 28S RNA (Uchiumi and Kominami, 1992) and constitutes a major part of the GTPase-associated center in eukaryotic 60S subunit. Unlike other protein factors in the ribosome, the P1 and P2 in eukaryotic ribosomal stalks are highly dynamic and undergo a cyclic process of assembly and disassembly during translation (Briceno et al., 2009). A recent study has shown that altering the level of P1/P2 in the ribosomal stalk can affect translational efficiency by reducing the ribosomal subunit joining capacity (Martinez-Azorin et al., 2008). Since our data show that the translation inhibitory regions of GW182 interact with P0 which is an incomplete ribosomal stalk, it is reasonable to deduce that not the whole ribosome is associated with GW182. The presence of additional protein markers from

either the small or large ribosomal subunit as well as rRNA will need to be examined in future studies. If only part of the ribosome is associated with the inhibitory regions of GW182, it will further confirm that GW182 can potentially disrupt the integrity of the 80S ribosome.

It is possible that ribosomal stalk and the joining of the ribosomal subunits are the potential targets for the translational repression effect of GW182-containing complex. Supporting this hypothesis, a recent study has showed that eIF6, a ribosomal inhibitory protein known to prevent productive assembly of the 80S ribosome, is important for miRNA-mediated translational repression (Chendrimada et al., 2007). However, to further explore this hypothesis, more experimental evidence is needed. First, one needs to show the interaction between GW182 and P0 in a more direct manner. Will the interaction with P0 and the repression effect of GW182 truncated constructs depend on the presence of Ago2? If not, will it depend on other protein factors that have been reported important for miRNA function, such as eIF6 or RCK/p54 (Chu and Rana, 2006)? Answer to all these questions in further studies may provide a better understanding of this translational repression effect. Secondly, although the bioinformatics analysis predicted that GW182 shares similarity to P1 protein, we have not shown whether GW182 contributes to the repression effect by competing with P1 to interact with P0 and eventually impairs the integrity of the ribosomal stalk. However, our data has shown some evidence supporting this scenario because P1 and P2 are absent in the pulldown complex. Although to show the direct competition between GW182 and P1 may be difficult, it is possible to first examine which region of P0 is required for its interaction with GW182. While the N-terminal half of P0 is known to attach to the 28s rRNA, its C-terminal half, more specifically, aa230-290, is shown important for interacting with P1/P2 (Santos and Ballesta, 1995). Therefore, it is feasible to generate truncated constructs of P0 representing the N-terminal and C-terminal

halves and examine their association with GW182. If the interaction of P0 and GW182 is through its C-terminal half and even the P1 interacting site, it can potentially explain the absence of P1/P2 in the complex. If the interaction is not through the C-terminal half, it implies there is potentially other factor(s) responsible for the absence of P1/P2. In this case, eIF6 may become a good candidate and the previous experiments examining the importance of eIF6 may help to interpret this issue.

In summary, we have characterized the repression regions in GW182 and confirmed again that the interaction with Ago2 and the repression effect are two separated aspects of GW182. We have also proposed a potential mechanism that GW182 may attack the ribosomal stalk to execute translational repression effect in the miRNA-mediated repression, which opens a wide range of testable possibilities for future studies.

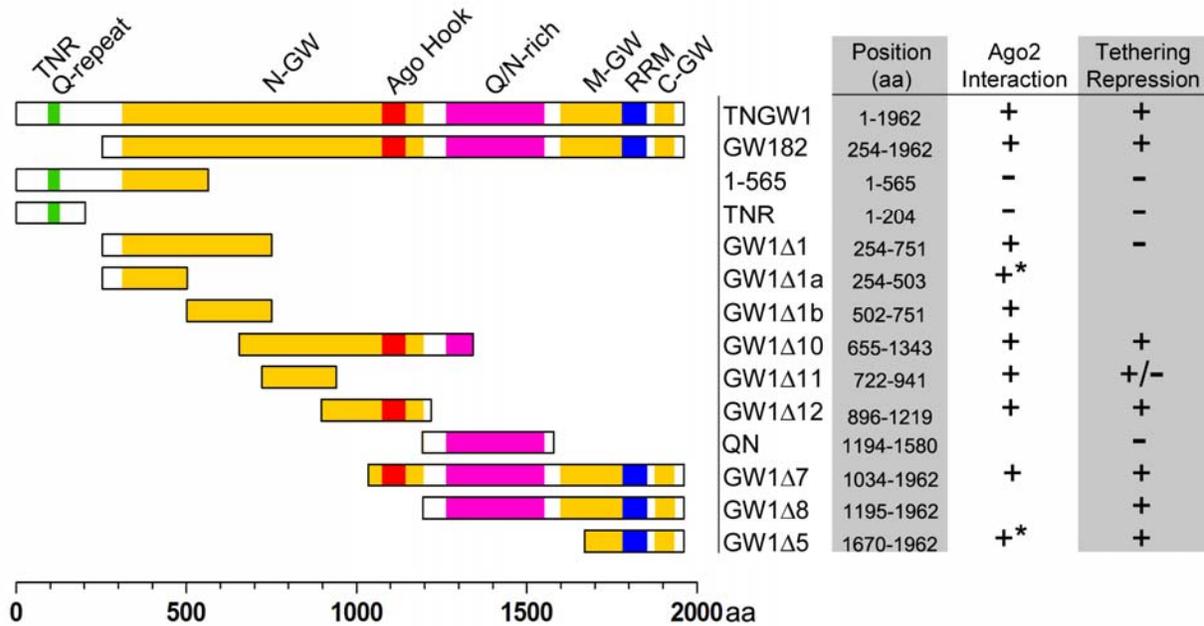


Figure 4-1. Schematic of human GW182 truncated constructs used in this study with a summary of their interaction with Ago2 and the repression ability in the tethering assay. The amino acid residues of the constructs are referenced to TNGW1, the newly reported isoform of GW182. TNR Q-repeat (aa93-127); Ago hook (aa1076-1144); Q/N rich (aa1264-1553) and RRM (RNA recognition motif, aa1780-1853); N-GW, M-GW and C-GW represent N-terminal, middle and C-terminal glycine/tryptophan (GW) repeats. Asterisks show indicated the GW182 truncated constructs which weakly interacted with Flag-Ago2 (Fig. 4-3A) but strongly interacted with GST-PIWI (Fig. 4-3B).

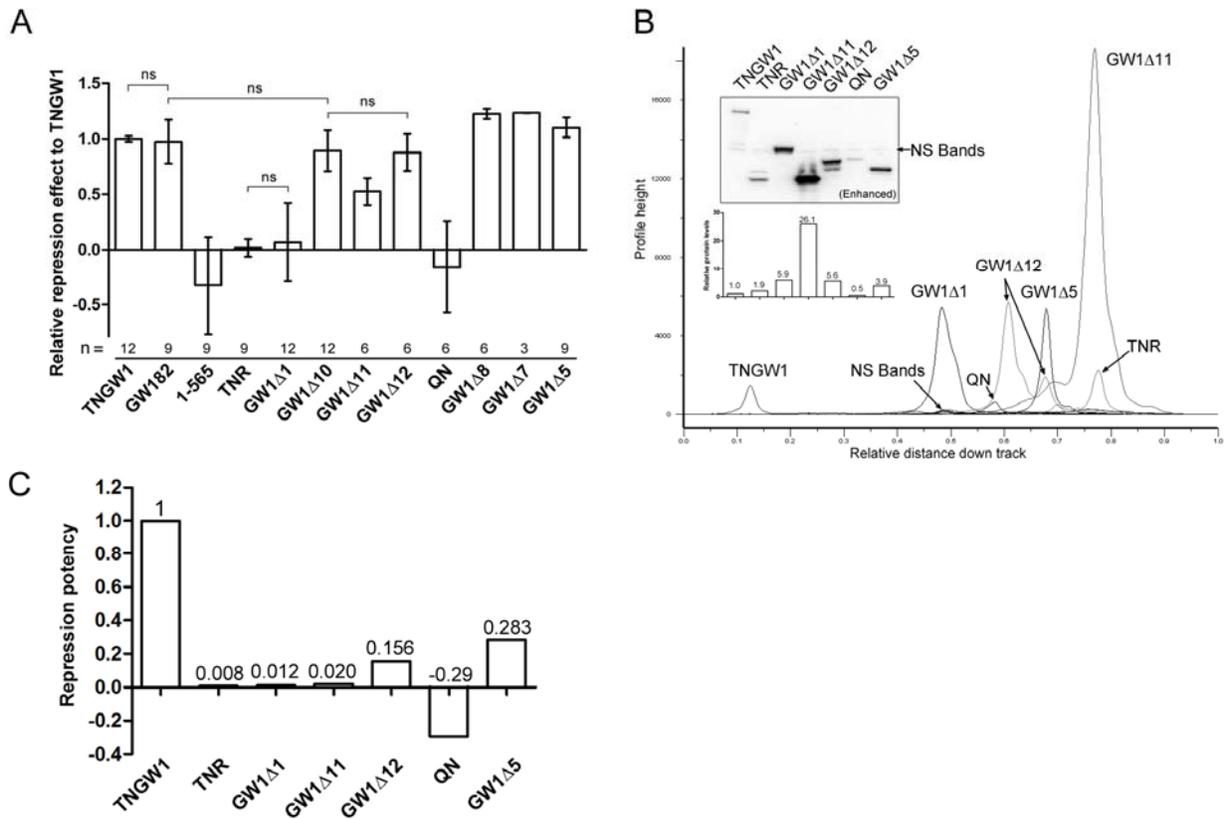


Figure 4-2. Identifying two non-overlapping regions harboring repression effect in GW182. A) Comparison of repression effects caused by GW182 full length isoforms and their truncated constructs in dual luciferase tethering assay. Different NHA-tagged GW182 constructs were tethered to either FL-5Bb or RL-5Bb reporters. Their repression effects were normalized to RL or FL control, respectively. Then all relative repression effects were standardized to the repression effect caused by TNGW1, which was assigned as 1. Error bars indicate standard deviations in each group; “n” numbers indicate times for repeating the experiments; “ns” indicates no significance by t test. B) Quantification of protein levels of different NHA-tagged constructs in tethering assay. 293 cell lysates from representative tethering assays were loaded equally and separated by SDS-PAGE. The NHA-tagged constructs were detected by ECL western blot assay using the monoclonal antibody to HA tag. The image was captured by Geliance 600 chemi imaging system (upper inset) and analyzed by GeneTools software. The protein levels relative to the level of NHA-TNGW1 were shown in the lower inset in the figure. The protein amount of the non-specific band (NS Band) was subtracted from the measurement of GW1Δ1. C) Comparison of the repression potencies of different NHA-constructs based on the repression effect per protein molecule. The repression potency of each tethering construct was calculated by its average repression effect (panel A) divided by its relative protein amount (panel B). The calculated potency was standardized to the potency of TNGW1, which was assigned as 1. The actual value of repression potency was shown on the top of its corresponding column.

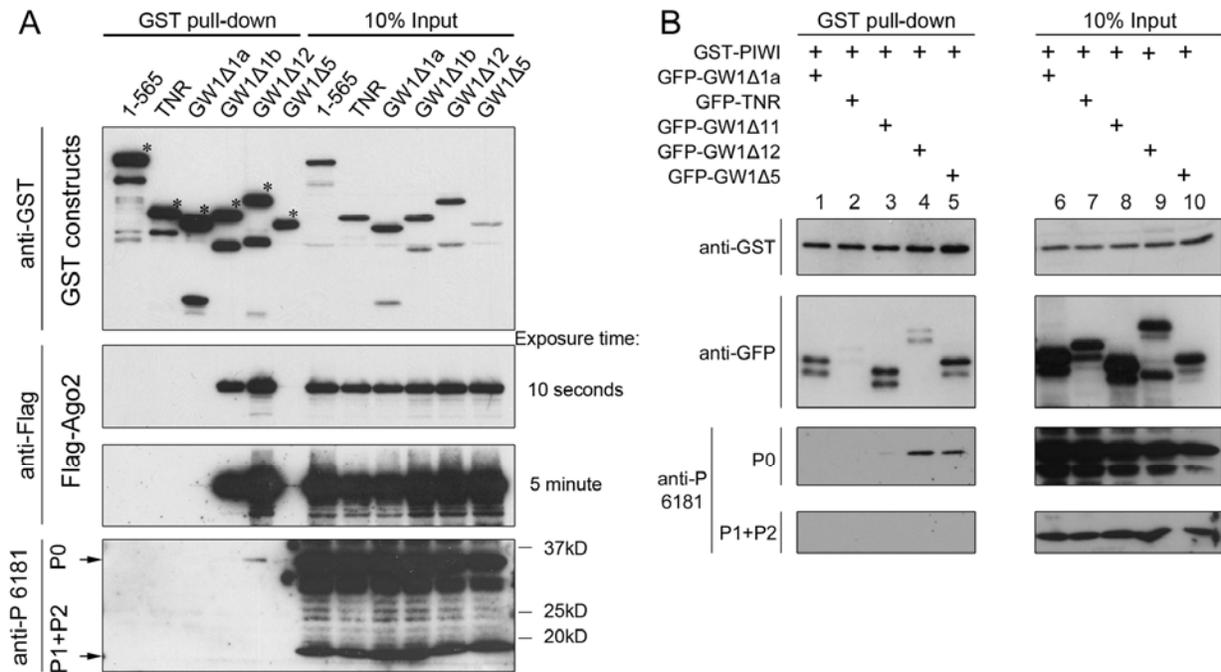


Figure 4-3. Endogenous acidic ribosomal protein P0, but not P1 or P2, was associated with GW182:Ago2 complex containing GW1Δ5 or GW1Δ12 in GST pull-down assays. A) P0, but not P1 or P2, was co-precipitated when GST tagged GW1Δ12 and GW1Δ5 pulled down Flag-Ago2. Different GST tagged GW182 constructs were co-expressed with Flag-Ago2 in HeLa cells. GST pull-down assay was performed cell lysates and the GST and Flag tagged constructs were detected by corresponding antibodies in western blot. Endogenous acidic ribosomal P proteins (P0, P1 and P2) were detected by human anti-P serum 6181. Asterisks showed the corresponding GST tagged constructs in the pull-down assay. B) P0 was co-precipitated when GST-PIWI pulled down GFP tagged GW1Δ12 and GW1Δ5. GST-PIWI was co-expressed with different GSP tagged GW182 truncated constructs in HeLa cells. Similar GST pull-down assay was performed on cell lysates were and the GST-PIWI and GFP tagged constructs were detected by corresponding antibodies in western blot. Human anti-P serum 6181 was used to detect ribosomal P proteins.

CHAPTER 5 DISCUSSION AND CONCLUSIONS

GW182 is the Repression Trigger of MiRNA-Mediated Gene Silencing

Although miRNAs often silence the gene expression through translational repression, the detailed molecular mechanism of this process remains unclear. In a recent review, Filipowicz and his colleagues summarized the potential mechanisms involved in the translational repression (Filipowicz et al., 2008). One consensus among those mechanisms is that more protein factors must be recruited to achieve silencing effect in miRNA pathway than that in siRNA pathway. Because the incomplete complementary sequence between the miRNA and the mRNA interferes with the catalytic site of the Ago2, the slicing function is hindered in miRNA-mediated gene silencing. Under this circumstance, Ago proteins function like an important guiding factor in the process by interacting with miRNA and recognizing its complementary mRNA. More importantly, they can recruit the downstream factors to trigger and secure the repression effect.

Multiple protein factors have been reported important for miRNA-mediated gene silencing, including GW182 (Liu et al., 2005a; Zhang et al., 2007; Eulalio et al., 2008b), RCK/p54 (Chu and Rana, 2006) and eIF6 (Chendrimada et al., 2007). GW182 is the only factor that has the conserved importance in miRNA function in mammal, *Drosophila* and *C. elegans*. It tops the list of translational inhibitory factors because it is the factor most closely associated with Ago proteins. The interaction between GW182 and Ago proteins has also been reported in different species. A conserved sequence residing in GW182, containing glycine/tryptophan motif, is reported important for the Ago:GW182 interaction (El-Shami et al., 2007; Till et al., 2007). In our study, at least 4 individual regions in GW182 are able to interact with Ago2 protein. Furthermore, one of the regions in N-terminal half of GW182, GW1 Δ 1a, interacts with Ago2 independent of the tryptophan in its sequence. These findings further emphasize that GW182

harbors a strong ability to interact with Ago proteins. Similar interaction has not been reported in RCK or eIF6 to date. Therefore, we believe that GW182 and Ago protein work closely in RNAi activities. In agreement with this concept, the interaction between GW182 and Ago2 is reported important for miRNA-mediated translational repression (Eulalio et al., 2008b).

The next reasonable question raised based on this finding is, whether GW182 can induce repression by itself when it is brought to the miRNA target site in the 3'-UTR of an mRNA. Using the tethering assay established by the Filipowicz laboratory (Pillai et al., 2004), we show that GW182 can induce translational repression independent on the presence of Ago proteins. In the following study, we even narrow down two regions in GW182 able to induce repression when tethered to the 3'-UTR of the reporter mRNA. The repression regions show a different pattern compared to the Ago-interacting regions, which implies the Ago-interacting ability is distinct from the inhibitory effect of GW182. At the same time, the two independent inhibitory regions indentified in GW182 do not show significant similarity in sequence. It implies that GW182 may be able to trigger the translational repression through more than one mechanism. Based on all these discussion, we propose that GW182 works downstream of the Ago proteins as the repression trigger in miRNA-mediated gene silencing.

However, more studies need to be carried on to understand how GW182 trigger the repression effect. In our study, the two inhibitory regions of GW182 are associated with the ribosomal stalk protein P0, but not P1 or P2. It implies that the assembly of the ribosomal stalk is interfered by the presence of GW182 constructs. Therefore, whether GW182 silences translation by attacking the assembly of ribosomal stalk is a reasonable next question to address. More evidence needs to found to clarify whether the association between GW182 and P0 is the cause, or the result of the repression. We will be able to gain a better understanding if we can

further characterize how direct this interaction is. More specifically, through what regions, or domains, they interact with each other? Is the P1-like sequence in GW182 responsible for the interaction with P0? If we disrupt this interaction, will GW182 lose its repression effect? We are looking forward to examining these hypotheses and answering these questions in future studies.

The relationship of GW182 and other repression factors will also need to be examined. With the knockdown-tethering assay as used in Chapter Two and Three, it is feasible to examine whether RCK or eIF6 is required for the GW182 repression effect. Based on the results, the roles of these factors can be placed at the appropriate stages in the cascade of translational repression.

The Redundancy of RNAi Factors and Their Potential Link to the Different Outcomes of miRNA-Mediated Gene Regulation

There are four Argonaute proteins, sharing over 90% identities with each other, in the mammalian system. Except for the slicing activity of Ago2, the functional bias of each paralog remains unclear. All four Ago proteins have been reported to induce repression effect when they are tethered to luciferase reporter as shown in our current study as well as by other researchers (Pillai et al., 2004). We have also showed that all four Ago proteins are able to interact with GW182. A recent study from the Tuschl's laboratory has showed that the protein factors and the mRNA associated with different Ago proteins are highly similar (Landthaler et al., 2008). Another study has reported that mouse embryonic stem cells deficient for Ago1-4 are completely defective in miRNA silencing function (Su et al., 2009). However, they have also showed that reintroduction of any single Ago into Ago-deficient cells is able to rescue the endogenous miRNA silencing function. All these data imply that there is substantial functional redundancy within the human Ago family.

Similar phenomenon is observed in the GW182 family. In our current study, we have identified a novel isoform of GW182 as TNGW1. We have showed that both TNGW1 and GW182 colocalize with other GWB component in cells and are able to induce repression effect in tethering assay. Studies from different groups have showed that the other two paralogs of GW182, TNRC6B and TNRC6C, are also able to induce translational repression in tethering assay and function in the miRNA pathway (Meister et al., 2005; Till et al., 2007; Zipprich et al., 2009).

As redundancies are observed in these important RNAi factors, the miRNA-mediated activity shows a broad variety of outcome. Even though most mRNAs targeted by miRNAs are silenced in translation, the subsequent mRNA degradation can happen in very different speeds (Eulalio et al., 2008b). In addition, some miRNA-targeted mRNA may be re-activated into translational stage under the serum-starved stress condition (Bhattacharyya et al., 2006a; Vasudevan et al., 2007). It is suggested that certain protein factors are important for these special regulations. The Ago and GW182 families can probably contribute to the potential different outcomes of miRNA-mediated regulations since they contain multiple paralogs that carry the same fundamental function in RNAi. The functional bias of each paralog needs to be further characterized in future study. Special attention needs to be paid to the functional importance of each paralog in different RNAi activities related to different cell types, different stages of cell cycle and different stress conditions. We are the first group providing evidence related to the heterogeneity of GWB in terms of its components (Jakymiw et al., 2007). In this current study, we also showed that the novel isoform, TNGW1, is distributed in only a subset of GWB. It implies that there are potential functional differences in GWBs containing different components. The cell imaging data may be more sensitive compared to biochemistry data,

because it describes individual variation instead of the behavior of a whole population. As novel technologies emerge rapidly nowadays, we are not surprised that distinguishable function will be found related to each paralog in the Ago and GW182 protein families.

Working Model of GW182 in miRNA-Mediated Gene Silencing

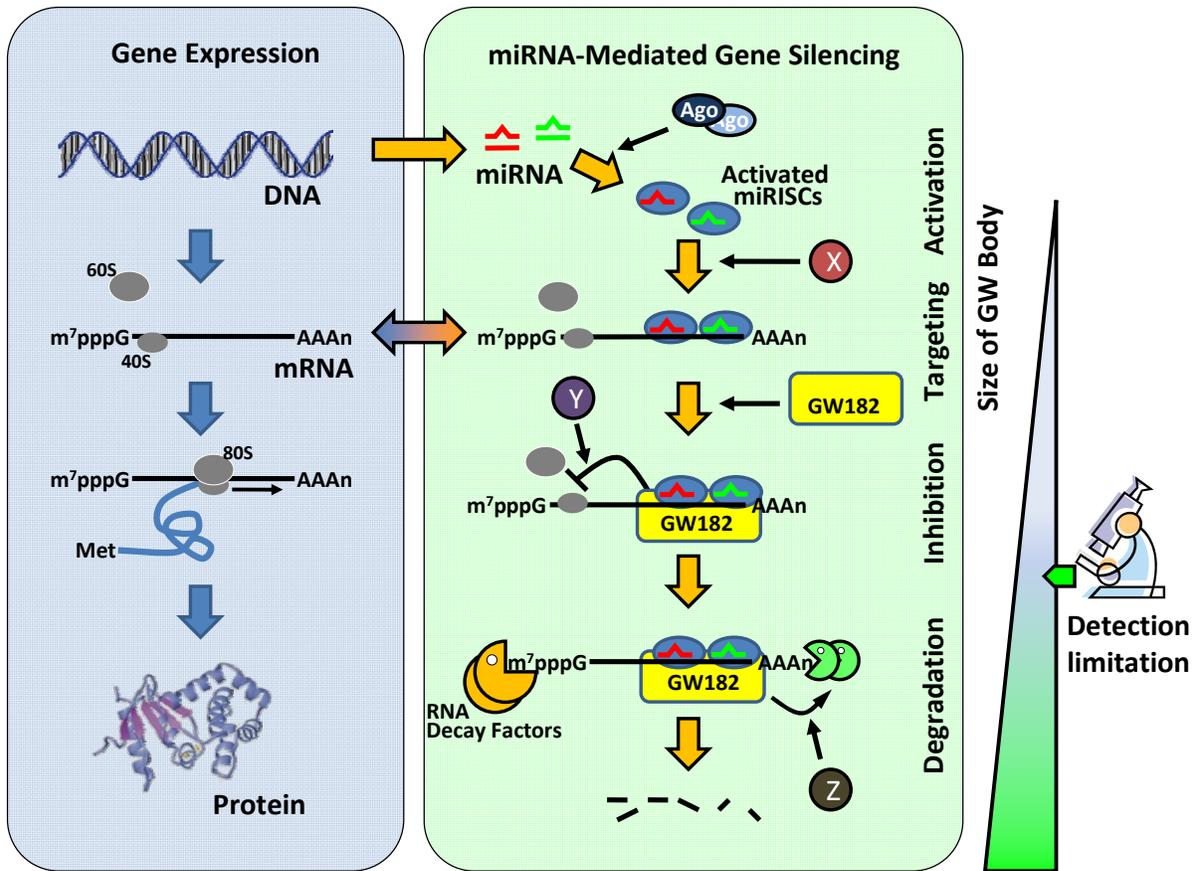
Based on our previous work and current studies, we propose a new working model, revised from our previous model (Jakymiw et al., 2007), that illustrates the functional importance of GW182 in miRNA-mediated gene silencing (Fig. 5-1). GW182 is an important factor in trigger the translational inhibition. Study from another group shows that GW182 is also able to induce the subsequent degradation of the targeted mRNAs (Behm-Ansmant et al., 2006; Eulalio et al., 2008b). Therefore, GW182 is apparently important for both miRNA pathway and GWB formation because this cascade is disrupted if GW182 is knockdown. It is likely that more protein factors may be involved in the pathway and potentially contribute to the regulation of the miRNA-mediated silencing. We hypothesize that there are unknown factors, including X, Y and Z, that contribute to the miRNA-mediated gene silencing at different stages of the cascade. The identification of these factors may potentially fill in the blank of the poor understanding in the fine controls in miRNA-mediated gene regulation and the reason for its potential different outcomes.

Furthermore, based on the current understanding and the findings from our study, we propose the concept of a four-stage cascade in the miRNA-mediated gene silencing pathway and correlate them to the formation of GWB. It is controversial that whether the formation of GWB is important for miRNA function. From this model, the conclusion for this issue may depend on two major factors: the current detection limitation and which factor is selected as the detection marker of GWB. Given the heterogeneity of GWB observed (Jakymiw et al., 2007; Li et al.,

2008), it is even very difficult to define the integrity of GWB. Therefore, whether the formation of GWB is absolutely important for miRNA function can still be debated.

In summary, our current characterization of the functional domains in GW182 has helped to advance the understanding of miRNA-mediated gene silencing at the molecular level. We have proposed an interesting model how the translational repression effect is triggered by GW182 and this opens a wide-range of future research to further dissect the mechanism. Our findings also provide a more comprehensive model of miRNA-mediated regulation contributing to future translational research in RNAi-based therapy.

Figure 5-1. A model illustrating the functional importance of GW182 in miRNA-mediated gene silencing. In the central dogma, genomic information in DNA was transcribed into mRNA. When the mRNA initiates translation, the 40S ribosome small subunit is loaded to the mRNA and recruits the 60S large subunit to form the 80S ribosome. The coding sequence is then translated into its functional protein. In the circumstance of miRNA-mediated gene silencing, miRNAs are transcribed from genomic DNA and transported into cytoplasm. The antisense-strand of the miRNA is incorporated into an Ago-containing complex, named miRNA-inducing silencing complex (miRISC) and activate its function. The activated miRISC can target to mRNA in sequence-specific manner. Some factors (X, such as RCK/p54) may facilitate the targeting at this stage. Note that it is possible that more than one miRNA can target to the same mRNA in a time. Therefore, there could be multiple miRISC binding to the 3'-UTR of the mRNA. The targeted mRNA is extracted from the gene expression pathway (left panel) at this stage. However, it could still return to expression under specific condition, such as stress. When GW182 is recruited to the 3'-UTR of the mRNA, it can bind to multiple Ago proteins and trigger the translational repression by interfering the joining of 80S ribosome. At this stage, the expression of mRNA is mainly silenced. There could be potentially more factors (Y, such as eIF6) involved in the process and securing the repression effect at this stage. The silenced mRNA is eventually led to the degradation stage when GW182 induce the deadenylation of mRNA. Multiple 5'-3' RNA decay factors (Pacmans) are recruited to degrade the mRNA. There may or may not be protein factors (Z) which can control the degradation speed in this step. Overall, after the miRNA is released to cytoplasm, the miRNA-mediated gene silencing can be separated into four stages, which include activation, targeting, inhibition and degradation. GW182 may play an important role in the inhibition and inducing the degradation. Along these stages, it accompanies with protein factors recruitment and aggregation, which reflects as the formation of GWB. Knockdown of any factors before the inhibition step, including blocking the biogenesis of miRNA, can significantly impair the silencing effect as well as GWB formation. However, knockdown of the RNA decay factors may only affect the detectable GWB but not the gene silencing effect.



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BIOGRAPHICAL SKETCH

Songqing Li was born in Guangzhou, China, 1978. He studied clinical medicine at Sun Yat-sen University, a famous medical school in China, and received Bachelor of Medicine in 2001. After he graduated, he had practiced as an OB/GYN doctor in the second affiliated hospital of the university for three years. His passion to understand the mechanisms behind diseases drove him to pursue a higher degree in basic science research. Songqing was admitted by the Interdisciplinary Program in Biomedical Sciences in College of Medicine at the University of Florida in 2004. At the same year, he married his love, Shang Li Lian, and established their family in America. In summer of 2005, Songqing joined the laboratory of Dr. Edward K.L. Chan and started his scientific adventure. With the guidance of his mentor, he devoted himself to the study of characterizing the expression and the functions of GW182, which is a protein factor that is important in the RNA interference pathway. He received Ph.D. degree in Medical Sciences-Molecular Cell Biology in May 2009.